Author's response to reviews

Title: Use of 16S ribosomal RNA gene analyses to characterize the bacterial signature associated with poor oral health in West Virginia

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Author's response to reviews: see over
Dear BMC Journal Editors,

We appreciate your favorable review of our manuscript. This letter includes point-by-point responses to each of the reviewers’ concerns and explains how these concerns were addressed in the revised manuscript. As requested, we have deposited the nucleic acid sequences generated during our studies in GenBank and the accession numbers are indicated in the revised manuscript. All changes introduced in the manuscript are underlined. We have tried to effectively address all concerns presented to us by the reviewers and editors, and we hope that you will find our revised manuscript acceptable for publication in BMC Oral Health.

Reviewer 1

Major compulsory revision:
1) The authors need to specify why it was important to study these diverse groups of subjects as currently it is unclear in the manuscript.

As a funded pilot project, the number of samples that we could analyze based on 16S rRNA sequencing was limited, and we reasoned that acquiring data from two independent population pools (age 23-48 or age >70) might help extend an understanding of the relevance our findings in relation to West Virginians in general. Observing similar disease associated bacterial patterns in the two populations helped validate the conclusions of this preliminary study, and a statement describing this rationale has been included on page 6 and is underlined.

Minor essential revisions:
2) The authors must not over interpret these data on such small groups of subjects, particularly as for the sequencing data the number of clones (with data) examined ranges from 55 to 133.

This was also a concern of reviewer 2, who provided specific suggestions as to how to ‘tone down the talk of how significant and important their data are”. The revised manuscript has been modified to include these recommended modifications, and these changes are underlined on pages 5 and 15.

3) The authors state ‘Comparisons of bacterial populations ......provided a means of assessing how bacterial populations associated with periodontal disease compared with those associated with poor caries outcome later in life.’ Are the authors really trying to determine microbial differences in high and low disease states in the young and the elderly with periodontis and caries in just 12 subjects?

This statement has been replaced on page 6 and with the statement clarifying why two diverse population groups were included in our studies (refer to (1) above).

4) Was statistical advice taken on the number of subjects required to be enrolled into the study? If it was then please add these details to the text of the manuscript.

The number of subjects examined in this study was determined by our funds. We tried to maximize the cost effectiveness of subject selection by restricting COHRA participants to low or high disease states, with emphasis on high disease states. The nature of the Cognitive Study limited acquisition of samples to availability. The goal of this pilot project was to assess whether bacterial types associated with high oral disease in West Virginia might provide insight into the etiology of the dental problems of this state. These preliminary studies suggest that this is true, which provides justification for obtaining funds for an in depth and systematic evaluation of bacterial patterns in relation to disease outcomes in West Virginia. Details underlying subject selection in this study are a component of underlined text on page 6.
5) Did the authors carry out examiner calibration to ensure that assessment of the clinical parameters in the two study groups was comparable?

Oral evaluations were performed by calibrated researchers in both the COHRA and Cognitive studies. This is referenced for the COHRA study (Polk et al., 2008), with documentation of calibration now noted and underlined on page 6. Documentation of calibration for the Cognitive study is on page 7. Comparable calibration in the two study groups was also ensured by Dr. Richard Crout, a co-author on this manuscript, who coordinated clinical evaluations for both the COHRA and Cognitive studies.

6) Was plaque collected with a curette for the COHRA study group? Please state in the text of the manuscript.

Subgingival plaque in the COHRA study was sampled using a curette, as referenced in Polk et al., 2008. This text has been added to the Methods section in the revised manuscript on page 6 and is underlined.

7) The COHRA group of patients had subgingival plaque collected from the first four molars and the elderly patients had plaque collected from six sites. Do the authors think that these differences may alter these data?

Samples were obtained from the first or most anterior molars in both studies, with two additional sampling sites included in the Cognitive study. Samples from all sites were pooled for analyses. Since comparable sampling sites were included in both studies and comparable results were obtained, there is no reason to suspect that differences in sampling sites affected our data.

8) Were any additions made to the DNA extraction method to improve lysis of Gram-positive cell walls? Please provide details if carried out.

No additions were introduced into the UltraClean Soil DNA Isolation Kit for lysis of Gram-positive bacteria. We did spend considerable time when the project was initiated exploring methods of extraction and isolation of DNA from subgingival plaque samples to optimize DNA extraction. Methods associated with the UltraClean Soil DNA Isolation Kit consistently provided the highest yields of PCR amplifiable genes encoding 16S rRNA and were also found to be effective with Gram-positive Group A Streptococcus.

9) Please provide the strain details for the E. coli strain used for 16S cloning.

