Author's response to reviews

Title: Molecular Identification and Antifungal Susceptibility Profile of Aspergillus flavus Isolates Recovered from Clinical Specimens in Kuwait

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Author's response to reviews: see over
Title: Molecular identification and Antifungal Susceptibility Profile of *Aspergillus flavus* Isolates Recovered from Clinical Specimens in Kuwait

Dr. Philippa Harris
Executive Editor
BMC-series Journals
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Dear Dr. Harris,

Many thanks for forwarding us the comments of the reviewers. We have modified the manuscript in the light of these comments/suggestions. Our point-by-point response, highlighted in red font, is presented below:

**Reviewer 1**

Major comments:

1. The authors need to write more in Discussion about why they have used the methodology in their study. For example, in Molecular characterization section of Methodology they should explain why they have used identification of B-tubulin gene and calmodulin gene, while they have detected the species using DNA sequencing which suffices for finding the species.

   **Response:** We used sequencing of B-tubulin and calmodulin gene fragments since sequencing of ITS region of rDNA alone is not discriminatory for isolates belonging to closely related species of *Aspergillus* section *flavi*.

2. In antifungal susceptibility testing, they have compared three different methods neither of which is standard. They should have used a standard method to make the comparison.

   **Response:** Unfortunately, we could not perform broth microdilution assays due to non-availability of enough funds and difficulty in obtaining pure powder of the test drugs. This limitation has been indicated in the discussion.

3. Since the study was carried out over an 18 year period, we expect some genetic differences and different susceptibility patterns during this long period. But the authors have included no details about this important fact. So more discussion about it is needed.

   **Response:** Requested information has been added.

4. In Discussion Paragraph 2, it is said that none of the isolates came from a proven case, but in Conclusion of Abstract it is said that 92 clinical isolates were identified. These sound contradictory and requires clarification.

   **Response:** The sentence has been deleted.
5. In Conclusion section, it is said that the work was the first in the Middle East. But it is not so, and one references as follows are introduced:


**Response:** The above reference of Badiee et al. has been added in the revised manuscript. Unfortunately, this reference was not available in PubMed when the manuscript was written.

6. The Discussion section is actually the inclusion of many references some of which belonging to the same authors. It is of no significance in terms of the findings of the study itself and thus the Discussion needs to be carefully revised taking into account the results of the study in Kuwait.

**Response:** The discussion has been shortened/modified taking into consideration comments of the reviewers.

**Reviewer 2**

Major Compulsory Revisions

1. The title should be amended: I suggest using molecular identification rather that characterization (in the title and the manuscript).

**Response:** The title has been modified as suggested.

2. In the introduction, the authors should mention the epidemiological importance of A. flavus as a human pathogen (far before A. fumigatus) in Northern Africa, which climate is probably more like Kuwait than Northern countries.

**Response:** The suggested changes have been made

3. As fungal taxonomy is in progress, it is very important for the scientific community that the DNA sequences of these isolates are deposited in GenBank and that their GB accession no are mentioned in the manuscript. For the same reason and for the preservation of well characterized strains that might be used in further taxonomic or epidemiological studies, all the studied strains should ideally be sent to a fungal collection.

**Response:** The representative sequences have been deposited in the GenBank and accession numbers included in the revised manuscript.

4. The authors should explain how they collected these isolates. Indeed, they state that these 99 isolates have been collected over 18 years, which seems quite a long period of time.

**Response:** A sentence to explain how these strains were maintained for 18 years has been added.
5. In the results/molecular characterization section: It is not obvious from Figure 1 that all strains from Kuwait clustered together. This should be clarified.

**Response:** The Figure and its legend have been modified to address the point raised by the reviewer.

6. In the discussion section: the discussion should be shortened. It is not relevant mentioning *A. fumigatus* azole resistance. In contrast the authors should cite Hadrich et al. (Med Mycol. 2012 Nov;50(8):829-34.) who correlate amphotericin B MICs and fatal outcome in patients with *A. flavus* invasive aspergillosis. I agree that voriconazole has addressed the issue of Aspergillus amphotericin B resistance in “rich” countries (such as Kuwait). Yet, amphotericin B remains the first line antifungal (and voriconazole is often not available) in many “poor” countries. Unluckily, I should say, in these countries *A. flavus* is the most prevalent Aspergillus species.

