**Author's response to reviews**

**Title:** A molecular survey of S. mutans and P. gingivalis oral microbial burden in human saliva using Relative Endpoint Polymerase Chain Reaction (RE-PCR) within the population of a Nevada dental school revealed disparities among minorities.

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**Version:** 4 **Date:** 7 June 2012

**Author's response to reviews:** see over
Emilie Aime, BioMed Central Executive Editor

Re: MS: 1629430098694182
Research article - BMC Oral Health
A molecular survey of S. mutans and P. gingivalis oral microbial burden in human saliva using RE-PCR within the minority population of a Nevada dental school. Jay Erickson Davis, Nicholas Freel, Allison Findley, Keaton Tomlin, Katherine M Howard, Clifford C Seran, Patricia Cruz and Karl Kingsley

Enclosed, please find our revised manuscript entitled, A molecular survey of S. mutans and P. gingivalis oral microbial burden in human saliva using RE-PCR within the minority population of a Nevada dental school. This manuscript has been extensively and carefully revised based upon the specific comments of the reviewers. We believe that this manuscript is an innovative, important research study of particular interest to the readership of the BMC Oral Health. Detailed below is a response to each reviewer comment and suggestion:

Reviewer 1: General comments
The purpose of this study was to screen adult patients who are interested in orthodontic treatment for the presence/absence of two types of oral putative pathogen one specific for carries and other one specific for periodontal disease. Authors hypothesized that they can differentiate their study group based on gender and ethnicity for salivary presence of specific pathogen. Stimulated saliva was collected and bacterial DNA was extracted. Presence of specific bacteria was studied by using relative endpoint polymerase chain reaction. Subjects were divided into subgroups based on high/moderate/low expression (density of band on gel). They concluded that P.gingivalis as putative periodontal pathogen was highly expressed among minority patients. Similar result could not be detected for S.mutans.

Reviewer 1 – Comment 1
Inclusion/exclusion criteria... Were these subjects already in orthodontic treatment? or were they screened for orthodontic treatment? The authors concur that the subjects in the sample were not adequately described. Due to the retrospective nature of the saliva samples collected, the Methods section was revised to reflect that both General and Orthodontic (but not Pediatric) clinic patients were sampled, as follows:

Methods: Human Subjects
Briefly, subjects in this convenience sample were recruited in the Patient Waiting Area/Lobby by members of the UNLV-SDM Clinic during their dental visit on one of 15 clinic dates. Informed consent was required and was conducted onsite. Inclusion criteria: subjects had to be 18 years old or older and had to agree to participate. Subjects younger than 18 years of age, subjects that declined to participate, and subjects with prior diagnosis of oral cancer were excluded. The
Patient Waiting Area/Lobby is used for the UNLV-SDM General Patient, Orthodontic, and Pediatric clinics, therefore the sample would contain patients from both the General and Orthodontic patient clinics, although the exclusion of patients under 18 eliminated any Pediatric patients from participation in this study.

**Reviewer 1 – Comment 2**

**Why stimulated saliva?** The saliva collection protocol for the original pilot study (Turner et al., 2011 BMC Oral Health) was designed to allow for an adequate aliquot of material to work with and to closely match the saliva collection protocols used in similar studies. The Methods section has been revised to reflect this information, as follows:

*Methods: Saliva Collection Protocol*

In brief, healthy adults who agreed to participate were given a sterile 50 mL sterile polypropylene tube obtained from Fisher Scientific (Fair Lawn, NJ). Participants were then asked to chew on a small piece of paraffin wax for one minute and then to expectorate; Samples were stored on ice until transported to the laboratory for analysis, as previously described [Turner et al., 2011 BMC Oral Health].


**Reviewer 1 – Comments 3 and 4**

No information is given on patients' oral/periodontal health conditions. This should be included. Any smokers within this study group....Any systemic health issues that may affect oral/periodontal health? This study was a retrospective analysis of existing saliva samples, collected from UNLV-SDM patients (General and Orthodontic clinic). The authors concur that this should be more clearly explained, which may provide the reviewer and readers of *BMC Oral Health* with an explanation of why it was not possible to include any demographic information about smoking or other systemic health issues within this patient sample. The modification of the Methods and Discussion sections now reads:

*Methods: Human Subjects*

The current study is a retrospective examination of existing saliva samples. The protocol for this study was approved by the UNLV Office of Research Integrity – Human Subjects (OPRS#1104-3801M) on April 25, 2011. Saliva samples were originally collected under a separate protocol, approved by the UNLV Office of Research Integrity – Human Subjects (OPRS#1002-3361) on April 9, 2010.

