Author's response to reviews

Title: Clinical identification of bacteria in human chronic wound infections: culturing vs. 16S ribosomal DNA sequencing

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Author's response to reviews: see over
Dear Editor:

In this updated cover letter I have included point by point responses to reviewer comments as requested by Neil Nazareno on 1 October 2012 in his email to Dr. Wolcott. My responses are below in **bold**.

**Reviewer’s report**
**Title:** Clinical identification of bacteria in human chronic wound infections: culturing vs. deep 16S ribosomal DNA sequencing
**Version:** 1 **Date:** 21 May 2012
**Reviewer:** Lance Price

**Reviewer’s report:**

Dr. Wolcott and his colleagues are some of the leaders in the field of molecular analyses of chronic wounds, but sadly the research presented in the current manuscript does not move beyond their previous work and does not contribute any additional information to the field. In the revised manuscript, submitted 31 July 2012, we have made numerous revisions and have clearly described how this study does contribute additional information to the field. For example on page 4 we write, “Culture-free sequencing of bacterial DNA reveals qualitatively which bacteria are present, but it also reports a relative abundance score for each organism. Correlating this relative abundance score with the likelihood that the same bacterium would be detected by culture has not been performed until now.”

Another group of researchers, led by Dr. Zenilman, also conducted very similar work that was presented in PLoS One nearly three years ago (not cited in the current manuscript). In fact, the current manuscript is almost a step back considering they didn’t bother to culture the anaerobic bacteria that make up a considerable fraction of the wound microbiota. This is a pretty major oversight given that this was a study comparing culture to molecular detection. **We have now included citations of the reviewer’s, Dr. Price’s, publications within the manuscript (Citations 7 & 8).** The reviewer’s PLoS One publication is an excellent article. However, the report is only qualitative, and it does not attempt to correlate relative quantities of amplicons that were sequenced to the likelihood that the bacteria would be cultured. Our study is the first to report this. Our study is also different in that it examines samples from twice as many subjects. Our study was performed using standard of care culture methods; wounds are routinely cultured for only aerobic bacteria because of the low recovery of anaerobes. This has been previously described by many, including the reviewer in Citation 8:

“Obligate anaerobes, such as Clostridiales family XI, are particularly difficult to grow and were not identified using culture-based methods in the current study. Using 16S rRNA gene-based sequence analysis, we identified bacteria from Clostridiales family XI in 25 of the 32 wounds analyzed.”
This field is never going to progress if we don't move beyond these little surveys that reveal—with false surprise—that molecular detection is more sensitive than culture for characterizing mixed microbial communities. Researchers in this field have to start using molecular characterization of wound communities to answer important clinical questions. It is often difficult to convince clinicians that molecular microbial techniques may sometimes be more beneficial than the culture-based bacteriology methods that have been used for more than a century. The current study helps to correlate the relative quantitative results of a novel molecular microbial assay with the culture-based methods with which clinicians are familiar. No other study has done this.

I hope that the authors will read this review, file this paper in a drawer, and move on to more important work (which they are obviously capable of conducting). But, as is standard for our field, I'm sure they will continue submitting the paper until it is accepted somewhere. So, I offer the following suggestions:

1) the authors must report the number of sequences generated for each sample and show that the depth of coverage is sufficient to characterize the full scope of taxa within the wounds. It is unacceptable to leave out this variable in a paper comparing this method to the standard for pathogen detection. This is now reported in the first full paragraph of page 8.

2) the authors have to describe how the sequences were assigned to their respective taxa. This is another unacceptable omission. This is now reported in the second full paragraph of page 5.

3) the discussion should be reduced by at least 50%. We have made efforts to reduce the length of the discussion as much as possible.

