SCREENING OF ORTHODONTIC PATIENTS AND AGE-MATCHED NON- ORTHODONTIC SAMPLES FOR HIGH-RISK ONCOGENIC HUMAN PAPILLOMAVIRUS (HPV) STRAINS

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Abstract

SCREENING OF ORTHODONTIC PATIENTS AND AGE-MATCHED NON-ORTHODONTIC SAMPLES FOR HIGH-RISK ONCOGENIC HUMAN PAPILLOMAVIRUS (HPV) STRAINS

By

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Introduction: The human papillomavirus (HPV) is known to cause cancer in several tissues and organs, including the cervix and the oral cavity. Several oncogenic or high-risk strains have been identified, which include HPV16 and HPV18.However, other research has demonstrated that additional oncogenic, high-risk strains exist - including HPV31, HPV33, HPV35, HPV52 and HPV58, although much less is known about their prevalence. Based upon this lack of knowledge, the primary objective of this project is to address the prevalence of these strains among pediatric and adult patients through screening of saliva samples from the UNLV-SDM and orthodontic clinic populations.

Methods: The protocol for this study was reviewed and approved by the UNLV Institutional Review Board (IRB #1619329-1) titled "Retrospective analysis of Oral Health Status of Dental Population". Clinical samples from a saliva biorepository (N=253) were screened to provide age-matched pediatric and adult samples from the orthodontic and main patient clinics. Quantitative polymerase chain reaction (qPCR) screening of samples for high-risk HPV was performed using SYBR green master mix from Applied Biosciences and validated high-risk HPV primers.

Results: A total of N = 86 samples from the saliva biorepository met the quality and concentration standards and were screened for high-risk HPV. qPCR screening of adult samples revealed n = 10/45 or 22% were HPV31- or HPV33-positive. In addition, a total of n = 9/41 or 21.9% of pediatric samples were either HPV31- or HPV33-positive (or both). No samples harbored HPV35. Most samples were derived from patients within the recommended vaccination or catch-up age range (age 9-45 years).

Discussion and Conclusions: No previous studies from this institution have explored these high-risk HPV strains among this patient population. These results clearly demonstrate that a significant percentage of patients harbor additional high-risk HPV strains within the oral cavity, including HPV31 and HPV33. Although many studies promote the quadrivalent HPV vaccine (covering HPV6, 11, 16, and 18), these results suggest that oral healthcare providers may need to discuss the newer nine-valent vaccine, which includes HPV31 and HPV33. It might have a beneficial effect on this certain population to make a larger emphasis on this newer vaccine and the prevention potential to the general public.

Key words: Human papillomavirus (HPV); High-risk HPV; saliva screening; qPCR screening

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Chapter 1: Introduction

Background and Significance

Human Papillomavirus (HPV) is a non-enveloped, double stranded DNA virus that is epitheliotropic in nature [1]. There are between 150-200 different strains of HPV. Roughly 40 types cause cutaneous or mucosal infections. This includes infections of the skin, anogenital tract, and oral cavity - as HPV infects squamous cells in various cells and tissues. [1,2].
HPV can spread via skin-to-skin contact or through sexual intercourse [1-3]. It is estimated that there are over 42 million Americans who are currently infected with HPV [2].

With over 12% of the United States population currently infected, there are an estimated 13 million new HPV infections each year, just in the United States [2]. Most HPV infections (approximately 90%- 95%) are cleared by the immune system within 2 years [3]. However, some strains may cause infections that do not clear and can persist to cause precancerous abnormalities and eventual malignancy [1-4].

There are High Risk and Low Risk Strains of HPV based on their likelihood of causing cancer. The Low Risk HPV strains mostly do not cause disease and are often associated with warts, both anogenital and oral, including HPV6, 11, 40, 42, 43, 44, 53, 54, 61, 72, 73, and 81 [5]. The High Risk strains of HPV are associated with a variety of cancer HPV-related cancers including Cervical cancer, Oropharyngeal Cancer, Anal Cancer, Penile Cancer, and Vaginal Cancers. It is

well documented that HPV 16 and 18 are the main two strains relating to causing most of these cancers [6].