*Discussion*

Finally, and most importantly, the retrospective nature of this limited pilot study did not allow for other demographic information about smoking habits, systemic...
health issues, or oral disease risk to be collected, which may provide more information and additional insights in future studies of this population.

Reviewer 1 – Comment 5
What was the reason for RE-PCR instead of regular PCR or RT-PCR? Also what was the reason to create a standard by using human gingival fibroblast? I understand that this is necessary for RE-PCR but if the goal is to create a simple bacterial screening tool, RT-PCR should be sufficient to detect very small amount. The authors concur with the comments of this reviewer that the use of real-time PCR would be the most applicable and also most sensitive method for this type of screening. Unfortunately, due to the constraints of the laboratory, this equipment (and therefore this type of analysis) was not available to the study authors. A previous collaboration with the medical school allowed the use of real time PCR in a previous study, but the timing of this project and the goals of screening for oral pathogens, did not allow for this type of collaboration. The study authors had to work with the equipment and supply constraints, which allowed for the use of relative-endpoint PCR, as explained in the manuscript.

Reviewer 1 – Comment 6
Result of this study is not very surprising since higher risk of chronic and aggressive periodontitis in african americans is well documented. So the significance of this study should be discussed better. The authors concur with this comment and have revised the Discussion section to more accurately reflect the importance and context of these findings, as follows:

Discussion
Although periodontitis in US minorities, most notably African Americans, have been well documented, fewer studies have focused on Hispanics. The results of this study in Nevada, therefore, are particularly important to consider in Nevada, where recent estimates suggest that more than one-third of all state residents are minority and the vast majority of those (~80%) are Hispanic.
Reviewer 2 – Comment 1
The use of RE-PCR in the title (line 2) is misleading – do not use the initials RE but rather spell out completely Relative Estimation or Relative Quantitation. The authors concur with this comment and have revised the title accordingly, as follows:

A molecular survey of S. mutans and P. gingivalis oral microbial burden in human saliva using Relative Endpoint Polymerase Chain Reaction (RE-PCR) within the population of a Nevada dental school revealed disparities among minorities.

Reviewer 2 – Comment 2
The major concern for this article is the demographics of the study population. Although much time is spent on the proportions of male to female, whites or non-whites according to test group or general population of the school, many important factors are not presented. Minorities are all grouped together as if they can be considered a homogenous group, but are they Hispanic minorities, African-Americans, Asians, etc. Possibly of greater importance is the huge age range of 18 to 64yrs. with no further info. The clinical situation of the patients, both present and past disease, levels of oral hygiene, etc. are of utmost importance since comparisons are made and conclusions are drawn, but all of these may be based on erroneous assumptions. The authors must better define the groups and examine all differences that may have an impact on the ‘microbial burden’. The authors concur with the comments of this reviewer and have expanded and delineated the data analysis to include more specific information regarding ethnicity (separating Black, Hispanic, and Asian/Other), as well as providing more detailed information about age groups (Table 1). In addition, this study was a retrospective analysis of existing saliva samples, collected from UNLV-SDM patients (General and Orthodontic clinic). The authors concur that this should be more clearly explained, which may provide the reviewer and readers of BMC Oral Health with an explanation of why it was not possible to include any demographic information about smoking or other systemic health issues within this patient sample (Similar to Reviewer 1- Comment 3. The modification of the Methods and Discussion sections now reads:

