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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Author's response to reviews: see over
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 1
General comments: The MS is interesting and contains new information, material and methods at the end of the paper is in the biochemical tradition and different from clinical MS’s, nevertheless the referee is accepting this form.

Reviewer 1 - Comment 1: Throughout the MS subjects are called patients – they are NOT patients for this study, and some are just school teenagers. The authors concur with these comments and have changed all the appropriate references to “patient” samples throughout the text. The modified sections now read:

**Title**, Lines 1-3: 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.

**Results**, Lines 98-99: Detailed analysis of saliva samples revealed some variability between cell counts, DNA concentration and DNA purity between samples (Figure 1).

**Results**, Lines 109-111: Samples of extracted DNA were subsequently screened for the presence of HPV16 and HPV18 using PCR (Figure 2). This screening yielded three HPV-positive samples (n = 3/118), representing 2.5% of the total screened.

**Discussion**, Lines 146-149: Other evidence has suggested that although samples from females and males had similar rates of oral HPV (56 and 44%, respectively), the HPV-negative samples from that study were overwhelmingly female (82%) – providing some evidence of a possible male, gender-specific phenomenon similar to the current study results [46].

**Methods**, Lines 210-211: *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).
Methods, Lines 278-282: Following the acquisition of saliva samples and HPV screening results, demographic information from the samples were compared with the overall demographic profile of the local population using a chi-square ($\chi^2$) test, to determine if any characteristic (gender, race, age) was different than expected among the subjects evaluated in this study ($n = 118$). A probability level of alpha ($\alpha$) = 0.05 was used to determine statistical significance.

There were, however, some instances where the term patient is the most accurate descriptor and was retained in those places, where appropriate.

Abstract, Lines 30-33: Methods: This retrospective study utilized previously collected saliva samples, obtained from pediatric dental clinic patients (aged 2 – 11) and local school district teenagers (aged 12-17) for high-risk HPV screening ($n=118$) using qPCR for quantification and confirmation of analytical sensitivity and specificity.

Discussion, Lines 135-138: This retrospective analysis used existing saliva samples, collected from pediatric dental patients and local school district teenagers, to obtain novel data from this previously untested juvenile population thereby complementing the ever-growing body of evidence regarding oral HPV prevalence in children.

Reviewer 1 – Comment 2: The reason for saliva collection should be mentioned. The storage on ice (?) means what? The authors concur with the comments of this reviewer and have modified the text, as follows:

Methods, Lines 193-198: Saliva samples collected from a prior study of teenagers (14-15 years old) were derived from a convenience sample of selected schools within the Clark County, Nevada School District (CCSD; $n=48$); originally obtained for the purpose of surveying levels of oral cariogenic bacteria. Saliva samples collected from young children (2-11 years old) were obtained from a convenience sample within the UNLV-SDM pediatric dental clinic ($n=70$); originally obtained for the purpose of screening for heavy metal (lead or Pb) burden.

Methods, Lines 211-212: Samples were stored on ice until transport to a biomedical laboratory for analysis.

Reviewer 1 – Comment 3: Pediatric samples is the wrong term and not all age-related (ages 2-11). The authors have reviewed this comment and concur with the comments of this reviewer, however, not all experts agree on a standard definition for what defines the pediatric population. The United States governmental agencies, including the FDA, NIH and CDC, concur that this term
“pediatric” may refer to children and adolescents up to the age of 21. The authors have altered the text to address these concerns, as follows:

Results, Lines 94-96: Although data from the local population were unavailable for age-specific comparisons, the study sample was comprised more from younger children (ages 2-11: 59.3%) than from adolescents and teenagers (12-17: 40.7%).

There were, however, some instances where the term pediatric is the most accurate descriptor and was retained in those places, where appropriate. These include:

Abstract, Lines 30-33: Methods: This retrospective study utilized previously collected saliva samples, obtained from pediatric dental clinic patients (aged 2 – 11) and local school district teenagers (aged 12-17) for high-risk HPV screening (n=118) using qPCR for quantification and confirmation of analytical sensitivity and specificity.

