



## **Screening for Oral High-risk Human Papillomavirus (HPV) among a Pediatric Patient Population**

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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### **ABSTRACT**

**Introduction:** Human papillomavirus (HPV) is a known cancer-causing virus that has been primarily linked to cervical cancers. Recent studies have shown that HPV may also induce cancer in other tissues, including oral epithelia and mucosa. To determine the presence of oral HPV infection among pediatric patients, salivary samples were assessed to determine oral prevalence of high-risk HPV.

**Methods:** Using existing pediatric saliva samples from pediatric clinic patients (aged 6 - 16 years), this retrospective study involved isolating DNA for PCR screening for both high-risk strains of HPV (HPV16, HPV18). A total of n=98 patient samples were available for analysis.

**Results:** Demographic analysis of these samples revealed the majority were derived from females (63.2%) and mostly from Hispanics (54.1%). DNA was successfully isolated from 95.9% of samples (n=94/98) with an average concentration of 142.5 ng/uL. PCR screening demonstrated only a subset of patient samples harbored high-risk strains, HPV16 (n=9) and HPV18 (n=6), which

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represents 15.9% of the total sample population - and were concentrated among the older patients (>13 years old).

**Conclusions:** Although this retrospective study does not have a sufficient sample size to provide more robust analysis of other risk factors (race, sex, sociodemographic), these results to demonstrate that oral HPV infection may be successfully detected among a pediatric population. As more studies demonstrate oral HPV infection in children, these data are 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.

*Keywords: Human papillomavirus; saliva; oral screening.*

## ABBREVIATIONS

*Human papillomavirus (HPV); Institutional Review Board (IRB); Office for the Protection of Research Subjects (OPRS); University of Nevada, Las Vegas – School of Dental Medicine (UNLV-SDM); Polymerase chain reaction (PCR); Glyceraldehyde- 3- phosphate dehydrogenase (GAPDH); Limit of detection (LOD).*

## 1. INTRODUCTION

Human papillomavirus (HPV) is a known cancer-causing virus that has been primarily linked to cervical cancers [1,2]. The early proteins (E5,E6,E7) known to facilitate viral mRNA production and genome replication are also known to influence cellular phenotypes, promoting oncogenesis and allowing multiple pathways for bypass of traditional cell-cycle regulatory mechanisms [3]. For example, known interactions between early HPV virus proteins and both p16 and p53 have been demonstrated not only to mediate the oncogenic phenotypes, but also to provide clinical prognostic value for patient outcomes [4,5].

Recent studies have shown that HPV may also induce cancer in other tissues, including prostate, penile, oral epithelia and mucosa [6-8]. In fact, HPV infection has been identified as a clinically significant biomarker and prognostic indicator for oral squamous cell carcinomas [9-11]. These studies suggest that oral infection with high-risk HPV may significantly increase the risk of developing oral cancers, as well as limiting the long-term prognosis for these patients [10,11]. Due to the long time horizon for HPV-driven oncogenesis and oral tumor development, a greater focus has been placed on early detection and prevention efforts [12-14].

Previous studies from this group have attempted to assess the prevalence of HPV infection among the adult and pediatric clinic populations [15-17]. These studies appeared to support evidence that has suggested the incidence of oral HPV infection in the United States may, in fact, be

increasing [8,9,11]. However, other recent studies of microbial prevalence have recently increased the potential pool of oral samples available for further HPV screening and analysis [18,19]. To determine the presence of oral HPV infection among pediatric patients, salivary samples were assessed to determine oral prevalence of high-risk HPV.

## 2. METHODOLOGY

### 2.1 Study Approval

This project was reviewed and approved by the Institutional Review Board (IRB) and the Office for the Protection of Research Subjects (OPRS) at the University of Nevada, Las Vegas – School of Dental Medicine (UNLV-SDM) as a retrospective analysis under OPRS#880427-1 “The Prevalence of Oral Microbes in Saliva from the University of Nevada Las Vegas (UNLV) School of Dental Medicine (SDM) pediatric and adult clinical population”.