Table 1. Demographic analysis of salivary samples

<table>
<thead>
<tr>
<th>Variables</th>
<th>Orthodontic clinic</th>
<th>Saliva samples</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Female</td>
<td>n = 376 (61.3%)</td>
<td>n = 34 (60.1%)</td>
<td>$\chi^2 = 0.008$, d.f. =1</td>
</tr>
<tr>
<td>Male</td>
<td>n = 237 (38.7%)</td>
<td>n = 22 (39.2%)</td>
<td>$p = 0.9271$</td>
</tr>
<tr>
<td><strong>Race</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>n = 215 (35.1%)</td>
<td>n = 20 (35.7%)</td>
<td>$\chi^2 = 0.009$, d.f. = 1</td>
</tr>
<tr>
<td>Non-White</td>
<td>n = 398 (64.9%)</td>
<td>n = 36 (64.3%)</td>
<td>$p = 0.9234$</td>
</tr>
<tr>
<td>Hispanic</td>
<td>n = 331 (53.9%)</td>
<td>n = 30 (55.5%)</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>n = 60 (9.8%)</td>
<td>n = 5 (8.9%)</td>
<td></td>
</tr>
<tr>
<td>Asian/Other</td>
<td>n = 8 (1.3%)</td>
<td>n = 1 (1.8%)</td>
<td></td>
</tr>
</tbody>
</table>
### Age

| Age       | n = 426 (34.7%) | n = 0 (0.0%) | \( \chi^2 = 29.142, \text{ d.f.} = 1 \)  
<table>
<thead>
<tr>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>&lt;18</td>
<td></td>
<td></td>
<td>( p &lt; 0.0001 )</td>
</tr>
<tr>
<td>18 - 64</td>
<td>n = 800 (65.3%)</td>
<td>n = 56 (100.0%)</td>
<td></td>
</tr>
<tr>
<td>18-24</td>
<td>n = 159 (19.9%)</td>
<td>n = 13 (23.2%)</td>
<td></td>
</tr>
<tr>
<td>25-34</td>
<td>n = 283 (35.4%)</td>
<td>n = 23 (41.1%)</td>
<td></td>
</tr>
<tr>
<td>35-44</td>
<td>n = 257 (32.1%)</td>
<td>n = 17 (30.4%)</td>
<td></td>
</tr>
<tr>
<td>45-54</td>
<td>n = 101 (12.5%)</td>
<td>n = 3 (5.4%)</td>
<td></td>
</tr>
</tbody>
</table>

### Results

Similarly, there was approximately the same percentage of White (n = 20 or 37%) and Minority (n = 34 or 63%) patients in the study sample compared with Whites (n = 215 or 35.1%) and Minorities (n = 398 or 64.9%) in the overall Orthodontic clinic population (\( \chi^2 = 0.838, \text{ d.f.} = 1, p = 0.7722 \)). The overwhelming majority of non-White Minorities were Hispanic (n = 30/34 or 88.2%), which was similar to the overall percentage of Hispanics in the Orthodontic clinic population (n = 331/398 or 83.2%). However, because only saliva from adult patients was collected, there were no samples from patients under 18 years old in the study sample (n = 0 or 0%), which was statistically different from the ratio within the overall clinic (n = 426 or 34.7%) (\( \chi^2 < 0.0001 \)). Furthermore, the analysis of adults patients between 18 – 64 within age ranges revealed that approximately two thirds of the adults were either 25 – 34 or 35-44 (35.4% + 32.1% or 67.5%), which was similar to the distribution within the sample population (42.65% + 31.5% or 74.1% respectively).

### Results – S. mutans

Demographic analysis using chi-square revealed that the percentage of SM-positive samples from females (n = 7 or 53.8%) and males (n = 6 or 46.2%) was not significantly different (\( X^2 = 0.126, \text{ d.f.} = 1; p = 0.7224 \)) than their respective percentages in the overall sample (59.6 and 40.4%, respectively). Similarly, the percentages of SM-positive samples from Whites (n = 3 or 23.1%) and Minorities (n = 10 or 79.6%) was also not significantly different (\( X^2 = 0.906, \text{ d.f.} = 1; p = 0.3412 \)) from the overall sample (35.1% and 64.9%, respectively). In addition, the ages of SM-positive patients were not found to significantly different than those of the study sample (\( p=0.2784 \)).

### Results – P. gingivalis

Standards of genomic DNA extracted from PG samples containing 5.0 x 10^3 - 10^6 CFU/mL were used to establish detection threshold and saturation (\( C_T \) and \( C_S \)) cycle limits (Figure 3A). For the DNA from samples with the highest CFU/mL concentrations of PG (5.0 x 10^6 CFU/mL), \( C_T \) was observed at C15 and \( C_S \) at C35, similar to the results with the SM standards. \( C_T \) was established at approximately 20, 25 and 30 for each successful sample dilution (10^5, 10^4, and 10^3 CFU/mL, respectively), with \( C_S \) at correspondingly higher cycles (~C45 – C55). The previously established GAPDH EP cycle C30 was therefore found to be at \( C_T \).
for DNA samples with CFU/mL concentrations in the lowest category ($C_T = C30$), and above the $C_T$ for DNA samples from the higher categories ($C_T = C15 – 25$), as well as being below the upper limit for the highest concentration, $C_S = C35$). In addition, the ages of PG-positive patients were not found to significantly different than those of the study sample ($p=0.05$).

