Author's response to reviews

Title: Association of Atopobium vaginae, a recently described metronidazole resistant anaerobe, with bacterial vaginosis

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PDF covering letter
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Dear Editor:

Please find attached a revised version of our manuscript entitled “Association of \textit{Atopobium vaginae}, A Recently Described Metronidazole Resistant Anaerobe, with Bacterial Vaginosis”. We thank the reviewer's for their insightful comments and suggested revisions. As you will find in our responses to reviewer concerns (below), we have fully complied with nearly all suggested revisions and compromised with a few others including the concern each reviewer expressed regarding providing detailed cultivation descriptions in the methods section. We feel the revisions have improved the manuscript and hope it is now suitable for publication in BC Journal of Infectious Disease.

Please feel free to contact us directly with any questions regarding the manuscript. Thank you again for your time.

Sincerely,
Michael Ferris
(corresponding author)

\textbf{Response to Reviewer Concerns}

\textbf{Reviewer 1}

\textbf{Title:} Association of \textit{Atopobium vaginae}, a recently described metronidazole resistant anaerobe, with bacterial vaginosis

\textbf{Version: Date:} 28 November 2003
Reviewer: Gregor Reid

Response to reviewer's report:

General

1. In abstract, state number of patients, race and age range.

We have added numbers indicating, %race, age range and total patients to the abstract.

2. In Background, provide better reference than Hillier #2, for example citing Gardnerella, Prevotella and Mobiluncus associated with BV.

We added a new reference and species names from the typical list of "BV-associated" bacteria.

3. I don't follow the order of references. Is it in order of citation or alphabetical? The findings from ref 26 should be cited in first section of Background as it is the first use of PCR-DGGE for identifying vaginal microbes. In addition, Burton published in AEM 2003 and found DGGE to be useful to identify organisms not recovered by culture.

   The order is by citation in the text, we cite Muyzer at the first reference to DGGE because his was the original paper on DGGE/16S detection in microbial communities and the paper provides detailed information about the method. The Burton papers are cited as references to using DGGE to study vaginal flora later in the manuscript, but we feel they might not be the most appropriate references to direct readers to descriptions of the theory and practical aspects of DGGE. We have added references to Burton's two additional DGGE papers on vaginal flora which we were not aware of at the time our manuscript was written.

4. State which body approved the ethics.

   The IRB reviews were referred to in the last sentence in the second paragraph under “Subjects” in the methods section. Perhaps the wording was unsatisfactory. The wording has been changed to make it clear that both the LSUHSC and the University of Indiana IRB’s approved the research protocols.

5. I presume you have evidence that shipping does not spoil the detection of organisms.

   As stated in the methods section, samples for nucleic acid extraction were frozen until DNA isolation. This included shipping. Freezing is an accepted method of storing samples until DNA isolation.
6. P6 line 4 space needed.

We can't find this error on our copy.

7. Perhaps comment on the collection of a single swab as typifying the status of the flora, rather than taking swabs at days or weeks apart.

Of course a single sample (DGGE pattern) provides a "snap shot" of the bacterial community at the time of sampling. We agree that there is a need to better define the diversity and dynamics of the vaginal flora through longitudinal studies and realize the utility of DGGE and other molecular methods to approach this task. We intend to address these issues in a subsequent paper presenting a summation of our surveys of vaginal flora and prefer not to elaborate on the subject in the current manuscript. Our intent here was to focus on reporting to the community of researchers studying BV, the observation of a strong association between BV and the presence of a species with clinically notable (antibiotic resistance) properties that had gone unnoticed, presumably due to a reliance on traditional microscopic observations and cultivation methodologies.

It seems unlikely that a temporal analysis of specimens across this group of patients, rather than a blind, random sampling of single time points across these same patients, would alter the outcome. *A. vaginae* would still have been detected in a majority of BV-positive patients and rarely in patients clinically described as having normal vaginal flora.

