Author's response to reviews

Title: Screening and detection of human papillomavirus (HPV) high-risk strains HPV16 and HPV18 in saliva samples from subjects under 18 years old in Nevada: a pilot study.

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Screening and detection of human papillomavirus (HPV) high-risk strains HPV16 and HPV18 in healthy pediatric patient saliva samples from Nevada: a pilot study. Colton Flake, Jamal Arafa, Alex Hall, Eryn Ence, Katherine M Howard and Karl Kingsley

Enclosed, please find our revised manuscript, which 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 2 - Comment 1:
The background is focused on information about HPV and cervical and head and neck cancer. Since the prevalence of HPV in healthy children was studied, entirely different information should be included in the introduction, etc. prevalence of HPV in young people and adults, comparison of the age specific prevalence, differences caused by different methods used. The authors introduced changes into the discussion but still the background is the same, including the sentence ...“and malignancy process of nearly all cervical adenocarcinomas...”. And how about squamous carcinomas. I suggest to entirely rewriting the introduction.

Page 3: The higher prevalence of high-risk HPV strains in pre-cancerous and cancerous oropharyngeal tumors suggests that HPV may preferentially infect developing or established cancers, thereby modulating carcinogenic progression and ultimately influencing patient outcomes. Do the authors mean that in those? patients with head and neck cancers who get infected by HPV, the prognosis improves? So HPV is not etiological factor of a subset of head and neck cancers? Isn’t HPV the starting point of immortalization and transformation of epithelial cells? There were no changes made in this part of the background.

The authors have carefully reviewed these comments and have modified the Background and Introduction section to address each of these points specifically. The prevalence of HPV in healthy and diseased youth and adults is now integrated into this section, as is information regarding transformation of squamous epithelia by HPV. Moreover, the conflicting evidence regarding HPV-status of oral cancers and patient outcomes has been expanded to more accurately reflect the unclear nature of the findings to date. The revised text now reads:

The human papillomaviruses (HPV) encompass a closely related family of DNA viruses, which are capable of integrating into the human genome to drive transformation of infected epithelia [1-4]. Much of the epidemiological evidence for HPV-driven carcinogenesis, as well as the biological mechanisms, have been derived from studies of cervical cancers [5-7] that isolated high-risk HPV from both adeno- and squamous cell carcinomas [6-9]. Although high-risk HPV drives the transformation and malignancy process of nearly all cervical cancers, HPV infection is now known to modulate epithelial transformation in breast, lung, penile, anal, and also oral tissues [10-20].
The primary risk factors for oral carcinogenesis are tobacco and alcohol use, although new lines of evidence now suggest HPV may also be an independent risk factor [17-23]. The higher prevalence of high-risk HPV strains in pre-cancerous and cancerous oropharyngeal tumors suggests that HPV may preferentially infect developing or established cancers, thereby modulating carcinogenic progression and ultimately influencing health outcomes [24-26]. In fact, some evidence has suggested that high-risk HPV infection may be associated with improved response to treatment and higher survival rates [27-29], while conflicting reports have demonstrated significantly decreased patient survival [30-32] or have observed no discernable and statistically significant effects [33].

Despite the conflicting nature of these results, it is clear that HPV may be involved in modulating tumor responsiveness and carcinogenic progression, therefore many of these studies have provided valuable epidemiological information regarding which high-risk HPV strains are most often implicated in these cases [34-36]. These studies have revealed HPV16, and HPV18 to a lesser extent, accounted for the overwhelming majority (71-94.7%) of high-risk oral HPV detected [17,18,21-23,34-36]. Moreover, new evidence now suggests that these specific high-risk HPV strains (HPV16 and HPV18), may initiate oral carcinogenesis among the smaller fraction of oral cancer patients who do not consume alcohol or use tobacco [37,38].

Based upon the findings of oral HPV in non-tobacco and non-alcohol associated oral cancers, other recent efforts have focused more specifically to evaluate oral HPV prevalence and transmission within healthy populations, which also confirmed that HPV16 and HPV18 were the most commonly detected oral high-risk HPV strains [39-41] among healthy adults.