**Discussion**

Although periodontitis in US minorities, most notably African Americans, have been well documented, fewer studies have focused on Hispanics\(^{39}\). The results of this study in Nevada, therefore, are particularly important to consider in Nevada, where recent estimates suggest that more than one-third of all state residents are minority and the vast majority of those (~80%) are Hispanic\(^ {40}\).

**Discussion**

Finally, and most importantly, the retrospective nature of this limited pilot study did not allow for other demographic information about smoking habits, systemic health issues, or oral disease risk to be collected, which may provide more information and additional insights in future studies of this population.

**Reviewer 2 – Comment 3**

*The sample size determination as written in lines 101 to 103 is not clear. The authors could rather just use the determination as outlined in the Statistical Evaluation section (ln. 202). The double mention of sample size determination causes confusion.* The authors concur with the comments of this reviewer and have revised the second section to provide clarity that this section, in fact, refers specifically to the power calculation and not the sample size. The revised section now reads:

**Methods - Statistical evaluation and power calculation**

Data were analyzed and basic descriptive statistics, which included concentration averages, Pearson’s correlation ($r$) and coefficient of determination ($R^2$), were graphed using Microsoft Excel (Redmond, WA). The demographic comparisons, as well as the differences between the population sub-groups (Males, Females, Whites, Minorities) were measured using chi-square ($\chi^2$) test. A probability level of alpha ($\alpha = 0.05$) and two-tailed $p$-values were used to determine statistical significance.\(^ {21}\) As demonstrated in previous clinical studies of oral bacteria and orthodontic appliances, a mean CFU difference of approximately one log [standard deviation (SD) = approximately 1] will result in a clinically significant increase in oral bacterial species (SM) counts and disease (caries) risk. Based upon these data, the current sample size of 22 patients per group, would yield a statistical power > 0.8 for this study ($\alpha = 0.05$).\(^ {33-35}\)

**Reviewer 2 – Comment 4**

*Concerning the methodology used for DNA extraction, a couple problems arise. The GenomicPrep DNA isolation kit is suitable, according to the manufacturer’s sheet, for*
isolation of DNA from Gram negative bacteria but no mention is made for G+ bacteria such as S. mutans. Concerning the manufacturer, GE Healthcare seems to have taken over the original company (thus information mentioned in manuscript is wrong) and that in 2007 this product was replaced. (Moreover, the references 23-26 used to corroborate the methodology are concerning eukaryotic cells and not bacteria. These questions raise concerns over the overall reliability of the methods, which the authors must clarify. The authors concur with the comments of this reviewer that the kit is no longer sold by the original company; however, as mentioned previously - due to the constraints of the laboratory, the study authors had to work with the equipment and supplies which were already available and present at the time this project was proposed; The original kit was purchased from the aforementioned company and therefore maintain this should remain, with the caveat this is now available through GE Healthcare. In addition, GE Healthcare Life Sciences now advertises that this product, now sold as GenicPrep DNA isolation kit, is approved for isolating high quality DNA from both Gram-negative bacteria and Gram-positive bacteria, such as S. mutans.

Methods
DNA was isolated from each saliva sample using a standard volume, containing a minimum of 3.5 x 10^5 cells, using the GenomicPrep DNA isolation kit (Amersham Biosciences: Buckinghamshire, UK; now GE Healthcare), following the procedure recommended by the manufacturer as previously described.23-25

Reviewer 2 – Comment 5
Why fibroblast cell-lines are used, which are clearly used to enumerate the exfoliated cells found in the saliva, is never explained neither in the introduction or the discussion. It is something completely outside the scope of the title of the paper and proposed aim and as such does not offer the reader anything. In this regard the value of table 2 is unclear and should be removed. The authors have reviewed this comment and have revised the Results section to clearly direct the reader to the importance for using a control to determine cell number (and therefore RE-PCR band intensity) for comparisons of cell number and sample concentration. This now reads:

Results
DNA standards obtained from standardized control cells, human gingival fibroblasts (0.5 – 2.5 x 10^6 cells/mL), approximating the range of cell concentrations observed in the saliva samples (0.8 – 2.4 x 10^6 cells/mL) were used to establish the minimum threshold (C_T) and saturation (C_S) cycles required for calibration and concentration comparisons using relative endpoint PCR (Figure 1A).