Reviewer's report
Title: Clinical identification of bacteria in human chronic wound infections: culturing vs. deep 16S ribosomal DNA sequencing
Version: 1 Date: 1 June 2012
Reviewer: Luanne Hall-Stoodley
Reviewer's report:
Clinical identification of bacteria in human chronic wound infections: culturing vs. deep 16S ribosomal DNA sequencing

Significance
The paper by Rhoads et al. describes the analysis of 51 chronic wound samples obtained from patients with various types of non-healing wounds for comparison of culture and 16S rDNA sequencing methodology for identification of bacteria in the wound. The topic of chronic wounds (CW) is very important. CW already affect millions of patients yearly at enormous cost but further represent an increasing clinical problem as lifestyle-associated diseases such as obesity, diabetes and cardiovascular disease
continues to rise in the global population. An underlying problem with the treatment of CW has been the accurate diagnosis of the microbial etiology of the persistent infection. Several papers, including seminal work by the authors, have now shown that routine culture underestimates the presence of bacteria in the wound and that molecular methods give a more accurate depiction of the microbial burden. This is key to more accurate and precise diagnosis which will result in more rapid and targeted treatment. This manuscript compares culture methods and 16S deep rDNA sequencing.

Methods:
Chronic wounds from 51 subjects were sampled from a variety of different types of wounds following debridement of the wound as part of standard care and duplicate samples were tested in parallel. One half was analyzed by routine culture in a hospital-based microbiology laboratory and the other half was analyzed following DNA extraction from the sample, amplification of the 16S portion of ribosomal DNA pyroseequencing of 16S amplicons using Roche’s FLX Titantium platform. Samples were then analyzed for determining relative abundance of the bacteria taxa identified.

Strengths:
The problem of molecular identification of so many types of bacteria in clinical samples where few bacteria were previously detected by culture raises difficulty for clinicians trying to interpret these results. This study tries to address this difficulty by demonstrating that deep sequencing better resolved the bacteria present by showing that of the most abundant bacteria (dominant), only half were cultured, and far fewer proportions of bacteria were identified by culture in the major and minor classifications. The paper also discusses discrepancies between the 2 methods.

Weaknesses:
The authors need to more clearly address how the current paper differs from other publications (including their own) indicating that molecular methods find more organisms and do a better job at identifying them than culture. If the goal of the paper is to help clinicians become more comfortable with interpreting molecular diagnostic data then the manuscript needs to clarify the results for clinicians who may not have a very deep understanding of microbiology, particularly in terms of interpreting statistical data reporting bacterial abundance. We have worked to clarify the results and discussion so that both physicians and scientists can obtain useful information from the writing. The limitations of the study should also briefly be addressed/acknowledged. For example, the expertise and cost of the Roche FLX Titanium system is beyond many clinical microbiology labs’ resources and capabilities. We have added a paragraph discussing the limitations of the study (second full paragraph on page 11). Finally the message regarding how high throughput sequencing data improve the therapeutic approach to wound care is somewhat lost. In our revisions, we clarified the clinical importance of the study. That is, this study finds that clinicians can infer which of the bacteria that are detected using this molecular method are most likely to be detectable by culture, and which bacteria that are detected using this molecular method are most likely not to be detectable by culture due to the lower sensitivity of culture testing.
Accept with major revision.

Major revisions:
The results and discussion should be tightened up and shortened. Specifically how the data support each conclusion should be stated clearly. **We have tightened up and shortened the results and discussion. Also, we support each of our conclusions with data.**

Several parts of the study need to be clarified:

Clarify inclusion and exclusion criteria for patients. **We have clarified inclusion and exclusion criteria (last paragraph of page 4).**

Better clarify type of wounds (this may be relevant to the type of infection/microbial burden). **We have described the types of wounds that were sampled (last paragraph of page 4).**

Why was culture for anaerobic organisms not undertaken? Clinical microbiology labs generally do this if requested. Is this not routinely done for chronic wounds, especially in light of several papers in the literature showing that anaerobes are present in CW? **As discussed in this cover letter previously, anaerobes are often in chronic wounds. However, the anaerobes are often not successfully cultured in clinical laboratories, and chronic wounds are routinely cultured using only aerobic methods, as was done as part of this standard of care study.**

Results should consistently indicate percent followed by (the sample number/total number of samples) as in the abstract. **We have changed the formatting as suggested.**

