Author's response to reviews

Title: High-risk human papillomavirus (HPV) screening and detection in healthy patient saliva samples: a pilot study.

Authors:

Deidre O Turner (deidreoturner@yahoo.com)
Shelley J Williams-Cocks (scocks@medicine.nevada.edu)
Ryan Bullen (ryan.bullen@sdm.unlv.edu)
Jeremy Catmull (jeremy.catmull@sdm.unlv.edu)
Jesse Falk (jesse.falk@sdm.unlv.edu)
Danny Martin (daniel.martin@sdm.unlv.edu)
Jarom Mauer (jarom.mauer@sdm.unlv.edu)
Annabel E Barber (barber@medicine.nevada.edu)
Shawn L Gerstenberger (shawn.gerstenberger@unlv.edu)
Karl Kingsley (karl.kingsley@unlv.edu)

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Author's response to reviews: see over
Enclosed, please find our manuscript entitled, *High-risk human papillomavirus (HPV) screening and detection in healthy patient saliva samples: a pilot study*. This manuscript has been extensively revised based upon the comments of the reviewers. We believe that this manuscript is an innovative, important research study of particular interest to the readership of *BMC Oral Health*. Detailed below is a response to each reviewer comment and suggestion:

**Reviewer 1**

*General comments:* The manuscript by Turner et al. entitled ,,High-risk human papillomavirus (HPV) screening and detection in normal, healthy patient saliva samples: a pilot cluster randomized study“ deals with the prevalence of HPV-16 in the saliva specimens of healthy adults. Altogether 102 specimens of 151 enrolled subjects were evaluated by means of type-specific PCR for the presence of HPV-16. The specificity of the reaction was confirmed by quantitative real-time PCR with type-specific primers targeted to E6 region of HPV-16. The major aim was to assess HPV prevalence in healthy adults in Las Vegas, Nevada, USA. None-invasive method of saliva sampling has been used. In this state recently increasing rate of oral cancer has been documented despite the decline in the rates of well-known risk factors – smoking and alcohol consumption. The authors claim this as a pilot study.

*Comment 1-1: The introduction is unnecessary long. It needs to be more focused.*

- We concur with the comments of this reviewer and have modified the text in the Background/Introduction section to reduce the overall length and provide more direct focus. We believe the targeted reduction in length, combined with the revised text addresses this specific concern.

   Paragraph two deleted:

   *More than one hundred types of HPV have been identified and classified. These HPV types may be the primary oncogenic or etiologic cause of cancer or are associated with other dermatologic disorders, including the development of warts [12,13]. The HPV strains determined to be oncogenic...*
have been classified as high-risk, with HPV16 and HPV18 the most prevalent - accounting for the overwhelming majority of all HPV-associated cancers [14]. Other HPV strains, more commonly associated with genital and anal warts, or other skin and epithelial disorders, have been classified as low risk. These include HPV types 6 and 11, among many others [15]. Although the majority of these HPV strains were originally identified in cervical lesions, more recent evidence has demonstrated their presence in other tissues including colorectal, penile, breast, lung, and oral tissues [16-27].

Paragraph four deleted:
The role of HPV in the oral cavity, however, may differ by anatomic site and also by the particular strain of HPV infection [35]. For example, low-risk HPV strains 6 and 11 have been identified in benign laryngeal papillomas, common warts (verruca vulgaris), and condyloma acuminatum [36-38]. These strains have also been found in uncommon cancers, such as Ackerman’s (verrucous carcinomas) [39] and Buschke-Lowenstein tumors [40]. Conversely, high-risk strains HPV-16, and to a lesser extent HPV-18, are found in nearly half of all oral squamous cell carcinomas and epithelial lesions [33,41,42].

Paragraph six partially deleted:
Despite these findings, rates of oral cancer in the United States (US) have been rising among some subgroups within the population, and in specific geographic areas [34,53-56]. The steady decrease in the number of current smokers in recent years, combined with an ever-increasing percentage of never smokers in the US [33], suggests that other risk factors are likely responsible, in part, for these observed increases.

Comment 1-2: would also suggest including studies done by using oral lavage as non-invasive method of specimen collection on healthy controls in the introduction (see Herrero et al., 2003; Smith et al., 2004; Koppi et al., 2005; Zhao et al., 2005; D’Souza et al., 2007; Tachezy et al., 2009).