Reviewer 2 – Comment 6
The culture methodology for the bacterial reference strains is incomplete and cursory, i.e. ‘thawed, streaked, and cultured on their respective agar plates from Difco (Sparks, MD) according to the protocol recommended by the supplier.’ No information on
culture conditions, length of incubation, etc. Basic information must be provided. The authors concur with the comments of this reviewer, and have revised the methods to reflect the basic information requested, as follows:

Methods
The oral bacteria cell lines Streptococcus mutans (S. mutans or SM) 25175 (NCTC-10449) and Porphyromonas gingivalis (P. gingivalis or PG) BAA-1702 (FDC-381) were also obtained from ATCC. In brief, cells were thawed, streaked, and cultured on their respective agar plates from Difco (Sparks, MD) according to the protocol recommended by the supplier. In brief, bacteria were plated and grown at 37 °C overnight on Trypticase soy agar; SM plates were supplemented with 5% defibrinated sheep’s blood and PG were supplemented with 1% yeast extract on from Difco (Sparks, MD). Single plate colonies were then inoculated into liquid broth; Trypticase soy broth for SM and supplemented tryptic soy broth for PG from Difco overnight at 37 °C. Aliquots of bacterial cell suspensions were then used to inoculate growth standards.

Reviewer 2 – Comment 7
Reference concentrations for P. gingivalis for disease risk were never presented, but rather seem to be based on levels determined for S. mutans. In figure 3 the legend (lines 562-563) mentions risk, “DNA standards revealed samples with moderate to very high concentrations; Very high (n = 4), high risk (n = 4), moderate (n = 2).” Citations for this determination are needed. The authors concur with the comments of this reviewer and recognize that no previously determined standards exist; therefore, the Results and Discussion sections have been modified to reflect that the RE-PCR results for PG are only being reported in categories (very high, high, moderate) using the SM results as a standard, but are not to be used to delineate PG into specific “risk” categories, as follows:

Results
RE-PCR using PG primers at C30 also resulted in a strong, positive curvilinear correlation (R² = 0.8507) between band intensity and DNA standards from PG samples with increasing CFU/mL concentrations (Figure 3B). Using these parameters, screening of the saliva samples revealed a modest, but significant, percentage of these samples (n = 10/56 or 17.8%) were found to harbor PG levels corresponding to the moderate, up to the high, range – although the majority of samples were below the limit of detection (n = 46/56 or 78.2%) and therefore representative of average or below average CFU/mL concentrations (Figure 2C). More specifically, plotting the PG-positive band intensities alongside the DNA standards suggests that four samples corresponded with very high concentrations, four to high, and the remaining two to moderate CFU/mL concentrations, although the lack of previously established standards does not allow for these results may not be categorized into high and moderate risk categories (Figure 3B).

Discussion
Other research studies have demonstrated elevated PG levels ranging from 5 –
19%, which suggests the results of this study are among the highest yet reported. More specifically, the finding that a significant percentage (14.3%) of samples had high or very high levels of PG, most of whom were minorities, may suggest that many of these patients had underlying periodontal conditions that might be more readily exacerbated by orthodontic treatment and therapy. In addition, the finding that a similar percentage of samples were found to be at high or very high risk for caries disease (SM > 10^5 CFU/mL), may suggest a similar, but distinct, percentage of patients may require ancillary treatments, interventions, or additional oral health education in order to complete orthodontic treatment. However, care must taken when interpreting these results, as there have been no previously established PCR-based assessments of periodontal disease risk corresponding with PG levels, as there are for SM. These data are also consistent with previous studies, which found similar percentages of elevated SM levels ranging from 14 – 40%, which may result in complications involving oral infections and orthodontic treatments interruption.