### 2.2 Inclusion and Exclusion Criteria

The initial collection of samples involved both Informed Consent and Pediatric Assent from patients aged seven years and older. Inclusion criteria were patients of record at UNLV-SDM who also agreed to participate. Exclusion criteria included any person not a patient of record at UNLV-SDM and any parent or child who declined to participate.

### 2.3 Study Protocol

In brief, all participants were given a sterile container and were asked to provide between 2 –

5 mL of unstimulated saliva. Each sample was assigned a randomly generated, non-duplicated identifier, which allowed for samples to be archived without any patient-specific information associated with them. Concurrent collection of basic demographic information included only Sex, Race or Ethnicity and Age at the time of collection. No specific patient-identifying information was collected or associated with any sample and no patient-specific information was subsequently available to any research member.

## 2.4 DNA Isolation

Each sample was stored in a biomedical laboratory at -80°C until processed for analysis. In brief, DNA was isolated using the GenomicPrep DNA isolation kit from Amersham Biosciences using the manufacturer recommended protocol. In brief, this involved phenol:chloroform extraction with centrifugation and phase separation. DNA was resuspended at stored in DNA rehydration solution at 4°C. Quality and quantity of DNA samples were assessed using spectrophotometric absorbance at 260 and 280 nm (A260:A280). Recommended quantity for each sample for subsequent PCR screening was 1.0 ug with quality (A260:A280 ratio) of 1.65 or higher.

## 2.5 PCR Screening

Screening for human papillomavirus (HPV) was accomplished using polymerase chain reaction (PCR) screening using the Fisher ExACTgene complete PCR kit and a Master cycler gradient thermocycler from Eppendorf. In brief, one (1.0) ug of DNA was used for each reaction, which consisted of denaturation at 94°C for three minutes and thirty (30) amplification cycles of denaturation at 94°C for thirty (30) seconds, annealing at the appropriate primer temperature, and thirty (30) seconds of final extension at 72°C. All PCR reactions were separated using gel electrophoresis and visualized using ultraviolet illumination of ethidium bromide-stained gels that were captured by a Kodak Gel Logic 100 Imaging System. The following PCR screening primers were synthesized by SeqWright:

### Positive control:

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

GAPDH 5'-ATCTTCCAGGAGCGAGATCC-3' (sense); 20nt; 55% GC; Tm=66°C

GAPDH 5'-ACCACTGACACGTTGGCAGT-3' (antisense); 20nt; 55% GC; Tm=70°C

Optimal PCR Tm: 65°C

### Screening primers:

HPV16 5'-ATGTTTCAGGACCCACAGGA-3' (sense); 20nt; 50% GC; Tm=66°C

HPV16 5'-CCTCACGTCCGAGTAACTGT-3' (antisense); 20nt; 55% Tm=67°C

Optimal PCR Tm: 65°C

HPV18 5'-ATGGCGCGCTTTGAGGATCC-3' (sense); 20nt; 60% GC; Tm=71°C

HPV18 5'-GCATGCGGTATACTGTCTCT-3' (antisense); 20nt; 50% GC; Tm=64°C

Optimal PCR Tm: 63°C

## 3. RESULTS

The demographic breakdowns of the samples available for this study were analyzed (Table 1). This analysis revealed that more than half were derived from female patients (63.2%), which was significantly higher than their overall percentage from the clinic population,  $p=0.0093$ . The analysis of the racial and ethnic breakdown of the samples revealed the majority of samples were derived from minority patients (59.2%) that were mostly Hispanic (54.1%), which is similar to the overall percentage from the clinic population,  $p=0.7001$ . The average age of the study sample was 10.7 yrs. (ranging between 6 and 16 years old), which was significantly lower than the overall demographics of the pediatric clinic which averaged 13.5 yrs. (ranging between 2 – 17 yrs.),  $p=0.0018$ .