• We concur with the comments of this reviewer and have modified the text to include these specific studies using oral lavage as a non-invasive method of specimen collection. The modified text is as follows:

Background (Page 5, Lines 95-97):
In addition, other studies have begun to report less invasive saliva and oral lavage-based testing methods to successfully screen for oral HPV among healthy adults, revealing prevalence rates between 2.8 to 25% [15,52-60].


Comment 1-3: Some parts of the introduction are quite confusing (e.g. The role of HPV in the oral cavity, however, may differ by anatomic site and also by the particular strain of HPV infection [35]. For example, low-risk HPV strains 6 and 11 have been identified in benign laryngeal papillomas, common warts (verruca vulgaris), and condyloma acuminatum [36-38]). It is important to distinguish studies by anatomical site. Laryngeal papilloma is not disorder of the oral cavity.

- We concur with the comments of this reviewer and have modified the text to reduce the overall length and provide more direct focus (Comment 1-1). We believe the removal of this discussion adequately addresses this specific concern.

Comment 1-4: In the sentence below the localization of tumors has to be specified (underlined). Recent epidemiologic and case-control studies have demonstrated that patients with HPV-positive tumors had significantly better response rates to chemotherapy and chemoradiation treatments when compared with HPV-negative tumors [28,46-48].

- We concur with the comments of this reviewer and have modified the text to more specifically identify these as oral cancers. The modified text is as follows:

Introduction (Page 4, Lines 78-82):

HPV may then subsequently function to modulate the malignancy process in developing or establish oral cancers, as has been observed in studies of HPV infection in other developing cancers [29-39]. For example, recent epidemiologic and case-control studies have demonstrated that patients with HPV-positive oral tumors had significantly improved survival rates [12,40,41] and therapeutic response rates when compared with HPV-negative controls [42].

Comment 1-5: Methods: No information of controls of eventual carry over contamination during the extraction of DNA as well as incorporation of positive and negative controls for PCR reactions are provided. The region which targets primers used for qualitative PCR is not specified. For quantitative PCR information about the primers and method in general is missing. Also, no information of construction of a calibration curve for HPV-16 quantification is provided.

- We concur with the comments of this reviewer and have modified the text to provide more specific information about the qPCR procedures, controls, primers, and method. The modified text is as follows:

Methods (Pages 8-10, Lines 171-205):

Quantitative PCR (qPCR)

DNA samples were then processed using qPCR to provide more specific and sensitive quantification. Primers and probes were designed using Roche
Universal Probe library (UPL) assay design software to amplify the region overlapping E6 and E7 gene sequence of HPV16 (GenBank accession no. K02718) and the human β-actin housekeeping gene (GenBank accession no. M10277). All primers were purchased from Sigma-Aldrich (St. Louis, MO.) and probes purchased from Roche Applied Science (Indianapolis, IN.)

HPV16 E6/E7 forward primer 5’-CAACTGATCTCTACTGTATGAGCAA-3’, HPV16 E6/E7 reverse primer 5’-CCAGCTGGACCATTCTATTTCA-3’, HPV16 E6/E7 hydrolysis “Taqman” probe 5’-(fam)-AGGAGGAG-(dark quencher dye)-3’ (UPL probe #63) was used to amplify the 73 base pair (bp) region between the 535 nucelotide (nt) position and 607 nt position. Human β-actin forward primer 5’-GTGGGGGTCTCTGTGGTGTG-3’, human β-actin 5’-GAAGGGGACAGGCAGTGA-3’, human β-actin hydrolysis “Taqman” probe 5’-(fam)-GGGAGCTG-(dark quencher dye)-3’ (UPL probe #24) amplified the 61 bp region between 2642 nt position and 2702 nt position.

The real-time reaction mixture was prepared in a LightCycler® 480 multiwell Plate 96 containing 1x LightCycler® 480 Probes Master (Roche Applied Sciences), 1 µM of each respective primer set (forward and reverse), 0.2 µM of respective probe, and 2 µl of DNA template; in a 20 µl final reaction volume. The probes master mix contained reaction buffer, dNTP mix (including dUTP in place of dTTP), 3.2 mM MgCl2, and Taq DNA polymerase. The real-time PCR assay was performed on a LightCycler 480 system (Roche Applied Science) with the following cycle parameters: pre-incubation for initial enzyme activation at 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 10 seconds (ramp rate 4.4 °C/second), 60 °C for 30 seconds (ramp rate 2.2 °C/second) and 72 °C for 1 second (ramp rate 4.4 °C/second). Following amplification phase, a cooling step was performed at 40 °C for 30 seconds (ramp rate of 2.2 °C/ second). Acquisition of the fluorescence signal was performed using Mono Hydrolysis Probe setting (465-510 nm) following the 72 °C extension phase of each cycle. All samples were carried out in triplicate.