**Reviewer 2 – Comment 8**

Concerning the conclusions, these must all be changed to reflect the findings of the study. For example, the fact that “Low-income and minority patients are increasingly seeking orthodontic treatment” may be true but not determined by the finding of the study just as the fact that ‘oral health literacy’ may be different. The authors concur that the conclusions must be determined by the findings of this study and have therefore removed three sections, which relate specifically to the Discussion and context of the results (Low-income and minority patients are increasingly seeking orthodontic treatment; Oral health literacy may be different among these patient populations; Appropriate care and oral health education may be needed for similar patient populations). The revised conclusions now read:

**Conclusions**

- UNLV-SDM treats a racially and ethnically diverse patient population
- Nearly 25% of UNLV-SDM patients screened had elevated risk of caries (S. mutans)
- Almost 20% of these patient had elevated *P. gingivalis*, levels
- Virtually all of these patients were Minorities (90%)

**Reviewer 2 – Comment 9**

The authors must be careful when discussing the literature to differentiate the findings based on saliva samples and those from plaque, either super- or sub-gingival. The authors concur that the Discussion section did not differentiate the findings based on saliva or plaque collection, and have revised the results to more clearly outline that some studies may involve saliva, others plaque, and sometimes both. The revised Discussion now reads:
Discussion
These data are also consistent with previous studies, which found similar percentages of elevated SM levels ranging from 14 – 40% in both saliva and plaque, which may result in complications involving oral infections and orthodontic treatments interruption.5-9
Reviewers' Comments and Revisions

**Reviewer 3 – Comment 1**

The RE-PCR methods used in this study seem to be appropriate for detection of *P. gingivalis*, which is considered to be difficult to isolate and quantify. On the other hands, the detection rates of *S. mutans* using those methods were quite low. Thus, it is possible to speculate that detection of *S. mutans* isolated on *Mitis-Salivarius* agar might be more sensitive than that with RE-PCR. In other words, it is easier to isolate *S. mutans* as compared to *P. gingivalis*. Therefore, the authors should describe the specific advantages of RE-PCR to strengthen the objective of the present study, especially for detection of *S. mutans*. The authors concur with the comments of this reviewer and have modified the Discussion section to more accurately reflect this specific advantage, as follows:

**Discussion**

However, the ability of this study to detect log-scale significant differences in CFU/mL between samples, provides strong evidence for sufficient statistical power to make broader inferences, which significantly mitigates any limitations based upon sample size.\(^{33-35}\) In addition, the use of relative-endpoint (RE) PCR to provide quantitative comparative data has been successfully used in many previous study, which may suggest this method may be particularly appropriate to assess salivary microbial burden when more resource-intensive equipment and facilities for real-time PCR are not available; removing\(^ {23-25}\) the barriers regarding the difficulty of both isolation and culture of PG, which might otherwise complicate studies examining oral microbial concentrations.\(^ {4,5}\)

**Reviewer 3 – Comment 2**

The authors focused on analyses of patients who visited the orthodontic clinic. This reviewer is concerned about differences in bacterial species between orthodontic and non-orthodontic patients. The authors should justify the subjects selected for analysis in the present study. We concur with this comment regarding patient selection, similar to Reviewer 1 – Comment 1, that the subjects in the sample were not adequately described. Due to the retrospective nature of the saliva samples collected, the Methods section was revised to reflect that both General and Orthodontic (but not Pediatric) clinic patients were sampled, as follows:

**Methods: Human Subjects**

Briefly, subjects in this convenience sample were recruited in the Patient Waiting Area/Lobby by members of the UNLV-SDM Clinic during their dental visit on one of 15 clinic dates. Informed consent was required and was conducted onsite. Inclusion criteria: subjects had to be 18 years old or older and had to agree to participate. Subjects younger than 18 years of age, subjects that declined to participate, and subjects with prior diagnosis of oral cancer were excluded. The Patient Waiting Area/Lobby is used for the UNLV-SDM General Patient, Orthodontic, and Pediatric clinics, therefore the sample would contain patients...
from both the General and Orthodontic patient clinics, although the exclusion of patients under 18 eliminated any Pediatric patients from participation in this study.