Abstract, Lines 39-42: With increasing evidence of oral HPV infection in children, this study provides critical information of significant value to other dental, medical, oral and public health professionals who seek to further an understanding of oral health and disease risk in pediatric populations.

Results, Lines 129-131: All three HPV-positive samples were from the CCSD (teenage) cohort (n = 3/48 or 6.25%), while none were observed in the UNLV-SDM (pediatric) cohort (n = 70).

Discussion, Lines 135-138: This retrospective analysis used existing saliva samples, collected from pediatric dental patients and local school district teenagers, to obtain novel data from this previously untested juvenile population thereby complementing the ever-growing body of evidence regarding oral HPV prevalence in children.

Conclusions, Lines 171-174: This study, therefore, provides critical information of significant value to other dental, medical, oral and public health professionals who seek to further an understanding of high-risk HPV prevalence among children as part of a broader understanding of oral health and disease risk in pediatric populations.

Methods, Lines 196-198: Saliva samples collected from young children (2-11 years old) were obtained from a convenience sample within the UNLV-SDM pediatric dental clinic (n=70); originally obtained for the purpose of screening for heavy metal (lead or Pb) burden.

Reviewer 1 – Comment 4: The presence of HPV in saliva is not (yet) an infection, this is rather part of the oral microbiota. The authors concur and have modified the text, as follows:
Discussion, Lines 134-135: The main goal of this study was to screen normal healthy children and teenagers in Nevada for the presence of high-risk oral HPV.

Discussion, Lines 154-158: This study represents a significant turning point in public health efforts to elucidate oral HPV detection in a geographic area known for higher than average (and previously increasing) rates of oropharyngeal cancers [23,24]. First, the retrospective nature of this study limited the inferences that could be made, unlike other recent prospective studies of oral HPV in children and adolescents [37,46,47].

Discussion, Lines 160-163: It is hoped that future investigations involving oral HPV will provide additional insights with more detailed behavioral information, as well as data regarding housing, education, income and other socioeconomic indicators [38,48-50].

Conclusions, Lines 167-174: Although previous work has focused on oral HPV transmission from mother to newborn during birth and while nursing [47], there is growing evidence to suggest that following the perinatal period, oral HPV transmission through close personal contact, such as shared eating utensils, toys, kissing and bathing may account for oral HPV transmission (primarily HPV16) in children and adolescents [51-54]. This study, therefore, provides critical information of significant value to other dental, medical, oral and public health professionals who seek to further an understanding of high-risk HPV prevalence among children as part of a broader understanding of oral health and disease risk in pediatric populations.

Reviewer 1 – Comment 5: Please avoid phrases like “most were White” for two out of three, or “all three were males and none were female”. The authors concur with these comments and have modified the text, as follows:

Discussion, Lines 143 – 146: The results of the current study were markedly different, however, because two of the three HPV-positive samples were obtained from Whites and all were from males – whereas the prior study of adults found oral HPV among females and minorities only.

Results, Lines 124-127: Although the relatively small proportion of HPV-positive samples does not allow for more broad inferences, a descriptive analysis of the demographic information regarding these samples revealed that all three were derived from males (n=3/57 or 5/3%) and none were derived from females.

Reviewer 1 – Comment 6: Budgetary constraints within the University system may influence the research activity, but this argument should not serve as an excuse for the number of subjects, etc. The authors concur that this should never be used as an “excuse” for the sample size – but had originally intended to explain one of the study limitations. This section has been removed entirely, and now reads:
Discussion, Lines 156 – 160: However, this study had several limitations that must be acknowledged. First, the retrospective nature of this study limited the inferences that could be made, unlike other recent prospective studies of oral HPV in children and adolescents [37,46,47]. In addition, no detailed behavioral or socioeconomic data were available, such as parental income or smoking behaviors, due to the nature of this retrospective pilot study.