The CaSki (American Type Culture Collection; Manassas, VA) cervical adenocarcinoma cell line was used to develop standard curves for both the HPV16 (600 copies/genome) and β-actin (2 copies/genome) genes. DNA extracted from CaSki cells were serially diluted tenfold starting at 50 ng to 0.0005 ng [65] Quantification was achieved using Cycle Threshold (C_T) measured with the second derivative maximum method (LightCycler 480 Software version 1.5.0.39; Roche Applied Science). Saliva samples > 0.001 copy/genome were considered HPV positive. Specificity analysis was performed on qPCR assay against HPV18 and found to be 100% specific (data not shown).
Comment 1-6: *Results: Expression of statistical significance is rather unusual (p < 0.1, p > 0.05), either exact value or information - significant or not significant, should be used.*

- We concur with the comments of this reviewer and have modified the results to include the exact p-values and significance. The modified text from the Results section is as follows:

**Results (Pages 10-11, Lines 217-223):**

*The patients from whom samples were collected and screened were not statistically different from the overall UNLV-SDM clinic population with respect to gender, race, or age (Table 1). More specifically, the total number of females and males in the sample was roughly equal (52.3% and 47.7%, respectively) and not significantly different than the overall clinic population (p = 0.589). There were slightly more White patients in the study sample (48.3%) than in the overall UNLV-SDM population (40.8%) (p = 0.133). In addition, there were slightly fewer 18 – 64 year olds in the sample (80.8%) than in the overall clinic (85.3%) (p = 0.354).*

- In addition, Table 1 was modified to provide exact $\chi^2$ and p-values.

Comment 1-7: *The numbers if in the middle of sentence, can be expressed as numbers not by words, e.g. 102, or even better 102/151 specimens.*

- We concur with the comments of this reviewer and have modified the text to remove all word references to numbers greater than ten. We believe this adequately addresses this specific concern.

Comment 1-8: *The use of quantitative PCR for this study gives no additional value. Since primers for both types of PCR were targeted to E6 region, the qualitative PCR should detect also the integrated form of HPV-16 and therefore not increase the number of false negative samples.*

- The authors have spent considerable time reviewing this comment and have concluded that the inclusion of pPCR data provides two specific benefits for the scientific readers of this manuscript. First, it provides confirmation of the original screening, even though it will most likely confirm only the integrated form of HPV16. However, this also provides quantitation of relative copy number, which may be of specific importance when comparing results to other studies evaluating oral HPV infection, viral load and copy number/genome. The text has been modified to incorporate evidence that demonstrates this specific relevance, as follows:

**Background (Page 4, Lines 80-86):**
For example, recent epidemiologic and case-control studies have demonstrated that patients with HPV-positive oral tumors had significantly improved survival rates [12,40,41] and therapeutic response rates when compared with HPV-negative controls [42]. Several in vitro studies have recently investigated possible mechanisms that may account for these phenotypic changes in oral cancers [25-27]. Evidence is now accumulating that HPV infection of oral cancers correlates with increased survival rates and better prognosis among some patients due to these changes in cellular responsiveness [40-45].

Comment 1-9: Furthermore, only the positive samples and 10 negative samples on the qualitative PCR were rescreened by quantitative real time PCR. The later method is more sensitive and therefore in theory it can detect higher prevalence of HPV. The use of this method would make sense only if all samples are tested. However, as it is presented in this study it has no value. Also the information about the number of copies below the cutoff limit is redundant.

- We concur with the comments of this reviewer and have spent considerable time rescreening all samples by quantitative real time PCR. In addition, only a range of copy numbers was given, to avoid redundancy. The revised methods and results sections are as follows:

Methods (Page 8, Lines 172-173):
DNA samples were then processed using qPCR to provide more specific and sensitive quantification.

Results (Page 11, Lines 235-236):
DNA samples were then processed using quantitative qPCR to provide quantitative assessment, as well as measurement of sensitivity and specificity (Figure 2).