Reviewer 3 – Comment 3
Why did the authors use saliva samples only containing a minimum of 3.5x10^5 cells? When considering the sensitive methods like PCR used in the present study, those amounts were quite large, as detection is possible with much smaller samples. The authors should clarify this point. The authors concur that this specific detail was not previously included in the Methods, and have revised the text to more accurately describe this recommendation by the manufacturer, as follows:

Methods
DNA was isolated from each saliva sample using a standard volume, containing the recommended protocol minimum of 3.5 x 10^5 cells, using the GenomicPrep DNA isolation kit (Amersham Biosciences: Buckinghamshire, UK; now GE Healthcare), following the procedure recommended by the manufacturer as previously described.23-25

Reviewer 3 – Comments 4 and 5
The authors should include more explanations relevant references regarding the RE-PCR methods as that is likely to be unfamiliar to most of the readers of the journal. The authors describe clinical aspects in most parts of the Discussion section. However, that section should focus on the results obtained in the present study, especially with the RE-PCR methods employed. In addition, the advantages and weak points of RE-PCR should noted, as those would be beneficial for readers who are unfamiliar with the technique. The authors concur with the comments of this reviewer and have highlighted some specific studies, completed by this group, that have utilized RE-PCR for comparison and quantitation (although not for bacteria, specifically). The revised Discussion section now reads:

Discussion
However, the ability of this study to detect log-scale significant differences in CFU/mL between samples, provides strong evidence for sufficient statistical power to make broader inferences, which significantly mitigates any limitations based upon sample size.33-35 In addition, the use of relative-endpoint (RE) PCR to provide quantitative comparative data has been successfully used in many previous study, which may suggest this method may be particularly appropriate to assess salivary microbial burden when more resource-intensive equipment and facilities for real-time PCR are not available; removing the barriers regarding the difficulty of both isolation and culture of PG, which might otherwise complicate studies examining oral microbial concentrations.4,5
Reviewer 3 – Comment 6
There too many subheadings in the Methods section. Those could be merged in the revised version for the readers to better understand the entire Methods section. The authors concur with the comments of this reviewer and have combined the following four sections into two sections, thereby combining the most related material for the ease of the reader:

- Human subjects and Saliva Collection protocol
- Sample size and Statistical evaluation, and power calculation

Reviewer 3 – Comment 6
(Results) Lines 215-254: A subtitle is needed for this portion. In addition, most of the information described here should be presented in the Methods section. The authors have added a subtitle “demographic analysis”, as requested by this reviewer. However, the remainder of the information presented are results of the demographic analysis and are therefore not “methods” but findings – based upon this information, the authors have decided to retain these results in their current location.

Demographic analysis
Fifty six (56) saliva samples, collected from UNLV-SDM patients between June and October 2010, were selected at random for this study. Demographic analysis revealed this sample was not statistically different from the demographic composition of the orthodontic clinic patient population with respect to gender or race (Table 1).

Reviewer 3 – Comment 7
Line 360: The word “addition” in this sentence should be written as “additional”. The authors apologize for this oversight and have made this correction as requested:

Discussion
However, an additional limitation may be that the sample population of this study consisted solely of adult patients, which does not provide any information regarding the adolescent orthodontic population (<18), although these younger populations have been the focus of intense study in previous research efforts because they have been the more traditional orthodontic patients until very recently.38

Reviewer 3 – Comment 8
Table 1: The total number of subjects may be incorrect. Please check again the number of subjects in each group. The authors apologize for this oversight and have corrected Table 1 as follows:
Table 1. Demographic analysis of salivary samples

<table>
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<td>n = 237 (38.7%)</td>
<td>n = 22 (39.2%)</td>
<td>$p = 0.9271$</td>
</tr>
<tr>
<td><strong>Race</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>n = 215 (35.1%)</td>
<td>n = 20 (35.7%)</td>
<td>$\chi^2 = 0.009$, d.f. = 1</td>
</tr>
<tr>
<td>Non-White</td>
<td>n = 398 (64.9%)</td>
<td>n = 36 (64.3%)</td>
<td>$p = 0.9234$</td>
</tr>
<tr>
<td>Hispanic</td>
<td>n = 331 (53.9%)</td>
<td>n = 30 (55.5%)</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>n = 60 (9.8%)</td>
<td>n = 5 (8.9%)</td>
<td></td>
</tr>
<tr>
<td>Asian/Other</td>
<td>n = 8 (1.3%)</td>
<td>n = 1 (1.8%)</td>
<td></td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;18</td>
<td>n = 426 (34.7%)</td>
<td>n = 0 (0.0%)</td>
<td>$\chi^2 = 29.142$, d.f. = 1</td>
</tr>
<tr>
<td>18-64 years</td>
<td>n = 800 (65.3%)</td>
<td>n = 56 (100.0%)</td>
<td>$p &lt; 0.0001$</td>
</tr>
<tr>
<td>18-24</td>
<td>n = 159 (19.9%)</td>
<td>n = 13 (23.2%)</td>
<td></td>
</tr>
<tr>
<td>25-34</td>
<td>n = 283 (35.4%)</td>
<td>n = 23 (41.1%)</td>
<td></td>
</tr>
<tr>
<td>35-44</td>
<td>n = 257 (32.1%)</td>
<td>n = 17 (30.4%)</td>
<td></td>
</tr>
<tr>
<td>45-54</td>
<td>n = 101 (12.5%)</td>
<td>n = 3 (5.4%)</td>
<td></td>
</tr>
</tbody>
</table>