Reviewer 1 – Comment 7: The references should appear in the BMC style: Abbreviations of all journals. The authors apologize for this oversight and have modified the references, as follows:


Reviewer 1 – Comment 8: Materials should be declared by (Company, City, Country, State). Do not use country abbreviations for the US, the rest of the world does not understand, Goettingen is the correct city but not the
Methods, Lines 210-211: 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).

Methods, Lines 218-226: All samples were centrifuged for 10 minutes at 2,100 g (RCF) and the 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 Axiolab 40 inverted microscope (Carl Zeiss, Inc: Thornwood, New York, USA) and a hemacytometer (Fisher Scientific: Fair Lawn, New Jersey, USA). To determine if any samples harbored the HPV virus, DNA was isolated from the saliva sample using a minimum of 3.5 x 10⁵ cells using the GenomicPrep DNA isolation kit (Amersham Biosciences: Buckinghamshire, United Kingdom), using the procedure recommended by the manufacturer as previously described [10,42,57,58].

Methods, Lines 230-234: DNA from each sample was then used to perform PCR with the Fisher exACTGene complete PCR kit (Fisher Scientific: Fair Lawn, New Jersey, USA) and a Mastercycler gradient thermocycler (Eppendorf: Hamburg, Germany) using the following primers for HPV16 [10,55], HPV18 [10,55,56], and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [57] (SeqWright: Houston, Texas, USA):

Methods, Lines 244-248: The PCR reaction products were separated by gel electrophoresis using Reliant 4% NuSieve® 3:1 Plus Agarose gels (Lonza: Rockland, Maine, USA). Bands were visualized by UV illumination of ethidium-bromide-stained gels and captured using a Kodak Gel Logic 100 Imaging System and 1D Image Analysis Software (Eastman Kodak: Rochester, New York, USA).

Methods, Lines 255-256: These primers were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and probes from Roche Applied Science (Indianapolis, Indiana, USA).

Methods, Lines 281-283: The CaSki (American Type Culture Collection; Manassas, Virginia, USA) cervical adenocarcinoma cell line was used to develop standard curves for both the HPV16 (600 copies/genome) and GAPDH (2 copies/genome) genes.

Reviewer 1 - Comment 9: Fig. 2C is completely unclear. Min.?; 63%; and so many = ? The authors concur and have modified the text, as follows:

Figure Legends, Lines 578-585: Figure 2. Saliva sample screening: analysis of qPCR HPV results. A) Three samples were found to harbor HPV DNA: HPV16-positive samples (S5, S38); One sample was HPV-positive (S7). B) qPCR analysis revealed HPV-positive samples had values well above the established cutoff value (> 0.001 copies/genome). C) All three HPV-positive samples were male, representing 2.5% of the total (n = 118). Two of the three HPV-positive samples were from White participants.
All three HPV-positive samples were from the CCSD cohort (ages: 12-17); Both HPV16-positive samples were obtained from 14-year old participants, while the HPV18-positive sample was obtained from a 15-year old.

Reviewer 2
General comments:
The major aim was to assess HPV prevalence in healthy children and teenager 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.

Reviewer 2 – Comment 1: 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. The authors have designed and implemented this project based upon the only other study of oral HPV ever completed in Nevada (Turner et al., BMC Oral Health 2011, 11:28). The original study of healthy adults screened for the most prevalent high-risk HPV strains found in the oral cavity, HPV16 and HPV18. Based upon this evidence, the current project also screened for HPV16 and HPV18, as these two account for the vast majority of all HPV strains detected in the oral cavity. The text of the manuscript, however, has been modified to more accurately reflect why HPV16 and HPV18 were chosen for the current study of oral HPV screening in children, as follows:

**Background, Lines 64 – 69:** Many of these studies have provided valuable epidemiological information regarding HPV in oropharyngeal tumors and case-matched controls [27-29]; demonstrating HPV16, and HPV18 to a lesser extent, accounted for the overwhelming majority (71-94.7%) of high-risk oral HPV detected [15,16, 19-21,27-29]. More recent efforts, however, have focused specifically on estimating oral HPV prevalence and transmission within healthy populations, also demonstrating that HPV16 and HPV18 were the most commonly detected oral high-risk HPV strains [30-32].