Each saliva sample was then processed to extract DNA for the screening protocol (Table 2). These results demonstrated that DNA was successfully isolated from  $n=94/98$  samples, yielding an overall recovery rate of 95.9% - well within the manufacturer suggested range of 90-99%. The average concentration of DNA was 142.5 ng/uL, which ranged between 71 – 585 ng/uL. Measurements for the purity of the DNA isolated using the ratio of the absorbance at 260 nm and 280 nm revealed an average purity of

1.68, which is comparable and slightly above the manufacturer recommendation of 1.65.

The saliva samples with sufficient DNA quantity and quality were then screened using the PCR positive control gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) – a commonly used human glycolytic pathway enzyme (Fig. 1). This data demonstrated that from the total sample pool (n=118), only n=98 had a reliable, identifiable PCR signal band intensity for the positive control (GAPDH), resulting in a final yield of 83.1% (n=98/118). These samples were then selected for further screening for the HPV strains of interest.

Each of DNA isolates with sufficient DNA quality and quantity that also produced a positive signal for GAPDH (n=98) were then screened for the presence of HPV16 and HPV18 (Fig. 2). These data demonstrated that only a subset of patient samples harbored high-risk strains HPV16 (n=9) or HPV18 (n=6). These results represented 15.9% of the total sample population and were highly concentrated among the older patients from this study (> 13 years old).

#### 4. DISCUSSION

The primary objective of this study was to determine the presence of oral HPV infection among pediatric patients using an existing saliva repository to screen for high-risk HPV. This study

was successful in screening nearly one hundred samples for HPV16 and HPV18, the main high-risk HPV strains found in the oral cavity of both children and adults [20]. These data support the growing body of evidence that high-risk HPV stains may, in fact, infect the oral cavity of pediatric patients, which may be acquired through a variety of means including fomites, shared eating utensils, and close personal contact [16,17].

In addition, these data support the findings from other groups that demonstrated oral HPV frequency and prevalence between 5 – 12% among other pediatric patient populations [21,22]. As more research in the area of pediatric oral HPV infection develops, these detection methods may be utilized in other health related fields to provide greater understanding of pediatric risk and disease potential [23,24]. Furthermore, these data will be critically important to oral health researchers and dental professionals as they provide clinical guidance and health advice for parents and pediatric patients considering HPV vaccination [25].

Despite the significance of these findings, several limitations to this study must also be considered. First, this was a retrospective study of samples from an existing saliva repository and may not necessarily reflect the oral HPV prevalence of the current patient population –