The E. coli strain used for 16S cloning was TOP10 Chemically Competent cells, a component of the TOPO TA Cloning Kit from Invitrogen. This information has been added to the Methods section and is underlined on page 8.

10) Page 10: The authors state ‘As expected, increased bacterial diversity was evident in plaque from individuals diagnosed with high oral disease.’ Please provide a reference to support this statement.

This reference has been added and is underlined on page 10.

11) The authors describe bacterial diversity in the text of the results and discussion, but perhaps sometimes they mean species richness. The species richness is the number of different species identified whereas the diversity would include the number of individual species as well as the numbers present for each species. If the authors wish to describe diversity then they should consider using a diversity index (e.g. Shannon-Weaver Index or the Evenness score) to measure this and add these details to the text. It would allow a better diversity comparison to be carried out which may be of interest to readers of this manuscript.

Our paper draws extensively on the results in Figure 1, which shows identified bacterial genera and the number of isolates for each, which by definition is ‘diversity’. As a result, our use of the term ‘diversity’ in the manuscript is correct most of the time. However, in response to this concern, we have reviewed the manuscript for text that would more accurately employ the description ‘species richness’ or other places were the usage was vague and could be improved by a rewording. This text and the changes that have been made are listed below and underlined in the revised manuscript:

Page 3: High disease exhibited substantially increased bacterial diversity and included a large proportion of Clostridiales cluster bacteria (Selenomonas, Eubacterium, Dialister). ‘Diversity’ was not changed.

Page 9: This approach provided a level of detail that was informative for classifying bacterial
sequences.
‘Diversity’ was replaced with ‘sequences’.

Page 10: As previously reported [6], increased bacterial diversity was evident in plaque from individuals diagnosed with high oral disease. ‘Diversity’ was not changed.

Page 11: The bacterial diversity in low disease COHRA samples was highly consistent with a recent study, which identified Streptococcus, Veillonella and Capnocytophaga in 100% of the plaque samples obtained from individuals with healthy oral cavities [27]. ‘Diversity’ was not changed.

Page 11: In comparison, DAA and DZ had bacterial patterns that included increased diversity and species richness of Selenomonas, Eubacterium and Dialister, in addition to Parvimonas, Campylobacter, Lachnospiraceae and Megasphaera, similar to that observed in high oral disease COHRA participants. ‘Higher frequencies’ was replaced with ‘species richness’.

Page 13: In plaque from high disease, HOMIM analyses confirmed the presence of genera from the Clostridiales order, including Selenomonas, Eubacterium and Dialister and increased bacterial diversity (Figure 3B). ‘And increased bacterial diversity’ was deleted.

Page 13: Importantly, conclusions from HOMIM analyses confirmed those from 16S rRNA sequencing, and identified increased genus richness and high intensity signals for Selenomonas, Eubacterium and Dialister in association with a decline in oral health. ‘Diversity’ was replaced with ‘genus richness’.

Page 14: The 16S rRNA tree represented in Figure 4 shows the broad diversity of Selenomonas phylotypes recovered from a single plaque sample from high disease (DA). This contrasted with the low diversity of Veillonella phylotypes recovered from a low disease sample (DB). In plaque from high oral disease, where fewer Selenomonads were present, diversity was again evident in Eubacterium and Dialister phylotypes within the Clostridiales cluster (not shown). ‘Diversity’ usage was retained in this sentence.

Page 14: Low disease Veillonella have nearly identical sequences typical of a single species (>97% 16S rRNA gene sequence identity), as represented in Figure 4. ‘Limited genetic diversity’ was replaced with ‘nearly identical sequences’.

Page 14: The breadth of this bacterial group is large (minimum ~85% identity), and this allowed us to recognize that Clostridiales were repeated colonizers in plaque of individuals having high oral disease, as represented by the phylogenetic diversity of Selenomonas in Figure 4. ‘Diversity’ was replaced with ‘breadth’.

12) The authors highlight a potential ‘atypical bacterial phylogenetic signature’ however, are they confident that this is the case, as they did not examine non-West Virginia subjects in this study.

The use of the term ‘atypical’ to describe the bacterial population we identified in plaque samples from high oral disease in West Virginia was based on the paucity of bacterial species previously reported to be associated with high oral and the prevalence of Clostridiales cluster bacteria, which are less frequently reported to be associated with high oral disease. Based on the current literature, the term ‘atypical’ (but not unique) accurately describes the bacterial pattern we identified in the plaque of West Virginians with high oral disease.

Discretionary revisions:

13) Why was plaque placed into 100-500µl and not a standardized volume used?

