Author's response to reviews

Title: Molecular characterization, biofilm analysis and experimental biofouling study of Fusarium isolates from recent cases of fungal keratitis in New York State

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Version: 3 Date: 21 December 2006

Author's response to reviews: see over
December 21, 2006

Scott Edmunds PhD
Assistant Editor
BMC-series Journals
BioMed Central
Middlesex House
34-42 Cleveland Street
London W1T 4LB

Dear Dr. Edmunds:

Please find enclosed a revised version of our manuscript first submitted to BMC Ophthalmology on November 2, 2006. The details are:
MS: 1329119781120793
Molecular characterization, biofilm analysis and experimental biofouling study of Fusarium isolates from recent cases of fungal keratitis in New York State

We have extensively revised this manuscript in view of the referees’ comments. The revised version includes additional experiments, new or revised figures and photographs, and revised text. A point-by-point disposition of the referees’ comments is also enclosed.

We earnestly hope that this version will be accepted for publication at the earliest. Many thanks,

Sincerely,

Vishnu Chaturvedi, PhD
Director, Mycology Laboratory
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Response to Referees’ Reports

Referee 1
Reviewer’s report
Title: Molecular characterization, biofilm analysis and experimental biofouling study of Fusarium isolates from recent cases of fungal keratitis in New York State

Version: 1

Date: 29 November 2006

Reviewer: Hajib Madhavan

Reviewer’s report: General

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

Discretionary Revisions (which the author can choose to ignore)

The manuscript is a good comprehensive genotypic research work done to characterize Fusarium species causing keratitis. However, there are certain queries raised which the authors need to clarify on the points listed below to make the paper acceptable.

ABSTRACT: Page No. 2

Result:
• The split up of Fusarium solani and Fusarium oxysporum isolates from corneal scraping, contact lens and the lens solution need to be indicated clearly in the abstract. The authors have shown these facts in the Figure legend 2 which provides difficulty to the reader in understanding the summary of the work done. This could be overcome if the split up of the specimens is provided in the abstract itself.

- We agree and the details of the fungal isolations are now provided (p.19, Table 1).

- The authors have isolated F. solani from an opened bottle of MoistureLoc solution. Details of the number of days after the bottle was opened and the number of days of its usage after it was opened before it was submitted to the laboratory need to be included in the paper.

- The patient was using the MoistureLoc bottle for approximately three-months when it was handed over for microbiology examination. This information is now provided (p. 4, last paragraph).

- The sentence, “A number of F. solani and F. oxysporum genotypes were present in the isolated strain” is not clear. The sentence has to be reframed properly.
- Corrected by specifying the number of genotypes seen (p.10, first paragraph).

**MATERIALS AND METHODS: Page No. 4**

- The number of *Fusarium* isolates included in the paper along with the source from which these have been isolated should be clearly indicated in the text under materials and methods or in a tabular form. Of course, the details of the same are provided in Figure legend 2, it is difficult for the reader to understand the details from the figure legend.

- A new Table 1 is included to address this concern (p. 19).

- The antibiotic and its concentration incorporated in Sabouraud’s dextrose agar for isolation of *Fusarium* species are needed to be mentioned.

- Done (p. 4, last paragraph).

- The authors have targeted Internal Transcribed Spacer region, 28SrDNA and *Fusarium* specific partial beta tubulin and elongation factor. The references for the same have to be indicated against each target separately.

- Done (p. 5, second paragraph).

- Brief methodology on DNA extraction, characterization using microsatellite and minisatellite probes needs to be provided.

- Done (p. 5, second paragraph).

**Experimental biofouling: Page No. 5**

**Quantitation of Fusarium cells**

- While performing Biofouling studies, an inoculum size of 5x 10^2 - 10^5 cells were inoculated on to MoistureLoc solution. How was the quantitation done? Was it done by counting the number of spores or by comparing the turbidity of hyphae using spectrophotometer? The specific reasons for choosing an inoculum size of 5 x 10^2 - 10^5 may please be mentioned.