Results (Pages 11-12, Lines 239-242):
Analysis of qPCR results in copy number/genome for HPV revealed striking differences in copy numbers between HPV-negative (range: 0.0001 – 0.000004 copies/genome) and HPV-positive (range: 70 – 111), which were easily distinguished using the cutoff value (>0.001 copies/genome).

Comment 1-10: Since this study is aimed on assessment of HPV prevalence in the healthy population the method of detection of multiple HPV types or at least multiple HR HPV types would be more appropriate. In conclusion the presented data are truly pilot data. The HPV prevalence should be tested by a method which allows detection of multiple HPV types and is also used in some other studies to allow for comparison.
We concur with the comments of this reviewer and have spent considerable time rescreening all samples for the second most commonly detection high-risk oral HPV strain, HPV18. The revised methods and results sections are as follows:

Methods (Pages 7-8, Lines 149-159):
DNA from each sample was then used to perform PCR with the Fisher exACTGene complete PCR kit (Fisher Scientific: Fair Lawn, NJ) and a Mastercycler gradient thermocycler (Eppendorf: Hamburg, Germany) using the following primers for HPV16 [26,27], HPV18 [27,32], and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [64], synthesized by SeqWright (Houston, TX):
HPV16 forward primer, ATGTTTCAGGACCCACAGGA;
HPV16 reverse primer, CCTCACGTCGCAGTAACTGT.
HPV18 forward primer, ATGGCGCGCTTTGAGGATCC;
HPV18 reverse primer, GCATGCGGTATACTGTCTCT;

Results (Page 11, Lines 228-232):
The extracted DNA was subsequently screened for the presence of HPV16 and HPV18 using PCR (Figure 1). From this screening, four patient samples were determined to be HPV-positive, which represented 2.6% of the total screened (n = 4/151). All four samples harbored HPV16 DNA and none were found to be positive for HPV18.

Comment 1-11: The study would greatly benefit from enlarging the number of subjects to be able to perform detailed statistical analyses and questionnaire for assessing possible risk factors which can eventually modify the presence of HPV in the oral cavity of healthy adults.

We concur with the comments of this reviewer and have devoted significant efforts into analyzing all the remaining samples for the revision. This was the main reason for the delay in completing these revisions by the original date requested. The total number of samples analyzed has been significantly increased (n = 151) from the original data submitted (n = 102). A review of the evidence reveals that many other studies had much smaller samples sizes (n = 12, 16, 16, 20, 50, 60, 70, and 97). Based on the increase in number of samples analyzed, combined with the review of other relevant studies, the text has been modified, as follows:

Discussion (Page 13-14, Lines 281-286):
This study had several limitations that should be considered. First, although the non-invasive nature of this study was sufficient for the recruitment and screening of a significant number of patients, the overall sample size was somewhat limited in comparison to the larger multinational studies previously mentioned [57,58,61]. The number of healthy adults screened for
oral HPV in this pilot study, however, compares favorably with a number of other reports – with sample sizes ranging from 12 to 97 [20,47-53].

- However, the authors were unable, in the short time frame allotted for revision, to develop a questionnaire, seek new approvals for expanding the study, recruit new patients, and screen new samples. Based upon the method for collection the original samples, no possibility exists to trace these samples back to specific patients to administer a questionnaire. The authors recognize this limitation and have modified the text as follows:

**Discussion (Page 14, Lines 286-290):**
Second, detailed demographic and behavioral data were not designated as critical to the initial goals of this pilot study, however, the inclusion of smoking and tobacco use, as well as more detailed information about other behaviors, housing, education, income and other socioeconomic indicator, as well as sexual practices may provide additional insights in future investigations [55,58,59,70].

**Reviewer 2**
General Comments: The authors collected oral specimens from healthy patients at a US dental school with the goal of evaluating oral HPV infection and risk factors for infection.

Comment 2-1: How was the sample size decided upon? Given the known rarity of oral HPV16 infection, it is surprising the investigators did not choose to study more individuals. Please include a confidence interval around the 3.9% point estimate (1.1% to 9.7%). Also, in Table 2, I didn’t get replicate the significant p values for the gender and race variables—how did you obtain these p values?

