Author’s response to reviews
Title: Susceptibility Testing of Leishmania spp. against Amphotericin B and Fluconazole using the Sensititre™ YeastOne™ YO9 Platform

Authors:

Ruwandi Kariyawasam (ruwandi.kariyawasam@mail.utoronto.ca)

Priyanka Challa (priyanka.challa@mail.utoronto.ca)

Rachel Lau (rachel.lau@oahpp.ca)

Boggild Andrea (andrea.boggild@utoronto.ca)

Version: 1 Date: 18 May 2019

Author’s response to reviews:

May 18, 2019

Dr. Cecilia Devoto

Editor in Chief, BMC Infectious Diseases

RE: BMC-INFD-19-00311 Revision 1

Dear Dr. Devoto,

Please find attached our revised manuscript entitled “Susceptibility Testing of Leishmania spp. using the Sensititre™ YeastOne YO9 Platform”, which we wish to have considered for publication as a Technical Advance article in BMC ID. We thank you for the opportunity to revise our manuscript along the lines suggested in peer-review, and have responded to reviewer comments in a point-by-point fashion below.

Specific Comments, Reviewer 1:
1. Page number 2, Line number 22 to 25 "Standardized clinically-approved drug …………
a therapeutic agent a priori." RESPONSE: We thank the reviewer for this suggestion, to
which we have attended.

2. Page number 2, Line number 25 to 29 "Our objective was to adapt the readily available
……………… … for the treatment of CL." RESPONSE: We thank the reviewer for
identifying this source of confusion and have corrected the text accordingly.

3. Page number 3, Line number 43 to 46 "Given its current utility in …………………
concentration of the YeastONE Y09 plate. RESPONSE: We thank the reviewer for
noting this source of confusion and have amended the statement accordingly.

4. Page number 5, Line number 79 to 84 "Current clinical management ……………………
…… accessibility of the drug [3]. RESPONSE: We thank the reviewer for detecting this
source of confusion and have clarified the text accordingly.

5. There are lot more throughout the manuscript. RESPONSE: We thank the reviewer for
identifying the typos and run-on sentences. The manuscript has been re-reviewed by two
native English speakers from Canada, and all such errors have been corrected.

6. There are several typographical mistakes and lack of uniformity throughout the
manuscript.

    Few examples: i. Page number 2, Line number 39 and 40 the mean MIC values should be
written as μg/mL instead of ug/mL. RESPONSE: We thank the reviewer for detecting these and
have corrected them throughout.

    ii. Page number 6 line number 101 the unit for denoting the size of amastigote should
be 2μm instead of 2um. RESPONSE: Amended accordingly.

    iii. Page number 6 line number 111, 119 and 127, Sensistitre should be 'Sensititre'.
9. There are lot more throughout the manuscript. RESPONSE: We thank the reviewer for detecting these typos, which have now been corrected.

10. From the manuscript it is appeared that authors investigated the use of Sensititre™ YeastONE™ YO9 susceptibility plate for testing the susceptibility profiles of promastigotes of different Leishmania spp. But in majority sections of the manuscript they have focused on testing the potential utility of amphotericin B (AB) AB and Fluconazole (FZ) for the treatment of cutaneous leishmaniasis (CL). For example in page no. 3 line no. 25 to 29 the authors mentioned that "Our objective was to adapt the readily available Sensititre™ YeastONE YO9 plate used for routine antifungal susceptibility testing in yeast to determine drug susceptibility profiles in cultured isolates of Old World and New World isolates of Leishmania spp., in order to, as proof-of-concept, inform potential utility of AB and FZ for the treatment of CL". Whereas, in the other sections of the manuscript they have generalized that "Customization of the plate may provide the opportunity to evaluate higher concentrations of FZ and an expanded panel of drugs with efficacy against Leishmania spp." It is better to include proper explanation in support of such generalized claims. RESPONSE: We thank the reviewer for raising this query. Our main objective was to evaluate the Sensititre™ YeastONE YO9 plate for routine drug susceptibility testing of Leishmania spp. for AB and FZ. Given that all isolates exhibited MICs ≥256µg/mL for FZ, it is necessary to expand the concentrations of FZ above this range to truly determine the appropriate MIC which could not be captured in this Sensititre™ YeastONE YO9 plate. This explanation has now been included.