Reviewer 2 – Comment 2: The manuscript 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 concur and have modified the Discussion section to reflect these changes, as follows:

**Discussion, Lines 166-174:** This project successfully screened saliva samples for high-
risk HPV, confirming both HPV16 and HPV18 strains were present in a small subset. Although previous work has focused on oral HPV transmission from mother to newborn during birth and while nursing [47], there is growing evidence to suggest that following the perinatal period, oral HPV transmission through close personal contact, such as shared eating utensils, toys, kissing and bathing may account for oral HPV transmission (primarily HPV16) in children and adolescents [51-54]. This study, therefore, provides critical information of significant value to other dental, medical, oral and public health professionals who seek to further an understanding of high-risk HPV prevalence among children as part of a broader understanding of oral health and disease risk in pediatric populations.


Reviewer 2 - Comment 3: Although high risk HPV drives the transformation and malignancy process of nearly all cervical adenocarcinomas...and how about squamous cell carcinoma of cervix? The authors concur with the comments of this reviewer and have revised the text to reflect the findings from a large meta-analysis and other research, which clearly indicate high-risk HPV drives cervical squamous cell carcinomas, as well as adenocarcinomas, as follows:

Background, Lines 48 – 50: 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 [6,7].

Reviewer 2 - Comment 4: 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?

In addition, new evidence now suggests that some high-risk HPV strains, such as HPV16 and HPV18, may initiate oral carcinogenesis among the smaller fraction of oral cancer patients who do not consume alcohol or use tobacco. The etiological relationship is accepted for HPV 16 and oropharyngeal cancer.
A large number of studies (Mehta, Yu and Schantz, 2010; Badaracco et al., 2007; Schwartz et al., 2001) have found that HPV-positive oral tumors may be correlated with clinical outcomes – including increased survival. Although HPV is an etiological factor in many oral cancers, and is more likely to initiate oral cancers in those who do not consume alcohol or use tobacco, it may not necessarily be the starting point for immortalization and transformation for all oral cancers. The authors have reviewed the text carefully and have concluded the following section accurately describes these concepts, as follows:

Background, Lines 55 – 62: The primary risk factors for oral carcinogenesis have been tobacco and alcohol use, although new lines of evidence now suggest HPV may also be an independent risk factor [19-21]. 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 [22-24]. In addition, new evidence now suggests that some high-risk HPV strains, such as HPV16 and HPV18, may initiate oral carcinogenesis among the smaller fraction of oral cancer patients who do not consume alcohol or use tobacco [25,26].

Reviewer 2 - Comment 5: To this end, less invasive methods, such as oral lavage- and saliva-based screening have yielded significant results. What the authors mean by significant results? The authors concur this statement is vague and have revised the text to more accurately reflect that HPV status in healthy adults may be determined using methods that do not require painful or invasive procedures, including biopsy (as follows):

Background, Lines 70-72: To this end, methods less invasive than biopsies, such as oral lavage- and saliva-based screening have been used to determine oral HPV status not only in oral cancer patients, but among healthy adults [33-36].

Reviewer 2 - Comment 6 part I: The authors specify in details the amount of DNA gained from saliva’s, but since internal control for PCR and housekeeping gene for qPCR was used it is irrelevant information. The authors have reviewed these comments and determined that this information may, in fact, be useful to other authors who may be attempting to use the same protocol for similar purpose (also published in BMC Oral Health last year - Turner et al., 2011; Highly accessed). Although not all information is relevant for qPCR, any centers using PCR for this type of screening would need to ascertain the DNA concentration in order to process any samples.