- We concur with the comments of this reviewer, and have included in this revision a specific section in the methods that directly addresses the comments of this reviewer. The revised text is as follows:

**Methods (Page 6, Lines 121-128):**
Sample size
To determine an appropriate sample size, previous studies that screened for high-risk oral HPV in healthy adults were evaluated to determine the range of sample sizes, which varied greatly from 12 – 1,680 [47-62]. Previous research at UNLV and the UNLV-SDM clinic demonstrated low participation rates for invasive, blood-based screenings [63], but higher rates of participation using non-invasive biomonitoring and screening methods, including saliva collection (unpublished data). These studies had sample
sizes ranging from 16 – 200. Based upon this combined information the maximum sample size was estimated to be 200.

- The authors would like to specify that the total number of samples analyzed has been significantly increased (n = 151) from the original submission (n = 102). A review of the evidence reveals that many other studies had samples sizes significantly smaller (n = 12, 16, 16, 20, 50, 60, 70, and 97). Based on the increase in number of samples analyzed, combined with the review of other relevant studies, the text has been modified, as follows:

Discussion (Page 13-14, Lines 281-286):
This study had several limitations that should be considered. First, although the non-invasive nature of this study was sufficient for the recruitment and screening of a significant number of patients, the overall sample size was somewhat limited in comparison to the larger multinational studies previously mentioned [57,58,61]. The number of healthy adults screened for oral HPV in this pilot study, however, compares favorably with a number of other reports – with sample sizes ranging from 12 to 97 [20,47-53].

- Finally, the authors are unable to provide a confidence interval because these are descriptive statistics regarding the total sample, not an estimate of a mean value. The methods for deriving the p-values in Table 2 are incorporated into the revised text, as follows:

Methods (Page 10, Lines 206-213):
Statistical evaluation
Following the acquisition of saliva samples and HPV screening results, demographic information from each sample was compared with the overall demographic profile of the UNLV-SDM patient pool (N = 71,051) using a chi-square ($\chi^2$) test, to determine if any characteristic (gender, race, age) was different than expected among the patients evaluated in this study (n = 151). A probability level of alpha ($\alpha$) = 0.05 was used to determine statistical significance.

Comment 2-2: What is a ‘pilot clustered randomized study’? Please elaborate on this design feature, and describe what this is a pilot effort for. Use of the word randomized indicates allocation to an intervention (i.e.: vaccine, drug, etc).

- We concur with the comments of this reviewer, and have revised all sections of the manuscript to describe this only as a pilot study. These revisions directly address the comments of this reviewer.

Title (Page 1, Lines 1-2):
High-risk human papillomavirus (HPV) screening and detection in healthy patient saliva samples: a pilot study
Comment 2-3: The method of oral specimen collection is not standard—please provide references in support of its use.

- We concur with the comments of this reviewer, and have revised the Background and Introduction section to include studies that use oral lavage, and also whole saliva, to test for oral HPV. The revised text is as follows:

  Background (Page 5, Lines 95-97):
  In addition, other studies have begun to report less invasive saliva and oral lavage-based testing methods to successfully screen for oral HPV among healthy adults, revealing prevalence rates between 2.8 to 25% [15,52-60].

Comment 2-4: The beta-actin positivity was unusually poor (68%), indicating a problem with the laboratory methodology. Perhaps a new aliquot from the original sample should undergo DNA extraction. There exists published literature on the topic of DNA extraction for the purpose of oral HPV detection—perhaps a new method for DNA extraction should be considered. Additionally, since the investigators choose not to use a standard kit for HPV detection, how was their qPCR assay for HPV16 detection validated? Were cervical samples used?

- We apologize for the misunderstanding of the original text. Due to personnel and budget constraints, only 102 out of the original 151 samples collected were screened and analyzed. The authors would like to specify that the total number of samples analyzed has been significantly increased (n = 151) from the original submission (n = 102). In addition, the methods have been revised to provide more specific details about the validation for HPV16 detection, as follows:

  Methods (Page 10, Lines 198-205):
  The CaSki (American Type Culture Collection; Manassas, VA) cervical adenocarcinoma cell line was used to develop standard curves for both the HPV16 (600 copies/genome) and β-actin (2 copies/genome) genes. DNA extracted from CaSki cells were serially diluted tenfold starting at 50 ng to 0.0005 ng [65] Quantification was achieved using Cycle Threshold (C_T) measured with the second derivative maximum method (LightCycler 480 Software version 1.5.0.39; Roche Applied Science). Saliva samples > 0.001 copy/genome were considered HPV positive. Specificity analysis was performed on qPCR assay against HPV18 and found to be 100% specific (data not shown).