To this end, a recent pilot study evaluating oral HPV among healthy adults was performed in Nevada [42], a state recently documented with rising rates of oropharyngeal cancers, in stark contrast to the declining rates observed in neighboring states and the US more generally [43,44]. This study revealed the presence of high-risk strain HPV16, but not HPV18, among females and minorities, the only population subgroups demonstrated to have rising orpharyngeal cancer rates – despite the overall declining rates observed within the general population [45-47]. This study utilized a saliva-based screening procedure, part of a growing trend towards less invasive methodologies, such as oral lavage- and saliva-based screenings, to analyze oral HPV status among healthy adult patients [48-51].

Although prevalence rates ranged widely, these studies have suggested that oral HPV infection may be increasing, not only among adults, but more specifically among younger adults, teenagers, and children [52-54]. To this end, some studies have examined oral HPV in children or adolescents, although many of these focused primarily on children with underlying medical conditions, such as co-infection with human immunodeficiency virus (HIV) [55-57]. Most other research, however, has focused more specifically on elucidating the role of vertical HPV transmission in newborns, demonstrating the potential to acquire high-risk HPV during the delivery and birthing process, although these infections typically resolve [58-63].
However, new evidence is emerging that demonstrated high-risk oral HPV infection in normal, healthy children, with the highest rates observed among children under 7 years old (7.9%-8.7%), and declining rates observed among healthy adolescents (13-20 years old; 5.1%-5.2%) and healthy adults (3.5%) [64,65]. These observations may suggest that oral HPV infection may occur through close personal contact with family members or through contact with fomites and other vectors at daycare centers, preschool or in primary education settings, with most children immunologically competent to resolve these infections [66]. However, some infections may persist and their contribution to the development of oral cancers and other pathologies remains unclear.

Although a pilot study was recently conducted to evaluate oral HPV status, this involved only healthy adult patients - with no information obtained about oral HPV prevalence among children or teenagers. Based upon the previous evidence demonstrating some level of oral HPV infection in healthy children and adolescents, combined with the lack of data about this population more specifically, the goal of this current study is to provide more detailed information about prevalence of high-risk HPV strains HPV16 and HPV18 in the oral cavity of children and teenagers in Nevada.

Reviewer 2 - Comment 2: Please specify in more details the process of sampling. Even in a retrospective study there must be information available about the process of sampling (solution for oral rinse, volume and etc.).

The authors have carefully reviewed these comments and have modified the Methods section to address this point, specifically. The revised text now reads:

**Saliva Collection Protocol**

In brief, subjects who agreed to participate were given a small, sterile saliva collection container, 50 mL sterile polypropylene tube (Fisher Scientific: Fair Lawn, New Jersey, USA). Participants were then asked to chew on a small piece of paraffin wax for one minute and then to expectorate, in accordance with the previous pilot study protocol [42]; sample volumes varied from approximately 50 μL to 2.5 μL. Samples were stored on ice until transport to a biomedical laboratory for analysis. Each saliva sample was assigned a unique, randomly-generated number to prevent research bias. Demographic information regarding the sample was concurrently collected, which consisted of age, gender, and ethnicity only.

**Cell counting and DNA isolation**

All samples were centrifuged for 10 minutes at 2,100 g (RCF) and the cell pellet washed with 1X phosphate-buffered saline (PBS) (HyClone: Logan, Utah, USA) and resuspended in 5 mL of 1X PBS. Cell number was determined using Trypan Blue (Fisher Scientific: Fair Lawn, New Jersey, USA) using a Zeiss Axiovert 40 inverted microscope (Carl Zeiss, Inc: Thornwood, New York, USA) and a hemacytometer (Fisher Scientific: Fair Lawn, New Jersey, USA).
Reviewer 1
General comments (June 11): Were addressed in previous resubmission (July 13, 2012). No further editorial comments.

Reviewer 3 - General comments (Sept. 25): The authors have answered the different points sufficiently well to allow their manuscript to be published.

Reviewer 4 Comments (June 11): The manuscript is interesting because it describes concise evaluation on the recurrence of high-risk strains HPV16, HPV18 in young patients, (pediatrics and teenagers) on the Nevada's population. Although this manuscript is limited for some aspects (e.g. not detailed patients data), these limitations are considered and well discussed on the text. The molecular method and the statistical approach presented in this work as well as revision and extension of results are appropriate.

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 this 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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