11. The authors are required to provide a comparative account in support of their claim that Sensititre™ YeastONE™ YO9 susceptibility plate is an efficient and cost effective drug susceptibility checker. They need to include the current techniques used in research laboratories for susceptibility testing and compare the result with the data obtained from Sensititre™ YeastONE™ YO9 susceptibility plate to prove its efficiency. RESPONSE: We thank the reviewer for this request. There is no standardized drug susceptibility testing of Leishmania spp. in either the EUCAST or CLSI guidelines, and no current techniques are available in a clinical microbiology laboratory. None of the in-vitro drug susceptibility testing platforms (references 14-18) being used in research laboratories are commercialized systems, unlike the YeastONE YO9 plate. Thus, this commercialized platform meets a standard of quality associated with GCP/GLP manufacturing, as it is currently used for non-fastidious fungal infections. Cost and efficiency have now been
described in detail and in conjunction with point #9 to support the efficiency and cost-effectiveness of this plate.

12. One of the major limitation of this study is the susceptibility checking of amastigote form of Leishmania parasite. The authors also mentioned that fact. RESPONSE: We thank the reviewer for reiterating this point. We have addressed that the next step is to evaluate the Sensititre™ YeastONE™ YO9 using amastigotes to further demonstrate its clinical utility as a drug susceptibility assay for routine Leishmania spp. testing in the discussion section (lines 331-334).

13. In the background section of the main manuscript the authors claimed that "In-vivo systems including animal models have been used extensively to determine Minimum Inhibitory Concentrations (MIC)s in Leishmania spp., however such systems could never be scaled to the clinical microbiology laboratory [14-18], and are therefore of limited utility." None of the references they have cited include information about in vivo testing of MIC against Leishmania. RESPONSE: We thank the reviewer for noting this discrepancy. The sentence has been changed to refer to in-vitro systems.

14. The information given in the line no 105 to 110 "However, given that log-phase promastigotes are generally less susceptible to anti-Leishmania drugs than amastigotes [14-17], and detectable in a cell-free culture system incubated at room temperature, it has been proposed that promastigotes serve as a surrogate for determining the susceptibility patterns of isolates to pharmacotherapeutics independent of any cell-mediated parasiticidal mechanisms [14]." is not clear. RESPONSE: We thank the reviewer for raising this concern. Given that log-phase promastigotes are less likely to be killed by the antileishmanial agent compared to amastigotes, it is worth testing in-vitro models using promastigotes as proof-of-concept, particularly given the cost of amastigote-based assays, such as human or murine macrophage or those that are truly in vivo. As a first step, given the fiscal constraints of our publicly funded laboratory, we needed to establish proof-of-principle with a cell-free (ie, more economical) model. We do acknowledge the appropriate next step is to test amastigotes. The sentence has been altered to be more clear.

15. In the materials and method section (line number 163 and 164) authors mentioned "Concentrations of impregnated AB ranged from 0.12 µg/mL to 8 µg/mL, whereas FZ
ranged from 0.12 µg/mL to 256 µg/mL [19]." But from the cited reference it not clear why they have chosen such concentration range in the present study. RESPONSE: We thank the reviewer for raising this query. These are ranges of the concentrations of drug included in (and therefore directly impregnated into) the Sensititre™ YeastONE™ YO9 plate when purchased from Thermo Scientific. With a commercialized product, we had no jurisdiction over the concentrations of AB or FZ put into each well.

16. In the discussion section an elaborate comparative analysis of the efficiency of Sensititre™ YeastONE™ YO9 susceptibility plate with other methods of susceptibility testing are need to be included to enrich the manuscript. RESPONSE: We thank the reviewer for this request. Using in-vitro references 14-18, a comparison regarding time, technical expertise and cost has now been included to highlight the clinical utility of the Sensititre™ YeastONE™ YO9 plate in a clinical parasitology laboratory.