Discretionary Revisions
Comment 2-5: The Introduction is extensive and reads more like a teaching tool than of summary of the pertinent literature relating to this topic.
We concur with the comments of this reviewer (similar to Comment 1-1) and have modified the text in the Background/Introduction section to reduce the overall length and provide more direct focus. We believe the targeted reduction in length, combined with the revised text addresses this specific concern.

Comment 2-6: Introduction- the authors point to several publications where HPV has been detected in several tumors, including colorectal and breast. The authors should use caution in that PCR based detection of HPV in tumors or tissue does not imply causation, and may merely be the result of contamination. Similarly, in the following paragraph, the authors state “Of all HPV types, the high-risk strains HPV16 and HPV18 are the most commonly identified from biopsies of oral cancers [19-21], providing strong evidence that HPV may be an independent risk factor for oral cancer.” Finding HPV DNA in tumor is not indicative of causation—more quantitative assays (such as ISH) are needed.

The authors have reviewed the comments of this reviewer, and concur that the presence of HPV does not imply causation, but may indicate other mechanisms at work. The text has been revised to address this specific concern as follows:

Background (Page 4, Lines 76-82):
The comparatively low presence of high-risk HPV in normal tissues and much higher prevalence in oral cancers may suggest that HPV preferentially infects already developing oral cancers [12-14]. HPV may then subsequently function to modulate the malignancy process in developing or establish oral cancers, as has been observed in studies of HPV infection in other developing cancers [29-39]. For example, recent epidemiologic and case-control studies have demonstrated that patients with HPV-positive oral tumors had significantly improved survival rates [12,40,41] and therapeutic response rates when compared with HPV-negative controls [42]. Several in vitro studies have recently investigated possible mechanisms that may account for these phenotypic changes in oral cancers [25-27]. Evidence is now accumulating that HPV infection of oral cancers correlates with increased survival rates and better prognosis among some patients due to these changes in cellular responsiveness [40-45].

In addition, some evidence now suggests that HPV may, in fact, induce carcinogenesis in patients that do not smoke or drink. Although this does not provide definitive evidence in the form of a prospective, experimental study, the evidence should be discussed and the text has been revised as follows:

Background (Page 5, Lines 88-92):
It is likely that HPV may modulate the malignancy process in some tobacco- and alcohol-induced oral cancers, but may also be the primary oncogenic factor for inducing carcinogenesis in a subset of patients without these traditional risk factors. Some evidence has demonstrated that non-tobacco
and non-alcohol related oral cancers were six times more likely to harbor HPV infections than case-matched controls [46].

Comment 2-7: The authors may want to make the concluding paragraph of their introduction more prominent, as it summarizes the literature pertaining to the topic of this investigation. Further, the author say “little evidence has been provided....”, but then go on to reference several publications including a systematic review of the literature.

- We concur with the comments of this reviewer and have removed the references to “little evidence”. The modified text in the concluding paragraph of the Background now reads as follows:

Background (Page 5, Lines 101-107):
Based upon this information, the goal of this project was to perform a screening for the most prevalent high-risk HPV strains, HPV16 and HPV18, in normal healthy adults. This pilot study was performed in Nevada, a state recently documented to have increasing rates of oral cancer between 1997 and 2005 - despite declining rates of tobacco and alcohol use in the state, as well as declining rates of oral cancer nationally [23,24]. The long-term goal is to provide more detailed information about high-risk oral HPV prevalence to allow for more robust estimates of oral cancer risk.

Reviewer 3
General comments: This paper describes a screening for human papilloma virus type 16, in saliva samples from healthy patients.

Comment 3-1: Other authors have detected different HPV genotypes in oral samples, in particular genotype 18 [1-5]. How could these data influence your epidemiological analysis?

- We concur with the comments of this reviewer (similar to Comment 1-10, above) and have spent considerable time rescreening all samples for the second most commonly detection high-risk oral HPV strain, HPV18. HPV16 and HPV18 account for the vast majority of documented oral HPV infections in the previous studies that were reviewed. The revised methods and results sections are as follows:

Methods (Pages 7-8, Lines 149-159):
DNA from each sample was then used to perform PCR with the Fisher exACTGene complete PCR kit (Fisher Scientific: Fair Lawn, NJ) and a
Mastercycler gradient thermocycler (Eppendorf: Hamburg, Germany) using the following primers for HPV16 [26,27], HPV18 [27,32], and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [64], synthesized by SeqWright (Houston, TX):

- HPV16 forward primer, ATGTTTCAGGACCCACAGGA;
- HPV16 reverse primer, CCTCACGTCGCAGTAACTGT;
- HPV18 forward primer, ATGGCGCGCTTTGAGGATCC;
- HPV18 reverse primer, GCATGCGGTATACTGATCC;

Results (Page 11, Lines 228-232):
The extracted DNA was subsequently screened for the presence of HPV16 and HPV18 using PCR (Figure 1). From this screening, four patient samples were determined to be HPV-positive, which represented 2.6% of the total screened (n = 4/151). All four samples harbored HPV16 DNA and none were found to be positive for HPV18.

Comment 3-2: Several recent papers have presented epidemiological results regarding HPV16 in the oral cavity [4, 6]. The authors should discuss in the manuscript whether these results are comparable. Please underline this point.

- We concur with the comments of this reviewer and have revised the Discussion section as follows:

Discussion (Page 12, Lines 265-261):
These data demonstrated a prevalence rate of high-risk oral HPV (2.6%) virtually the same as the most recent multinational studies of healthy, cancer-free adults (3.1% to 5%) [61,62]. Over the past few decades, international studies have evaluated HPV prevalence in healthy adults using biopsy samples, which reported widely variable prevalence rates that ranged from 0–15% [47-51]. However, other recently published reports screening for oral HPV infection among healthy adults using saliva and oral lavage testing reported overall prevalence rates that were also close to this range (1.3%, 2.8%, 7%) [52-59].

Comment 3-3: The real time PCR methodology used for HPV quantisation is incomplete (primer sequences and PCR cycle are not reported).

- We concur with the comments of this reviewer (similar to Comment 1-5) and have modified the text to provide more specific information about the qPCR procedures, controls, primers, and method. The modified text is as follows:

Methods (Pages 8-10, Lines 171-205):
Quantitative PCR (qPCR)
DNA samples were then processed using qPCR to provide more specific and
sensitive quantification. Primers and probes were designed using Roche Universal Probe library (UPL) assay design software to amplify the region overlapping E6 and E7 gene sequence of HPV16 (GenBank accession no. K02718) and the human β-actin housekeeping gene (GenBank accession no. M10277). All primers were purchased from Sigma-Aldrich (St. Louis, MO.) and probes purchased from Roche Applied Science (Indianapolis, IN.)

HPV16 E6/E7 forward primer 5’-CAACTGATCTCTACTGTTATGAGCAA-3’, HPV16 E6/E7 reverse primer 5’-CCAGCTGGACCATCTATTTCA-3’, HPV16 E6/E7 hydrolysis “Taqman” probe 5’-(fam)-AGGAGGAG-(dark quencher dye)-3’ (UPL probe #63) was used to amplify the 73 base pair (bp) region between the 535 nucelotide (nt) position and 607 nt position. Human β-actin forward primer 5’-GTGGGGTCTCTGTGGTGTG-3’, human β-actin 5’-GAAGGGGAGCAGGCAGTG-3’, human β-actin hydrolysis “Taqman” probe 5’-(fam)-GGGAGCTG-(dark quencher dye)-3’ (UPL probe #24) amplified the 61 bp region between 2642 nt position and 2702 nt position.

The real-time reaction mixture was prepared in a LightCycler® 480 multiwell Plate 96 containing 1x LightCycler® 480 Probes Master (Roche Applied Sciences), 1 µM of each respective primer set (forward and reverse), 0.2 µM of respective probe, and 2 µl of DNA template; in a 20 µl final reaction volume. The probes master mix contained reaction buffer, dNTP mix (including dUTP in place of dTTP), 3.2 mM MgCl₂, and Taq DNA polymerase. The real-time PCR assay was performed on a LightCycler 480 system (Roche Applied Science) with the following cycle parameters: pre-incubation for initial enzyme activation at 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 10 seconds (ramp rate 4.4 °C/second), 60 °C for 30 seconds (ramp rate 2.2 °C/second) and 72 °C for 1 second (ramp rate 4.4 °C/second). Following amplification phase, a cooling step was performed at 40 °C for 30 seconds (ramp rate of 2.2 °C/ second). Acquisition of the fluorescence signal was performed using Mono Hydrolysis Probe setting (465-510 nm) following the 72 °C extension phase of each cycle. All samples were carried out in triplicate.