**Response:** In the light of the comments, the discussion has been modified and reference of Hadrich et al. (2012) has been included.

- **Minor Essential Revisions**

1. In the results/Disk diffusion test section: Figures 2 and 3 should be deleted. The regression curves equations should be deleted in the text (R square is enough).

**Response:** As suggested, Figures 2 and 3 have been dropped.

**Reviewer 3**

**Minor Essential Revisions:**

a. Page 2, line 28: Replace “report of emergence” with “report on emergence”.

b. Page 2, line 33: Replace “morphologic” with “morphological”.

c. Page 2, line 39: Replace “combined sequencing” with “combined analysis”.

d. Page 2, line 39: This sentence refers to the mean MIC90 values, please add it.

e. Page 2, line 42: “The zone of inhibition…” Please rewrite this sentence.

f. Page 4, line 72: “Aspergillus spp” should be corrected to “Aspergillus spp.”

g. Page 5, line 94: “rDNA was amplified with Aspergillus section Flavi-specific AFLF and AFLR primers” According to my best knowledge this primer pair is specific to *A. flavus*, not to the whole section.
h. Page 6, line 106: Please specify the software used for the phylogenetic analysis.

i. Page 6, line 106: Replace “Neighbor-joining” with “neighbor joining”.

j. Page 6, line 124: Replace “E-test” with “Etest” here and in the entire text.

Response: All the corrections and changes suggested above(a-j) have been incorporated.

k. Page 7, line 134: The European Committee on Antifungal Susceptibility Testing has recently published an updated list of break-points.

(\url{http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Antifungal_breakpoints_v_4.1.pdf}). I recommend to use these data.

Response: The clinical breakpoints proposed in EUCAST have been indicated.

l. Page 8, line 169: Replace “A. parvisclerotegenus” with „A. parvisclerotigenus” here and in other occurrences too.

Response: The taxon name has been corrected.

m. Page 9, line 172: “(A. fasciculatus CBS110.55), A. thomii…” Please remove the parenthesis.

Response: The parentheses have been removed.

n. Page 10, line 193: “About 11% (n=10) isolates showed MICs of >2 µg/ml.” Please make this sentence clearer. Add that these values were measured in the case of RPMI media.

Response: We have modified the sentence for greater clarity, as suggested.

o. Page 10, line 193: “inhibited 100% isolates” should be changed to “inhibited xxx% of the isolates” here and in other occurrences too. p. Page 10, line 203: “(range 0.008 -0.19 µg/ml)” The space should be deleted between the dash and the number.

Response: The suggested changes and corrections have been made.

q. Page 10, line 205: “A comparison of mean MICs revealed slightly lower values for environmental isolates for voriconazole and posaconazole, but higher values for amphotericin B.” I think this statement is not important because of the low number of environmental isolates.

Response: The statement has been deleted.

r. Page 11, line 223: Replace “All clinical and environmental isolates were identified as A. flavus strains based on partial sequencing of #-tubulin and calmodulin gene fragments.” with “All
clinical and environmental isolates were identified as A. flavus strains based on the analysis of partial #tubulin and calmodulin sequences.”

Response: As suggested, the above statement has been modified.

s. Page 13, line 266: Replace “capturing 100% isolates” with “involving 100% of the isolates”.

Response: The statement has been modified.

t. Please make the references uniform.

Response: The references have been made uniform.

Quality of written English: Needs some language corrections before being published.

Response: Improvement in the syntax has been made in some places in the text.