8. Please identify the organisms represented on the Figure 1 BV and Normal gels. Did the controls and BV patients have lactobacilli?

We have sequenced many of the DGGE bands over the course of our study. However, not all have been done. Our goal is to obtain sequences for bands that migrate to unique positions in the gradient, and once identified, to use band positions as identification criteria. We are aware of the pitfalls of DGGE including band co-migration, heteroduplex formation, multiple different 16S rRNA genes in the same organism, and are working through samples to define diagnostic band-pattern-types where we can be reasonably certain of the identities of the bands from past sequencing efforts. We realize that band position is not definitive and one can never be sure of a bands sequence without actually sequencing. In the long term, the advantage of DGGE is that eventually, after repeatedly detecting the same bands or similar band patterns, we can begin to use these patterns to rapidly assess the flora in large patient cohorts. That will be the topic of our next paper.

We prefer to not specifically illustrate the identities of non-*A. vaginae* bands as we intend to generate a more comprehensive paper of all the organisms.
identified in our study using these same samples. However, we have included a comment in the results to indicate that (in addition to A. vaginae) organisms such as G. vaginalis. Prevotella, Bifidobacterium, and others species were detected by sequencing DGGE bands.

9. Better define "simple type". What does "limited", "predominant", "typically" mean? Be more specific so that future studies can reference what you found.

We changed the names of the DGGE patterns to “normal (previously simple) type” and the “BV (previously complex) type.” Although admittedly subjective (as are all well-accepted current diagnostic schemes, i.e., Nugent score and Amsel criteria) our intent was to point out that there seems to be a DGGE pattern that distinguishes normal from BV samples. It is difficult to devise definitive labels for the patterns. For example the new term “BV type” includes 2 patients with intermediate Gram stains. Nevertheless, the patterns alone do seem to be indicative of normal or BV-positive vaginal samples as we were able to “call” them in a high percentage of cases. We are working on using cluster analysis software to "put numbers" on the similarities between banding patterns and hope to be able to more systematically define banding patterns in the future.

Undoubtedly there is a limit to the resolving power of DGGE banding patterns and we suspect that there will always be cases where clinically normal vaginal samples will not conform to the descriptions presented here. The same will likely be true of some BV-positive patient samples which will resemble normal patterns. Another issue is that we have a new diagnostic test for BV (i.e. DGGE) which could be better than the current “gold standard” tests. Whenever a new test is more sensitive and/or specific than the “gold standard” initial ambiguity is the result. Further research will be needed in order to clarify the appropriate use of terms.

10. The description of Vaginal Cultures is misleading if no data are presented. I would have preferred to have had that data included, but I understand the reason for not doing so. Thus, maybe leave out the culture description.

This point is a difficult one for us and we suspected that it might raise questions. In fact Dr.Schewbke asked for more detail on culture methods. In the paper as written we compromised by briefly describing our quantitative methods and indicating that the detailed culture methods are available from the authors on request (a long section on culture methods was part of the original draft of the manuscript but was taken out for the reason alluded to by Dr. Reid). However, since we describe successful cultivation of two isolates of A vaginae, we felt that readers would expect some mention of culture methods. Providing the detailed culture methods and all of the culture data would make the paper much longer and much more complex. We intend to write a subsequent paper which will focus on the discrepancies between DGGE/sequencing and culture. Not all of
the patients included here will be included in that report. That paper will contain the detailed culture methods. To include all of this here would detract from the major point of this paper which is the association of *A vaginae* and abnormal vaginal flora. As stated we will provide detailed culture methods to any one who requests them.

11. Is there any evidence that Atopobium is found more often after repeated antibiotic use – thus perhaps a selection for these organisms is due to repeated metronidazole use? Could it be they are a result of treatment rather than a cause of failures? Where do they originate - the gut, from a male partner, environment? If drug resistant strains are also in the patients' gut, this explain some findings.

We do not have detailed prior antibiotic histories from the patients enrolled in these studies. Dr. Reid’s point is valid and will require further study.