- The cells were counted using a hemacytometer. The inoculum size was similar to that reported by other published studies cited in the manuscript (Miller et al., 2001; Rosenthal et al., 2003).

- What were the specific reasons to use yeast extract peptone dextrose agar instead of Sabouraud’s dextrose agar? This needs to be clarified.

- Both media gave comparable results in this experiment. We have repeated the experiment with Sabouraud’s dextrose agar, and figures 5-6 in the revised manuscript summarize our observations.

- *Fusarium oxysporum* spelling has to be corrected Page No. 5 (last sentence).
Results:

• The authors have claimed that the recovery of *Fusarium solani* from MoistureLoc solution yielded 50 colonies per ml. How was it quantitated? Figure 1a representing the growth of *Fusarium solani* on YEPD medium shows the presence of fungal colonies in the margin of the plate also. This would raise a query whether it would have come as a plate contaminant. Can this be explained?

- One-hundred microliter aliquots of MoistureLoc solution, provided by the patient for microbiology examination, were plated and dispersed with the help of a mechanical spreader over the entire surface of the culture plates. The colony counts were multiplied by ten to arrive at colonies per ml estimations. The entire procedure was performed in a BSL 2 laminar flow biosafety cabinet. The culture plates were quality controlled for sterility and positive growth of reference strains of fungi. We have not encountered any contamination in these plates. The appearance of colonies on the margin could be due to spreader touching the plate wall. Moreover, all colonies in the margin and the center were examined microscopically, and were found to be similar.

• Figure 2 represents unrooted parsimony phylogram of EF 1 of Fusarium strains to explain the degree of relativity between Fusarium isolates characterized in the study. It would be of better understanding to the reader if the details of specimens from which the isolates have been recovered along with Mycology Identity Number is provided in the figure.

For example, Myc 23-06 (F. solani Corneal ulcer isolate)
Myc 237-06 (F. solani Contact lens case swab isolate)
Myc 238-06 (F. solani Contact lens cleaning solution isolate)

This type of representation in the figure would help the reader to understand that these three isolates were genotypically identical.

- Done

• *Fusarium solani* and *Fusarium oxysporum* isolates were compared with the laboratory isolates to determine the genetic similarity. Why were not the standard strains of *Fusarium solani* and *Fusarium oxysporum* used in the study?

- *F. solani* and *F. oxysporum* taxonomy is in a flux as both are recognized as species complexes with many sub-species. Our attempt in this study was to achieve clinically relevant identification of these fungi, which was accomplished by a combination of classical mycology and nucleotide sequencing.

Laboratory isolates unrelated to this outbreak were used to check robustness of two genotyping methods used in our study. We did not procure any 'standard strains' from the recognized culture collections as their inclusion would have been necessary only if the focus was exclusively on the taxonomy of the isolated strains. As mentioned in the manuscript, a number of publications have documented a mix of environmental and plant genotypes among *Fusaria* isolated from clinical specimens.
(p. 12, first paragraph). Therefore, no standard genotype can be associated with the clinical strains.

- The authors have attempted DNA sequencing to characterize the isolates. Though the sequences were deposited in GenBank, (materials and methods), the same has not been provided under results nor it was dealt in discussion. The authors can add on a few lines on the results of DNA sequencing related to sequence homology or nucleotide polymorphisms if present in the isolates under results and discussion.

- We appreciate the requirement for more experimental details. The revised manuscript includes these details (p. 9).

- Figure 4: Photograph on scanning electron micrographs of biofilm formation
The authors have well documented the formation of biofilm on the lens, but the duration (whether 48, 72 or 96 hours?) at which the growth of Fusarium species was documented is missing and the same needs to be provided in the figure legend.

- Done (p. 18, figure 4).

Figure 5:
- Figure 5a - h representing the growth of Fusarium species on shows the presence of fungal colonies in the side of the plate –Figure 5c, 5d, 5f, 5g, 5h. This would raise a query whether it would have come as a plate contaminant. The authors need to replace the present photographs with a good figure.

- The experiments were repeated, figure five was replaced with a better quality photograph, and a new figure 6 was included to summarize this data.