Reviewer 2 - Comment 6 part II: Why didn’t the authors used also primers for HPV 18? How the authors confirm the sensitivity of PCR when qPCR was type specific for HPV 16 only and one sample contained HPV 18? The authors have reviewed these comments and apologize for this oversight. The revised text now reads:

Methods, Lines 260 – 263: HPV18 E7 forward primer 5’-GACTCAGAGGAAGGAAAACGATGAAA, HPV18 E7 reverse primer 5’-
GTGACGTTGTGGTTCCGCT; HPV18 E7 probe 5'-TGGAGTTAATCATCAACATTTACCA was used to amplify the 25 bp region between the 715 and 739 nt position.

Reviewer 2 – Comment 7: Why didn`t the authors used system which allows for the detection of a wide number of HPV types when prevalence in healthy individuals was studied? This comment is similar to the previous comment (Reviewer 2 – Comment1); The authors have designed and implemented this project based upon the only other study of oral HPV ever completed in Nevada (Turner et al., BMC Oral Health 2011, 11:28). The original study of healthy adults screened for the most prevalent high-risk HPV strains found in the oral cavity, HPV16 and HPV18. Based upon this evidence, the current project also screened for HPV16 and HPV18, as these two account for the vast majority of all HPV strains detected in the oral cavity. The text of the manuscript, however, has been modified to more accurately reflect why HPV16 and HPV18 were chosen for the current study of oral HPV screening in children, as follows:

Background, Lines 64 – 69: Many of these studies have provided valuable epidemiological information regarding HPV in oropharyngeal tumors and case-matched controls [27-29]; demonstrating HPV16, and HPV18 to a lesser extent, accounted for the overwhelming majority (71-94.7%) of high-risk oral HPV detected [15,16, 19-21,27-29]. More recent efforts, however, have focused specifically on estimating oral HPV prevalence and transmission within healthy populations, also demonstrating that HPV16 and HPV18 were the most commonly detected oral high-risk HPV strains [30-32].

Reviewer 2 – Comment 8: Please specify in more details the process of sampling. The authors have reviewed this comment and have made some revisions to the Methods section to detail more specifically the process of sampling. Unfortunately, due to the retrospective nature of this study, some details are beyond the capability of these researchers to provide. The revised text now reads:

Methods, Lines 188- 198: The protocol for this study titled “Retrospective Evaluation of Microbial Presence in Existing Saliva Repository: A PCR-Based Molecular Survey of Oral Microbial Populations from Existing Saliva Samples” was filed, amended, and approved by the UNLV Office of Research Integrity – Human Subjects (OPRS#1104-3801M) on May 10, 2011. The existing saliva samples were collected during two previous studies within the UNLV School of Dental Medicine (SDM) during 2009-2010. Saliva samples collected from a prior study of teenagers (14-15 years old) were derived from a convenience sample of selected schools within the Clark County, Nevada School District (CCSD; n=48); originally obtained for the purpose of surveying levels of oral cariogenic bacteria. Saliva samples collected from young children (2-11 years old) were obtained from a convenience sample within the UNLV-SDM pediatric dental clinic (n=70); originally obtained for the purpose of screening for heavy metal (lead or Pb) burden.
Reviewer 2 – Comment 9: Why did the authors count the cells when they didn’t use a fixed input number for PCR? Using the procedures and protocol recommended by the manufacturer, the specifications of kit suggest a minimum number of cells – which was determined as follows:

Methods, Lines 218 – 226: All samples were centrifuged for 10 minutes at 2,100 g (RCF) and the 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). To determine if any samples harbored the HPV virus, DNA was isolated from the saliva sample using a minimum of 3.5 x 10^5 cells using the GenomicPrep DNA isolation kit (Amersham Biosciences: Buckinghamshire, United Kingdom), using the procedure recommended by the manufacturer as previously described [10,42,57,58].

Reviewer 2 – Comment 10: Figure 1 is redundant. The authors have reviewed this comment and do not see how this information is redundant and have chosen to retain this figure.

Reviewer 3 – General comments: A recent pilot study provided new information about oral HPV status in healthy adults from Nevada, a state recently documented with rising rates of oropharyngeal cancers. The goal of present study is to provide more detailed information about oral prevalence of high-risk HPV among children and teenagers in Nevada. The topic raised by the paper is interesting for the understanding of the natural history of HPV infection in oral cavity. A small subset of saliva samples (2.5%) were found to harbor high-risk HPV16 and HPV18.