12. The Discussion reads well, although 43 references seems a lot for this paper.

If the editors desire some of the references can be removed but lack of space limitation is an advantage of electronic publication and the references may prove useful to some readers.

13. Overall, this is an excellent contribution to the field and raises many questions about the vaginal flora. Sadly, too few studies are supported in microbial ecology. Given the importance of the vaginal microbiota in reproduction and the health of the host, more studies of this nature are needed.

We agree.
Declaration of competing interests:
NONE

Reviewer 2 report

Title Association of Atopobium vaginae, a recently described metronidazole resistant anarobe, with bacterial vaginosis
Version: Date 15 December 2003:
Reviewer: Jane Schwebke

Response to reviewer's report:

General
Discretionary Revisions (which the author can choose to ignore)

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

1. Please describe the training and experience of the person who interpreted the Gram stains – what type of quality assurance measures were in place.

Our laboratory contributed specimens to the original study that resulted in the development of the Nugent score. The technicians participating in this study still work for Dr. Martin and were responsible for the Gram stain readings presented in this paper.

2. Please add more detail to the vaginal culture section - media used exact concentrations used for organisms, etc

See response to point #10 in Dr. Reid’s review.

3. For the 2 patients with normal Gram stain scores and bands consistent with A. vaginae were the slides reviewed? What were the clinical data for those patients - signs, symptoms, Amsel criteria?

These slides were reviewed and appeared to be normal. We do not have data for signs and symptoms for these patients.

4. Although you state that you performed susceptibility studies on the ATCC control you only show the data for metronidazole - please provide the additional data.

These data are now included in Table 1.
5. page 12 - 55% of patients is barely a "majority of patients" - this is misleading
The word majority has been removed.

6. Why do you suppose that these bands are not present in all BV patients if the organism is important in pathogenesis

We suspect that traditional methods of categorizing the vaginal flora associated with BV mask the diversity of bacteria present in cases where the flora is considered "abnormal". This is consistent with the array of different DGGE banding patterns for the BV patients in fig 1, all of which would be grouped into the BV category by traditional standards. BV as currently diagnosed probably represents a spectrum of different variants of an abnormal vaginal ecosystem. Therefore A. vaginae could play a significant role in one of these variants but not in others. Of course this is all hypothetical at this point and subject to further research. We note that Dr. Schwebke implied in a 2001 publication that M. curtisii may be important in the pathogenesis of BV based on its presence by PCR in 64% of cases which is not greatly different than the proportion of our cases positive for A vaginae by DGGE.

7. page 13 - are the same patients presented in this manuscript going to be presented in the future manuscript? if so, this may represent duplicative publication and the results of the quantitative cultures should be included in this manuscript.

See our discussion of Dr. Reid's point #10.

8. page 14 - other organisms associated with BV are also metronidazole resistant such as Mobiluncus curtisii - this should be mentioned

This fact is now noted in the last paragraph of the paper.

9. If the organism is susceptible to clindamycin how do you explain similar cure and recurrence rates with clinda and metronidazole?

As noted above, we think that clinical BV represents a complex of diverse variations in the vaginal ecosystem. Therefore it is reasonable to hypothesize that some patients would fail treatment with one drug and others would fail treatment with another for different reasons.

10. Figure 1 - it would be good to show an example of a patient with intermediate flora by Gram stain as well.

The DGGE pattern for the intermediate cases could not be differentiated from those that met Nugent's criteria for BV. Furthermore, they were not identical to each other and there appears to be no way of distinguishing them from the BV cases. After we
have studied many more cases there may be features that distinguish those with intermediate Gram stains but we are not there yet. Therefore we did not want to make a special issue of showing a single intermediate DGGE pattern. Here we are trying to emphasize the difference between normal and abnormal flora only.

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)
Accept after minor essential revisions What next?:
An article whose findings are important to those with closely related research Level of interest:
interests
Acceptable Quality of written English: