Author's response to reviews

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

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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 the final reviewer comments and suggestions:

**Reviewer 4**
*Comment: The authors answered most of my comments. The article is suitable for publication.*

**Reviewer 3**
*Comment: The manuscript is acceptable for publication in its present form.*

**Reviewer 1**
*Comments: I have no further comments.*

**Reviewer 2**
*Comment 1: The authors use the term ‘oral cancer’ which encompasses oral and oropharynx tumors. The association with HPV is seen mainly for oropharynx tumors. The authors may want to state this.*

- 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:

  Abstract (Page 2, Lines 31-35):
  Recent epidemiologic evidence has suggested that HPV may be an independent risk factor for [oropharyngeal cancers](#). Evidence now suggests HPV may modulate the malignancy process in some tobacco- and alcohol-induced [oropharynx tumors](#), but might also be the primary oncogenic factor for inducing carcinogenesis among some non-smokers.

  Background (Page 4, Line s67-69):
  Recent epidemiologic evidence has suggested that HPV may also be an independent risk factor for [oropharyngeal cancer](#), revealing HPV in three times as
many pre-cancerous oral lesions, and almost five times as many oropharyngeal cancers compared with normal oral mucosa [12-14].

Background (Page 4, Line 71-74):
Although the traditional risk factors for developing oropharyngeal cancer remain tobacco use and heavy alcohol consumption, other risk factors, such as HPV, may play significant roles in determining whether develops and how quickly it may progress [14,19,22-28].

Background (Page 4-5, Line 76-91):
The comparatively low presence of high-risk HPV in normal tissues and much higher prevalence may suggest that HPV preferentially infects already developing oropharyngeal cancers [12-14]. Although it is possible that the low prevalence in healthy individuals might be attributable to other factors, including improper specimen collection or assay sensitivity, it is also possible that HPV may function to modulate the malignancy process in developing or established oropharyngeal tumors, 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 oropharyngeal 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 oropharyngeal cancers [25-27]. Evidence is now accumulating that HPV infection of some oropharyngeal cancers correlates with increased survival rates and better prognosis among some patients due to these changes in cellular responsiveness [40-45]. These studies highlight the need to understand not only the prevalence of oral HPV infection, but also the duration and persistence of such infections due to their potential to affect oropharyngeal tumor progression.

Background (Page 5, Line 93-97):
It is likely that HPV may modulate the malignancy process in some tobacco- and alcohol-induced oropharyngeal 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 oropharyngeal cancers were six times more likely to harbor HPV infections than case-matched controls [46].

Background (Page 5, Line 104-105):
Some of these oral HPV screening studies were performed in the US on normal, healthy adults, in addition to oropharyngeal cancer patients [57,58,61].

Background (Page 5-6, Line 109-113):
This pilot study was performed in Nevada, a state recently documented to have increasing rates of oropharyngeal cancer between 1997 and 2005 - despite declining rates of tobacco and alcohol use in the state, as well as declining rates of oropharyngeal 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 oropharyngeal cancer risk.

Methods (Page 6, Line 123-125):
Exclusion criteria: subjects younger than 18 years of age, subjects that declined to participate, and subjects with prior diagnosis of oropharyngeal cancer were excluded.

Discussion (Page 13, Lines 276-281):
As an initial pilot study, these data suggest a more comprehensive and in-depth analysis of this population may be necessary, as recent epidemiologic studies have shown that rates of oropharyngeal cancer have risen sharply among minority females in the US, despite overall declining rates [66]. More generally, rates of oropharyngeal cancer have risen among some minority subgroups [67], despite an overall decline among the general population in the US [23,24,68].

Conclusions (Page 15, Lines 310-314):
Many health disparities face females and minorities in the US, and with some evidence suggesting that smoking and oropharyngeal cancer rates may be increasing in these population subgroups, the results of this study may be of significant value to other dental, medical, and health care professionals to further an understanding of oral health and disease risk.

Comment 2: The following statement is problematic: “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 established oral cancers, as has been observed in studies of HPV infection in other developing cancers [29-29].

The low oral HPV prevalence in healthy individuals could be attributed to many things, including improper specimen collection and insensitive assays, and highlights the importance of persistence (Prevalence = incidence * duration). The authors jump to the conclusion that low prevalence in healthy oral tissue and high prevalence in oral cancer suggests that HPV preferentially infects already developing oral cancers, which may be inaccurate.

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

Background (Page 4, Lines 76-82):
The comparatively low presence of high-risk HPV in normal tissues and much higher prevalence may suggest that HPV preferentially infects already developing oropharyngeal cancers [12-14]. Although it is possible that the low prevalence in
healthy individuals might be attributable to other factors, including improper specimen collection or assay sensitivity, it is also possible that HPV may function to modulate the malignancy process in developing or established oropharyngeal tumors, as has been observed in studies of HPV infection in other developing cancers [29-39].

Background (Page 5, Lines 88-91):
These studies highlight the need to understand not only the prevalence of oral HPV infection, but also the duration and persistence of such infections due to their potential to affect oropharyngeal tumor progression.

Comment 3: In the intro, the authors state “…oral lavage-based testing methods to successfully screen for oral HPV…” How do they know these methods are successful? Against what gold standard have they been measured?

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

Background (Page 5, Lines 100-102):
In addition, other studies have begun to report less invasive saliva and oral lavage-based testing methods to identify HPV among healthy adult saliva samples, revealing prevalence rates between 2.8 to 25% [15,52-60].

Comment 4: Results (242) – the authors provide data on the analytic sensitivity/specificity of the assays. This should not be confused with clinical sensitivity/specificity where a gold standard is used to determine the performance of the assay. Analytic sensitivity is typically included in the methods section.

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

Methods (Page 10, Lines 204-213):
**Analytic sensitivity**
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]. This step allowed for relative quantification of the input DNA level and final quantity as the number of viral copies/genome/cell. 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 5: The authors refer to blood-based biomonitoring in Nevada (pg 12, line 249) but this is out of context and I don’t know what it’s referring to. HPV antibodies?

- We concur with the comments of this reviewer and have modified the text to provide a more clear description of the previous studies and the relevance. The modified text is as follows:

Discussion (Page 12, Lines 258-264):
Previous attempts at this institution and within the state to obtain blood-based samples for other types of screenings, such as lead (Pb) screening, have been largely unsuccessful due to patient apprehension and fear of blood collection [63]. To avoid these specific problems, this study utilized non-invasively collected saliva to perform this analysis. These efforts ultimately allowed for the collection and screening of dozens of patient samples; a marked improvement in patient participation rates over other previous, but similar, attempts to collect biological specimens from the local population.

Comment 6: With detection of 4 oral HPV infections, it is inappropriate to discuss the patient characteristics of those infected. The sample size for these inherent comparisons is simply inadequate.

- We have thoroughly reviewed and discussed the comments of this reviewer and have modified the text to provide a more nuanced description of the study results. The modified text is as follows:

Discussion (Page 13, Lines 274-279):
In addition, although the results of this study found oral HPV infection only among four patients, who were minority and female, the vast majority of female and minority patients in this study had no evidence of oral HPV infection. As an initial pilot study, these data suggest a more comprehensive and in-depth analysis of this population may be necessary, as recent epidemiologic studies have shown that rates of oropharyngeal cancer have risen sharply among minority females in the US, despite overall declining rates [66].
In summary, comments provided by the reviewer 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 and 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
the process of our revisions.

Respectfully submitted,

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