What next?: Accept after discretionray revisions

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

Statistical review: No

Declaration of competing interests: 'I declare that I have no competing interests'
Referee 2
Reviewer's report
Title: Molecular characterization, biofilm analysis and experimental biofouling study of Fusarium isolates from recent cases of fungal keratitis in New York State

Version: 1 Date: 1 December 2006

Reviewer: Mark Willcox

Reviewer's report:
General
This manuscript reports on findings associated with Fusarium isolated from contact lens wearers during recent outbreak in NY, USA.

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)
The authors should include further detail describing the numbers or amount of fungi in the biofilm experiments.

- More details are now added to the methods section to describe the set up of biofilm experiments (p. 7, first paragraph).

Details of the strains isolation site, subjects, etc should be added into a table.

- A new Table 1 is included (p. 19).

The authors should give more detail on the use of MoistureLoc in the biofilm analysis, so that others could repeat the experiments if needed.

- Relevant experimental details are provided in the revised version (p. 7, last paragraph).

The authors should show data for the statement that "MoistureLoc sufficed to destroy the biofilm".

- A new panel has been added to Figure 4 to illustrate absence of biofilm in MoistureLoc treated lens. The procedure is also explained in greater detail (pp. 8, 18).

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Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

Discretionary Revisions (which the author can choose to ignore)

What next?: Accept after minor essential revisions

Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: No

Declaration of competing interests: I have received grants from contact lens and contact lens solution manufacturers over the past 5 years.
Referee 3
Reviewer’s report
Title: Molecular characterization, biofilm analysis and experimental biofouling study of Fusarium isolates from recent cases of fungal keratitis in New York State

Version: 1 Date: 6 December 2006

Reviewer: Philip A Thomas

Reviewer’s report:
General
Comments for Authors
BMC Ophthalmology
Title of manuscript: Molecular characterization, biofilm analysis, and experimental biofouling study of Fusarium isolates from recent cases of fungal keratitis in New York State.
Authors: Dyavaiah et al.
The manuscript by Dyavaiah et al. is an interesting and generally well-written description of experiments performed on some isolates of Fusarium solani and Fusarium oxysporum isolated from patients in the Northeastern US during the recent outbreak of contact lens-associated Fusarium keratitis. The inferred research question, which the study proposes to answer, is whether the Fusarium isolates somehow had special properties that nullified the use of ReNu with Moisture Loc. The methods are appropriate and well-described, and have been described in sufficient detail to allow others to replicate the work. The data are sound. The manuscript adheres to the relevant standards for reporting and data deposition. A noteworthy observation made is that the much-maligned contact lens solution, ReNu with MoistureLoc, does exhibit anti-Fusarium activity (even when the solution used is several months old), provided the manufacturer's instructions are complied with. The discussion and conclusions are well-balanced and adequately supported by the data. In fact, the authors have been cautious in indicating that there is still much that we do not know about the actual factors that led to the outbreak. The title describes the aim of the study. The abstract accurately describes the salient observations made. I believe the manuscript has important information that readers of the journal would find relevant. However, I also believe that the manuscript would be strengthened by performing some revisions.

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)
No relevant controls were used. Controls would have helped to highlight unique characteristics of the strains of Fusarium solani and Fusarium oxysporum isolated from patients involved in this outbreak. The controls used could have been Fusarium strains isolated from corneal infections, but not isolated during this outbreak (maybe isolates from trauma-associated Fusarium keratitis from other centers), as well as Fusarium isolates from other, non-ocular infections. In addition, a comparison with an important non-Fusarium corneal fungal pathogen, for example Aspergillus fumigatus, might have yielded additional, interesting information.
- Adequate controls were included in the genotyping work and in the experimental studies. For example, molecular genotyping studies included three *Fusarium* strains unrelated to this outbreak including two strains from previous cases of fungal keratitis (p. 6, first paragraph). Similarly, experimental studies with biofilm and biofouling included negative controls as treatments without fungal inoculum. Future studies are clearly warranted to compare the relative virulence of *F. oxysporum* and *F. solani*. The genetic heterogeneity between *Fusarium* and *Aspergillus* would argue against their head-to-head comparisons in the context of our study objectives.