17. The manuscript is full of grammatical errors making it difficult to read. It requires a thorough editing upon revision. RESPONSE: Again, we thank the reviewer for raising this concern. The manuscript has been re-reviewed and edited for English throughout by two native English speakers, one of whom has senior level editorial responsibilities at 4 different peer-reviewed medical journals.

Specific Comments, Reviewer 2:

1. Since the objective of the paper was to demonstrate the utility of the Sensititre system to determine MICs for leishmania species (a new indication), a more thorough description of the methodological approach and analysis should be provided. For example, how was the colorimetric assay conducted? Alamar blue readings are typically measured by absorbance at 570nm or by fluorescence measurements. Data supporting the correlation between these data and microscopic analysis should be provided. RESPONSE: We thank the reviewer for raising this concern. The colorimetric assay does not require absorbance readings as per manufacturer’s instructions. All methodological approaches are described in the TREK Diagnostics manual, with changes to inoculation medium, concentration and MIC readings described in our paper. Additional methodological steps have been added for clarity and exposition.
2. Were the other drugs in the Sensititre system also measured? There are 9 antimicrobials present in Sensititre. If this was a custom plate, were other relevant antileishmanial drugs performed? Why wasn't this done? Justification should be provided on the narrow scope of drugs used for this study. Further, microscopic analysis and alamar blue assays are traditional assays used for proliferation and cytotoxicity studies. Therefore, a compelling rationale on the advantages of the Sensititre plate was used should be provided. RESPONSE: We thank the reviewer for this query. AB and FZ are the treatment options for CL that happen to be included in the Sensititre system. The other 7 antifungals including: anidulafungin, micafungin, caspofungin, 5-flucytosine, itraconazole, voriconazole and posaconazole, have little to no precedent in the literature as effective treatments for CL. Thus, these 7 other drugs were not evaluated for the purpose of this study. The advantages of Sensititre over traditional in-vitro methods have now been included in the discussion section of the paper.

3. The figures do not appear to be very informative. What is the point of showing pictures of plates since color changes are very difficult to determine with the naked eye? Besides we do not know what is in each well of the plates shown. Graphs of absorbance readings and correlations with microscopic data should be provided. RESPONSE: We thank the reviewer for this observation. Absorbance readings were not performed and not required as per Thermo Fisher Trek guidelines. The figures highlight the following: both plates are inoculated with L. amazonensis at 0 hours (left) and 96 hours (right). Column 12 and row 8 indicate AB and FZ, respectively. In Figure B, one can see no colour change in column 12 (indicating parasite killing) whereas row 8, all the wells have changed from blue to purple (compare same rows and columns in figures A and B) indicating parasite growth, thus indicating an MIC ≥ 256 for FZ. We are happy to remove the figures as desired at the Editorial level.

4. What is the point of figure 2? Are there statistical differences between the groups shown. If so where are the differences? The authors indicate that the Fishers exact test was used. What were the results of the tests? Any p values? RESPONSE: We thank the reviewer for raising this query. Figure 2 is a graphical representation of the difference in susceptible isolates between different groups (New World vs Old World; Visceralizing vs. Non-Visceralizing; Viannia vs. non-Viannia; ATCC vs Clinical) and there were no significant differences as described in the last paragraph (including p-values) of the Results section (Lines 268-274). Again, we are happy to remove this figure if it is felt to be unhelpful at the Editorial level.
I hope that we have addressed the reviewers' concerns satisfactorily. All authors have seen and approved this version of the manuscript, all contributed significantly to the work, and none has a conflict of interest with its publication. This manuscript has not been previously published and is not being considered for publication elsewhere. Please contact me at your convenience should you require additional information. I look forward to corresponding with you further, and I thank you for your consideration.

Sincerely,

Andrea K. Boggild, BSc, MSc, MD, DTMH, FRCPC
Medical Director, Tropical Disease Unit, Toronto General Hospital
Parasitology Lead, Public Health Ontario Laboratory
Associate Professor, Department of Medicine, University of Toronto