The statistical analysis needs to be better outlined and explained if the target audience is to be clinicians in the field. Better clarify software and analysis showing relative abundance and particularly regression analysis used. What is OR trying to show? If it is showing the difference in effect of diagnostic power of Roche platform over culture is this the best way to indicate effect size? **We have attempted to include methods and results in this study that both clinicians and scientists can appreciate. We have attempted to clearly describe the statistical software and analyses that were used. The usefulness of the OR is now described at the end of page 6.**

Figure legends should be more explanatory. Include titles for figure legends. E.g. Figure 4: Increased relative abundance of a bacterial genus increases the likelihood that the genus will be cultured. (This may help clarify the point each figure is trying to make.) **Figure legends are now more explanatory.**

Should figs 1 and 2 be combined to show that molecular methods although in bacteria which are much less abundant? Same for Figs 4 and 5? **The original Figure 1 was**
removed as suggested. We feel that the original Figures 4 & 5 (now Figures 3 & 4) should remain separate because one figure describes genera and the other describes species.

Citations need to be added to discussion to complement how research is supported by other published work or how it differs. If it differs, discuss why it might differ, e.g. different methodology, etc. Specifically relevant work by Kirketerp-Moller et al. 2008; Fazli et al. 2009; Thompsen et al. 2010 could be briefly discussed. **We have added citations and discussion of previous relevant research (first paragraph of page 7, second and third paragraphs of page 10) while still decreasing the total length of the Discussion.**

Minor suggested revisions:

Page 7: “These results can be unsettling for clinicians because ever since Robert Koch... Change to: “These results can be unsettling for clinicians who view culture as the gold-standard for bacterial identification. Our results suggest that culture testing may be insufficiently sensitive in some cases (or overly sensitive in other cases) and therefore require carefully interpretation.” **This has been removed.**

Page 9: Regardless of the cause of the bacteria’s aversion to growth in culture (change to …“failure of bacteria to grow”) **This has been changed.**

Page 10: “Because genetic detection of antibiotic resistance requires prior knowledge of the gene(s) (e.g. mecA, VanA, extended spectrum beta lactamas) …However, phenotypic testing of antibiotic resistance has a couple of shortcomings that are not often considered. “ It is not clear how the present molecular approach would overcome these shortcomings. Clarify how the present technology could improve over culture or remove from discussion. **This has been removed.**

Page 14: FLX Titanium technology is mispelt. **This has been corrected.**

Reviewer's report
Title: Clinical identification of bacteria in human chronic wound infections:
culturing vs. deep 16S ribosomal DNA sequencing
Version: 1 Date: 5 June 2012
Reviewer: Claire Jenkins
Reviewer's report:
Medical microbiologists have relied on culture of microorganisms to inform patient care with respect to diagnosis of infection and treatment for over a century. More recently molecular techniques, including 16S rRNA sequencing, have provided new insights into the complex microbiota associated with infection. The aim of this study was to compare culture and molecular testing of chronic wound infections.
The methods described in this study are clear and reproducible. They demonstrate (i) a close interaction between microbiologists performing traditional culture techniques and molecular microbiologists, (ii) a well-designed study plan and (iii) good statistical analysis.

Comparing results of culture with molecular methods is difficult because the two approaches are so different. However, the authors’ presentation of their results is comprehensible and unambiguous. The 16S data clearly shows how often obligate anaerobes, an important component of wound infections, are missed during routine culture. The authors raise some interesting issues that may have an impact on patient care and antimicrobial treatment regimes, such as the role of bacteria present in the wound at low-levels and the interactions between bacteria in a polymicrobial community. They also discuss the significance of “culture bias”, where robust bacteria may be selected for above the more fastidious genera.

This is well-designed, well written, thought provoking study addressing important clinical questions on the issues associated with interpreting results from molecular tests and their impact on patient care. Studies such as this represent a novel and valuable contribution to the field.

No major or minor compulsory revisions.

Discretionary revisions
1. The authors might discuss the precautions they took to avoid contamination during the DNA extraction and amplification processes. The design and set-up of a molecular testing laboratory is outside the scope of this study.

If you have any questions or concerns, please do not hesitate to contact me at rhoadsddd@upmc.edu or contact Randy Wolcott at randy@randallwolcott.com.

Thank you for your consideration,

Daniel D. Rhoads, MD, MT(ASCP)^CM