**Minor Essential Revisions** (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

**Minor essential revisions**

<table>
<thead>
<tr>
<th>Page No.</th>
<th>Line No.</th>
<th>Comment</th>
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<tbody>
<tr>
<td>3</td>
<td>1st paragraph, line 4</td>
<td>Add 'the' between 'by' and 'yeast'</td>
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<td>3</td>
<td>1st paragraph, line 7</td>
<td>Rephrase the beginning of the sentence to &quot;There had been no report until the beginning of 2006…….&quot;</td>
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<td>2nd paragraph 3rd line</td>
<td>'February' and not as written.</td>
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<tr>
<td>4</td>
<td>9 &amp; 10</td>
<td>&quot;Additional unopened bottles…. local pharmacies&quot;. Were these bottles procured before or after the worldwide recall of this product? If after, how were stocks available in the local pharmacies?</td>
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<td>- They were obtained before the recall of the product (p. 5, last paragraph).</td>
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<td>7</td>
<td>Experimental biofouling: &quot;A previous report…..&quot; Please cite the report.</td>
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<td>10</td>
<td>&quot;Moisture Loc solutions, purchased from local pharmacies&quot;. Same comment as for page 4, lines 9 &amp; 10.</td>
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<td>- The timeline of these purchases is now included (p. 8, first line).</td>
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<td>5</td>
<td>Results 3rd line</td>
<td>&quot;the supernatant but and not…..&quot; Remove 'but' or 'and'</td>
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<td><em>F. oxysporum</em> and not as spelt.</td>
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</table>
6 6th & 5th lines from end "Different batches of Moisture Loc, procured from local….”. Same comment as for pg. 4, lines 9 & 10.

- This has been clarified in the revision.

8 2nd paragraph 5th line "….. as long as recommended…". Add 'the' between 'as' and 'recommended'

- Done

8 2nd paragraph 6th line "….. the conclusions of many other…..". But only 2 reports are cited!

- Corrections were made in the citations.

8 5th line from bottom "inoculated F. solani….". Please add "with" between 'inoculated' and 'F. solani'

- Done

8 3rd line from end "recovered few colonies of ….". Please add 'a' between 'recovered' and 'few'.

- Done

8 Last sentence It might be appropriate to rephrase this sentence thus: "These results suggest that at least some occurrences of keratitis could have resulted from temporary contamination….”

- Done

10 Reference 5 "O'Brien", and not as written; add issue number, if you want to make this reference consistent with other references (which have issue numbers)

- Done

10 Reference 6 This reference is not written in the correct format.

- Corrected

10 Reference 9 Are the authors' names correct and complete? What about issue number?

- The author names have been corrected. The issue numbers are not required in citations according to the style format for BMC Ophthalmology. The issue numbers are now not shown in any reference.

11 Ref. 15 Add issue number
- Corrected
Discretionary Revisions (which the author can choose to ignore)

The authors could perhaps briefly provide their hypothesis of how *Fusarium* entered the contact lens solutions as a prelude to causing infection. They do hypothesize that not following the manufacturer’s instructions for using MoistureLoc might have predisposed to contamination, presumably from the environment or water supply. If so, why weren’t other organisms implicated? After all, the environment and the water supply probably contain other organisms, and not just *Fusarium*. A comment on this might be worthwhile.

- The last sentence in the discussion provides our hypothesis as to how this outbreak might have occurred. Certainly, more studies are warranted to examine the very interesting question as to why only *Fusarium* and not other microorganisms were involved in this outbreak. Additionally, the relative roles of *F. oxysporum* and *F. solani* in this outbreak also need to be examined. We do not have experimental data to propose any alternate hypotheses at this stage.

**What next?**: Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions

**Level of interest**: An article of importance in its field

**Quality of written English**: Acceptable

**Statistical review**: No

**Declaration of competing interests**: I declare that I have no competing interests