Reviewer 3 – Comment 1: The authors only reported the prevalence for HPV16 and 18 giving only a partial view of the high risk HPV types prevalence in oral cavity. Why the authors didn’t look for all the high risk HPV types using commercially available HPV assays? This comment is similar to the previous comment (Reviewer 2 – Comment1); The authors have designed and implemented this project based upon the only other study of oral HPV ever completed in Nevada (Turner et al., BMC Oral Health 2011, 11:28). The original study of healthy adults screened for the most prevalent high-risk HPV strains found in the oral cavity, HPV16 and HPV18. Based upon this evidence, the current project also screened for HPV16 and HPV18, as these two account for the vast majority of all HPV strains detected in the oral cavity. The text of the manuscript, however, has been modified to more accurately reflect why HPV16 and HPV18 were chosen for the current study of oral HPV screening in children, as follows:

Background, Lines 64 – 69: Many of these studies have provided valuable epidemiological information regarding HPV in oropharyngeal tumors and case-matched
controls [27-29]; demonstrating HPV16, and HPV18 to a lesser extent, accounted for the overwhelming majority (71-94.7%) of high-risk oral HPV detected [15,16, 19-21,27-29]. More recent efforts, however, have focused specifically on estimating oral HPV prevalence and transmission within healthy populations, also demonstrating that HPV16 and HPV18 were the most commonly detected oral high-risk HPV strains [30-32].

Reviewer 3 - Comment 2: The 3 samples HPV DNA positives should also be checked for RNA expression in order to see if these infections are active. The authors concur with this comment, however, the retrospective nature of this study has many inherent limitations. More specifically, most of the saliva samples contained barely enough saliva in order to process each sample for the DNA isolation - which did not allow for any additional assays to be completed. The authors will, however, include in the protocol for all future studies of this nature - a provision to allow for the collection of enough sample to enable these types of assays and analysis to be facilitated.

Reviewer 3 - Comment 3: The authors reported the case of 3 males which are HPV positives without giving the ages of the corresponding participants, this information is relevant so it should be furnished by the authors. The authors concur and have revised the results and figure legends, as follows:

Results, Lines 127-129: The two HPV16-positive samples were collected from White participants aged 14 (n = 2/35 or 5.7%), while the HPV18-positive sample was collected from a non-White participant aged 15 (n = 1/83 or 1.2%).

Figure Legends, Lines 520 – 527:
Figure 2. Saliva sample screening: analysis of qPCR HPV results. A) Three samples were found to harbor HPV DNA: HPV16-positive samples (S5, S38); One sample was HPV-positive (S7). B) qPCR analysis revealed HPV-positive samples had values well above the established cutoff value (> 0.001 copies/genome). C) All three HPV-positive samples were male, representing 2.5% of the total (n = 118). Two of the three HPV-positive samples were from White participants. All three HPV-positive samples were from the CCSD cohort (ages: 12-17); Both HPV16-positive samples were obtained from 14-year old participants, while the HPV18-positive sample was obtained from a 15-year old.

Reviewer 3 - Comment 4: The authors wrote pPCR instead of qPCR. The authors apologize for this oversight and have corrected the results section, as follows:

Results, Lines 112-119: Processing of DNA samples using qPCR provided quantitative assessments, as well as measurements of sensitivity and specificity. Analysis of copy number per genome for the housekeeping gene (β-actin) for the HPV-positive (range: 25 - 40 copies/genome) and HPV-negative samples (4 – 93 copies/genome) revealed values that were well above the cutoff value (> 0.1 copies/genome). Results of qPCR analysis revealed copy numbers of HPV-positive samples (range: 150 – 880 copies/genome) that were significantly higher than HPV-negative samples (range: 0.00148 – 0.0000016
copies/genome), which could be distinguished using the cutoff value (> 0.001 copies/genome).

Reviewer 4 Comments: 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 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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