Reviewer 4

General Comments

In this manuscript Al-Wathiqi, F. et al have investigated the in vitro susceptibility of 92 clinical (collected over a period of 18 years) and 7 environmental A. flavus isolates to 3 different classes of antifungal drugs commonly used for treating fungal infections, including those caused by members of the genus Aspergillus. These investigators initially characterized the isolates using morphological characteristics and subsequently confirmed their identity by molecular biologic techniques such as characterization of the ITS1-5.8S rRNA gene-ITS2 region as well as by partial sequencing of the #tubulin and calmodulin genes. The combined morphological and molecular biologic techniques confirmed that all the isolates these investigators used belonged to A. flavus taxon. Their in vitro susceptibility studies using E-test and disk inhibition assay revealed that both clinical and environmental isolates were highly susceptible to all the antifungal drugs they used except amphotericin B. A low but significant percentage of the clinical isolates were resistant to amphotericin B.

This is a well-designed, well performed and well written paper, and the results these authors obtained will be helpful to those studying drug susceptibility of A. flavus isolates. In general, A. flavus isolates have slightly elevated MIC values for amphotericin B compared to those obtained for A. fumigatus isolates, but they are highly susceptible to the triazoles and the echinocandins. It was interesting to note that the drug susceptibility of these A. flavus isolates has not changed significantly over a period of 18 years.

Response: We thank the reviewer for positive comments. No comments to respond to.

Discretionary Revisions:
One of the pitfalls of this study is the use of E-test and zone of inhibition to determine the antifungal activities of the echinocandins, since these authors ignored microcolony formation in their E-test and zone of inhibition. It is unclear how the microcolony formation would affect the interpretation of the results since the echinocandins are fungistatic agents. It is unfortunate that the authors did not perform broth dilution MICs for the echinocandins to compare with their E-test and zone of inhibition data. In addition to this deficiency, the authors should address few minor points (see below) before considering the publication of this manuscript.

**Response:** We share the concern of the reviewer. A statement indicating these limitations has been added in the Discussion.

**Minor Essential Revisions**

Line 28: Missing a comma after the word climate. ……geographic regions without and arid climate, including the Middle East.

**Response:** A ‘comma’ has been inserted.

Line 34: …… and their susceptibilities to six antifungal agents were determined……

**Response:** The sentence has been modified.

Line 35: The authors use the words Etest and E-test in the manuscript. Which one is accurate? Even if both versions are correct, be consistent in the usage.

**Response:** The usage of Etest has been made uniform.

Line 65: It should be ….. In 2008, Clinical and Laboratory Standards Institute……

**Response:** The correction has been made.

Line 114: What was the concentration of the saline used? 0.9%? Also,……vortexed for few seconds….. Be specific, 10 S, 15 S, 20 S?

**Response:** The required information has been provided.

Line 191: Insert the definite article ‘the’ after the word of. …… Of the 92 clinical isolates tested, 74.2%...........

**Response:** The correction has been made.

Line 194: ………at a concentration of #0.256 µg/ml on both test media. At this concentration…. 

**Response:** The correction has been made.

Lines 200-203: Why not include mean ±SD for the MIC values. For example, ..... amphotericin B 1.14 ±SD µg/ml etc…….
Response: The standard deviation values have been included in the text and also in Table 2

Line 315: Spelling mistake. ……to writing of the manuscript. All authors have……..

Response: The correction has been made

Table 3: Report the geometric mean values with standard deviations. For example 0.842 ± SD etc…….

Response: The data have been simplified and simple mean with standard deviations have been included in the revised Table 3

Figures 2 and 3: I am not sure about the significance of Figs. 2 and 3. The greater the antifungal activity of a drug is the larger the zone of inhibition. On the other hand, the better the antifungal activity of a drug is the lower the MIC value. Hence if we plot the E-test MICs (X-axis) vs. zone of inhibition (Y-axis) naturally you would get an inverse relationship.

Response: Figures 2 and 3 have been dropped. This is also suggested by Reviewer 2

We, sincerely hope that you will find the revised manuscript suitable for publication in BMC Infectious Diseases.

Looking forward to hearing from you.

Best regards,

Z. U. Khan