The CaSki (American Type Culture Collection; Manassas, VA) cervical adenocarcinoma cell line was used to develop standard curves for both the HPV16 (600 copies/genome) and β-actin (2 copies/genome) genes. DNA extracted from CaSki cells were serially diluted tenfold starting at 50 ng to 0.0005 ng [65] Quantification was achieved using Cycle Threshold (Cₚ) measured with the second derivative maximum method (LightCycler 480 Software version 1.5.0.39; Roche Applied Science). Saliva samples > 0.001 copy-genome were considered HPV positive. Specificity analysis was performed on qPCR assay against HPV18 and found to be 100% specific (data not shown).
Reviewer 4

General comments: The goal of this study was to perform HPV screening of normal healthy adults to assess oral HPV prevalence. HPV16 DNA was found in only 3.9% of the adult patients (4/102), which is very low compared to other recent studies reporting a rate of 20% in the oral cavity (SahebJamee et al., 2009). Three of the four HPV16 positive samples were from Hispanic patients. These results provide new information about oral HPV status, which may help to contextualize results from other studies demonstrating that oral cancer rates are increasing in the US among both females and minorities and in some geographic areas.

Minor Essential Revisions:

Comment 4-1: How can the authors explain the fact that the Hispanic women minority is more susceptible to HPV16 infection compared to the other minorities?

- We concur with the comments of this reviewer and have modified the text to provide more discussion about these findings. The modified text is as follows:

Discussion (Page 13, Lines 262-273):
In addition, the results of this study found oral HPV infection only among patients who were minority and female. Although the vast majority of female and minority patients in this study had no evidence of oral HPV infection, recent epidemiologic studies have shown that rates of oral cancer have risen sharply among minority females in the US, despite overall declining rates [66]. More generally, rates of oral cancer have risen among some minority subgroups [67], despite an overall decline among the general population in the US [23,24,68]. This may be explained, in part, by higher rates of tobacco and alcohol use, but may also be attributable to other factors including education, income, stress, diet, health literacy and exposure to oral infectious agents [22,25]. This pilot study provides preliminary information about oral HPV prevalence and the results suggest that further investigation may be warranted, particularly in light of the health disparities facing both females and minorities in Nevada and the US, in general [23,24].

Comment 4-2: Why did the authors use one microgram of DNA to perform the PCR? This unusual quantity is very high and could even inhibit the PCR and decrease the HPV16 prevalence, or generate unspecific signals. The authors should provide the picture of the agarose gel.

- We concur with the comments of this reviewer and have modified the Results to include these findings. The modified text is as follows:

Results (Page 11, Lines 328-231):
The extracted DNA was subsequently screened for the presence of HPV16 and HPV18 using PCR (Figure 1). From this screening, four patient samples
were determined to be HPV-positive, which represented 2.6% of the total screened (n = 4/151). All four samples harbored HPV16 DNA and none were found to be positive for HPV18.

Figure 1. Screening of patient samples for HPV. PCR using DNA extracted from patient samples (n = 151) was screened using HPV16- and HPV18-specific primers, which revealed four samples harbored HPV16 (2819, 2718, 2527, and 2430). No samples were found to harbor HPV18. DNA extracted previously from cervical adenocarcinoma cell lines, CaSki and GH354, was used as HPV16 and HPV18 positive controls, respectively.

<table>
<thead>
<tr>
<th>GAPDH</th>
<th>HPV18</th>
<th>HPV16</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+) control S: 2819</td>
<td>S: 2718</td>
<td>S: 2527</td>
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</tbody>
</table>

Comment 4-3: The oral cavity is susceptible to contaminations from many infectious agents in the external environment. In order to demonstrate a productive HPV16 infection, is it possible for the authors to check for the HPV16 RNA?

- We concur with the comments of this reviewer, however, the vast majority of the original samples were completely used in the initial screening and DNA extraction. Vanishingly small amounts, if any, remained – therefore we are unable to complete this specific request at this time. However, future studies will specify minimum collection amounts of saliva or oral lavage samples, in order to facilitate these experiments in future studies.

In summary, comments provided by the 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 to make this manuscript more interesting and relevant. 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 BMC Oral Health. We thank the editors of this journal for their patience and consideration during this process.

Respectfully submitted,
Karl Kingsley, PhD, MPH
Associate Professor of Biomedical Sciences