Volumes for suspension of subgingival plaque samples varied somewhat with time in both the COHRA and Cognitive studies, as described in the Methods section on page 6. The actual volume made no difference in analyses since the entire volume was subjected to DNA extraction procedures.

14) It would be ideal to have the clinical details for all 12 of the subjects.

As a pilot project, we did not have the financial resources to establish population pools from which to obtain subgingival plaque samples. We were fortunate in being aligned with two independent IRB approved dental projects at WVU that allowed acquisition of subgingival plaque for 16S rRNA gene analyses. As we had no control over acquisition of clinical data, we were limited to reporting clinical data that was provided to us by
15) As only 5 periodontitis patients were examined it may not be that surprising that only a minority of these subjects harboured ‘red-complex’ bacteria. The text could be altered to reflect this.

The general inability to detect ‘red-complex’ in our samples was unexpected and is what led to this manuscript. Including this text seems to diminish the significance of our primary finding, and we chose not to alter the text.

Reviewer 2 report:

Major Compulsory Revisions:
1) Make clear in the Discussion that their sample size needs to be expanded.

The following statement has been included in the Discussion on page 15 and is underlined:

“The results from this pilot project highlight the need for an expanded study that utilizes culture independent approaches to analyze bacterial populations in a larger number of West Virginians having low or high oral disease to confirm the relationship between bacterial profiles and disease.”

2) Emphasize in the Introduction that this study is preliminary.

To address this concern, the following edits/additions have been included in the Introduction on page 5, and changes are underlined:

“In this preliminary study we were able to identify significantly different 16S rRNA bacterial phylogenetic signatures in plaque from individuals having high or low oral disease, and the high disease signature was evident in two independent studies that span a wide range of age groups. Overall we found that communities rich in Veillonella and streptococci shifted to communities rich in Selenomonas and other Clostridiales in association with a decline in oral health, potentially linking an atypical bacterial signature with oral disease in West Virginians. The finding that an atypical bacterial signature may be linked to dental disparities observed in West Virginia highlights the need for further analyses of bacterial species associated with high and low oral disease in this population in order to understand the origin of this disparity.”

3) In the Conclusion that future studies are needed to confirm this result – especially note the need for more low disease and a focus on just the groups of interest.

This concern is also addressed in (1) above. Underlined text on page 15 includes modifications that were introduced to ‘tone down’ the importance of our results in view of the limited number of samples that were analyzed.

4) The other necessary aspect that needs to be dealt with prior to publication is the sequences need to be deposited in GenBank.

Nucleotide sequences generated in this study have been deposited in GenBank, and the accession numbers are included in the Methods section on page 8.

Discretionary Revisions:
5) The authors state in the abstract that their data provide “evidence that the oral environment selected for bacterial types associated with disease”. However, isn’t it also possible that these bacteria are causing the disease? So is it the oral environment selecting, or the bacteria changing the oral environment? Correlative data do not allow one to distinguish these two possibilities. Therefore, the best thing to say is that there is a correlation.

As indicated by this Reviewer, since the mechanism underlying the repeated colonization by Clostridiales cluster bacteria in the high disease environment is not known, the text in the abstract on page 3 has modified as follows:

“Phylogenetic trees constructed using 16S rRNA gene sequencing revealed that Clostridiales were repeated colonizers in plaque associated with high oral disease, providing evidence that the oral environment is somehow influencing the bacterial signature linked to disease.”

The term ‘influence’ was used instead of ‘correlation’ since the studies were not subjected to correlative statistics.
Text in the Conclusions section on page 15 has also been modified as follows to reflect the lack of understanding of the relationship between repeated colonizers and the oral environment:

“The breadth of the Clostridiales also allowed us to recognize that bacteria linked to oral disease were repeated colonizers of individuals with high disease, suggesting a functional relationship between the disease environment and these disease associated bacteria.”

6) In a similar vein, the last sentence of the Discussion (page 14) doesn’t make a lot of sense. Firstly, it is not clear whether the environment is selecting for the bacteria, or the bacteria are altering the environment and causing the disease. This would take more study. Secondly, what do the authors mean by “in situ evolution”? What do they mean by this and is this really an alternative possibility?

This sentence on page 14 has been modified, as indicated below, deleting the word ‘selection’ and defining in situ evolution occurs. The basis for this sentence is the fact that emerging pathogens are known originate in microbe rich environments, such as soil, intestines and presumably subgingival plaque, primarily through horizontal transfer of genetic elements.

“This finding is significant because it highlights the role of the oral environment (as opposed to in situ evolution due to horizontal gene transfer) in the generation of bacterial signatures associated with high oral disease in West Virginia.”