**Reviewer 3 – Comment 9**

*Table 2: The phrase samples (n) should be changed to samples n=52.* The authors apologize for this error and have corrected Table 2 to include all samples, n=56, as follows:

Table 2. Cell enumeration and DNA concentrations of saliva samples

<table>
<thead>
<tr>
<th>Cell count (cells/mL)</th>
<th>Average DNA concentration (ng/μL)</th>
<th>Samples (n=56)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 – 2.4 x 10⁶</td>
<td>886.47 +/- 167.9</td>
<td>20</td>
</tr>
<tr>
<td>1.6- 1.9 x 10⁶</td>
<td>814.89 +/- 137.6</td>
<td>20</td>
</tr>
<tr>
<td>0.8- 1.2 x 10⁶</td>
<td>843.94 +/- 138.2</td>
<td>16</td>
</tr>
</tbody>
</table>

**Reviewer 3 – Comment 10**

*The reviewer wonders whether there was any correlation between detection of P. gingivalis and S. mutans and subject age.* The authors concur with the comments of this reviewer that more information about the subject age should have been included in the results section of the manuscript (similar to Reviewer 2 Comment 2) and have included this analysis in the revised manuscript, as follows:
Results – S. mutans
Demographic analysis using chi-square revealed that the percentage of SM-positive samples from females (n = 7 or 53.8%) and males (n = 6 or 46.2%) was not significantly different ($X^2 = 0.126$, d.f. = 1; $p = 0.7224$) than their respective percentages in the overall sample (59.6 and 40.4%, respectively). Similarly, the percentages of SM-positive samples from Whites (n = 3 or 23.1%) and Minorities (n = 10 or 79.6%) was also not significantly different ($X^2 = 0.906$, d.f. = 1; $p = 0.3412$) from the overall sample (35.1% and 64.9%, respectively). In addition, the ages of SM-positive patients were not found to significantly different than those of the study sample ($p=0.2784$).

Results – P. gingivalis
Standards of genomic DNA extracted from PG samples containing $5.0 \times 10^3$ - $10^6$ CFU/mL were used to establish detection threshold and saturation ($C_T$ and $C_S$) cycle limits (Figure 3A). For the DNA from samples with the highest CFU/mL concentrations of PG ($5.0 \times 10^6$ CFU/mL), $C_T$ was observed at C15 and $C_S$ at C35, similar to the results with the SM standards. $C_T$ was established at approximately 20, 25 and 30 for each successful sample dilution ($10^5$, $10^6$, and $10^3$ CFU/mL, respectively), with $C_S$ at correspondingly higher cycles (~C45 – C55). The previously established GAPDH EP cycle C30 was therefore found to be at $C_T$ for DNA samples with CFU/mL concentrations in the lowest category ($C_T = C30$), and above the $C_T$ for DNA samples from the higher categories ($C_T = C15$ – 25), as well as being below the upper limit for the highest concentration, $C_S = C35$. In addition, the ages of PG-positive patients were not found to significantly different than those of the study sample ($p=0.05$).

In summary, comments provided by the editors and these reviewers were incorporated into the body of this manuscript, as appropriate. We have made every attempt to incorporate all of the reviewer comments and believe that these revisions adequately address the concerns of each reviewer. We would like to thank the editors and reviewers for their thoughtful consideration of this manuscript and strongly believe that this manuscript, as a result of their input and suggestions, is considerably strengthened and is of great scientific interest to the readers of the BMC Oral Health. We thank the editors of this journal for their patience and consideration during the process of our revisions.

Respectfully submitted,

Karl Kingsley, PhD, MPH
Associate Professor of Biomedical Sciences