**Table 1. Demographic analysis of study sample**

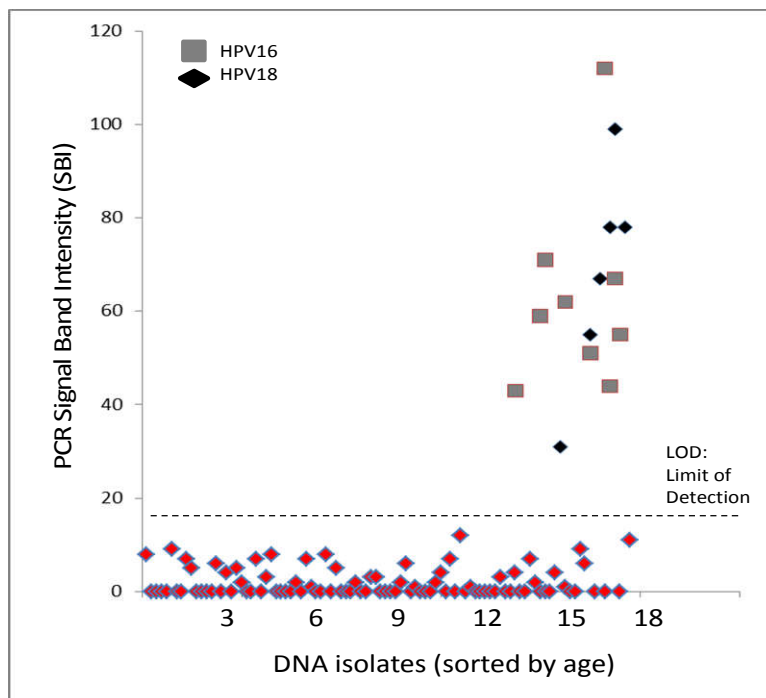
|                       | Study sample (n=98) | Clinic population | Statistical analysis                |
|-----------------------|---------------------|-------------------|-------------------------------------|
| <b>Sex</b>            |                     |                   |                                     |
| Female                | 63.2% (n=62)        | 49.1%             | $\chi^2=6.763$ , d.f.=1<br>p=0.0093 |
| Male                  | 36.8% (n=36)        | 50.9%             |                                     |
| <b>Race/Ethnicity</b> |                     |                   |                                     |
| White                 | 40.8% (n=40)        | 41.4%             | $\chi^2=0.148$ , d.f.=1<br>p=0.7001 |
| Minority              | 59.2% (n=58)        | 58.6%             |                                     |
| Hispanic              | 54.1% (n=53)        | 48.1%             |                                     |
| Black                 | 3.1% (n=3)          | 6.3%              |                                     |
| Asian/Other           | 2.0% (n=2)          | 4.2%              |                                     |
| <b>Age</b>            |                     |                   |                                     |
| Average               | 10.7 yrs.           | 13.5 yrs.         | Two-tailed t-test<br>p=0.0018       |
| Range                 | 6-16 yrs.           | 2 – 17 yrs.       |                                     |

**Table 2. DNA isolation and analysis**

| Study samples                        | DNA analysis    |
|--------------------------------------|-----------------|
| DNA concentration (average)          | 95.9% (n=94/98) |
| DNA concentration (range)            | 142.5 ng/uL     |
| DNA purity (A260:A280 ratio) average | 71 – 585 ng/uL  |
| DNA purity (A260:A280 ratio) range   | 1.68            |
|                                      | 1.32 – 1.93     |



**Fig. 1. PCR screening of saliva samples for positive control. Screening of all DNA isolates (n=118) for the positive control (human GAPDH) revealed positive results for n=98 samples (yield 83.1%; n=98/118) above the reliable limit of detection (LOD)**



**Fig. 2. PCR screening of saliva samples for high-risk HPV strains. Screening of DNA isolates (n=98) for high-risk HPV strains revealed 9.2% (n=9/98) harbored HPV16 and 6.1% harbored HPV18 (n=6/98), which were discernable above the reliable limit of detection (LOD). All positive samples were derived from patients > 13 years of age**

although the recent nature of this collection may reduce the potential for study bias [17,18]. In addition, the original saliva collection protocol involved a one-time, cross sectional sampling that does not allow for any temporal (before-and-after) analysis of HPV infection. For example, there is no data to demonstrate the length of time a particular sample harbored HPV or when the infection was acquired. Furthermore, as a retrospective study – there was no mechanism to identify or notify any patient of their oral HPV status, which may be a consideration for the design of future studies involving oral high-risk HPV prevalence. Another consideration is the fact that some of these patients, particularly over the age of 12, may have had one or more of the recommended HPV vaccinations, which might have influenced the findings of this study. Finally, although this study had a much larger sample size than other recent studies of oral HPV infection among pediatric patients [21,22], this study was limited to the number of previously collected pediatric saliva samples and could not be expanded to include additional samples.

## 5. CONCLUSIONS

Although this retrospective study does not have a sufficient sample size to provide more robust analysis of other risk factors (race, sex, sociodemographic), these results to demonstrate that oral HPV infection may be successfully detected among a pediatric population. As more studies demonstrate oral HPV infection in children, these data are 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.

## CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the author(s).

## ETHICAL APPROVAL

This project was reviewed and approved by the Institutional Review Board (IRB) and the Office for the Protection of Research Subjects (OPRS) at the University of Nevada, Las Vegas – School of Dental Medicine (UNLV-SDM).

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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