However, in addition to types 16 and 18, the International Agency for Research on Cancer (IARC) has identified 10 other High Risk strains: HPV31, 33, 35, 39, 45, 51, 52, 56, 58, and 59. [7]. Due to the limited focus on two specific strains of HPV (found in the majority of both cervical and oral cancer cases), the true extent of oral high-risk HPV infection and exposure may be unknown. Although many additional HPV strains (listed above) are also known to infect the oral cavity, studies in this population are extremely limited [8]. In addition, the ability for cross protective antibodies against these strains cannot be determined until the incidence, prevalence and range of HPV strains is more clearly understood.

Research Questions

Research question: What is the incidence and prevalence of various "overlooked " high risk oral strains of human papilloma virus?

- Null Hypothesis: Oral high risk HPV strains are uncommon and their prevalence are not statistically significant
- Alternative Hypothesis: Oral high risk HPV strains are common and more prevalent than previously thought and are statistically significant

Is there a relationship between age, race, and gender with these specific HPV strains?

● Null Hypothesis: There is no relationship amongst the given criteria and the HPV strains being investigated

● Alternative Hypothesis: There is a relationship/correlation with the given criteria and the HPV strains being investigated

Approval

The appointment of an advisory committee was submitted for approval and approved on June 29, 2022. The prospectus for this study was submitted for approval and approved on September 2, 2022. This study involved a retrospective analysis of previously collected saliva samples stored in an existing biorepository. No human subjects were recruited in this study.

Research Design

The methods used for sequencing the HPV strains will be replicated using materials and methods established in previous research studies here at UNLV [9,10]. The samples being used in this investigation are from previously collected saliva samples from voluntary participants (saliva repository).

Study approval

This study was reviewed and approved by the UNLV IRB under protocol [1717625-1] Retrospective analysis of microbial prevalence from DNA isolated from saliva samples originally obtained from the University of Nevada, Las Vegas (UNLV) School of Dental

Medicine (SDM) pediatric and clinical population on March 3, 2021.

Inclusion category

Samples from Patients Ages 7-26. This is based on the CDC's Advisory Committee on Immunization Practices (ACIP) The current ACIP recommended age range for the HPV vaccine is from ages 11 to 26. The target age is 11 to 12 years, although it may start at 9 years of age and can be given above the age of 26, specifically from 27 to 45 years of age, but is not recommended.

Exclusion category

Any patient sample over the age of 80. Any samples from patients outside the school of dental medicine at University of Nevada, Las Vegas

Saliva Samples

The following are the methods for handling saliva samples, which will be used [8-10]. Saliva samples will be centrifuged for 10 minutes at 2,100g (RCF) and the cell pellet washed with 1X phosphate-buffered saline and re-centrifuged. The pellet will be resuspended in 5mL of 1X phosphate-buffered saline. HPV DNA was isolated from the sample using the Genomic Prep DNA isolation kit. DNA purity and quantity will then be calculated prior to qPCR using the NanoDrop spectrophotometer.

Quantitative PCR (qPCR)

qPCR will be used to provide more specificity and sensitivity for the various HPV strains being tested. The validated primers for this study include: [8-10].

HPV 31

Forward primer: ATTCCACAACATAGGAGGAAGGTG

Reverse primer: CACTTGGGTTTCAGTACGAGGTCT

HPV 33

Forward primer: ATATTTCGGGTCGTTGGGCA

Reverse primer: ACGTCACAGTGCAGTTTCTCTACGT

HPV 35

Forward primer:TCGGTGTATGTCCTGTTGGAAAC

Reverse primer: CATAGTCTTGCAATGTAGTTATTTCTCCA

HPV52

Forward primer:GACATGTTAATGCAAACAAGCGAT

Reverse primer: CATGACGTTACACTTGGGTCACA

HPV 58

Forward primer:GGCATGTGGATTTAAACAAAAGGT

Reverse primer: TCTCATGGCGTTGTTACAGGTTAC

Statistical analysis

Descriptive statistics will be provided for the patient demographics. A chi-square test will be used to compare demographic characteristics (age, race, gender) and various oncogenic HPV strains. The mean age, range, and variance will also be calculated.

Anticipated Findings and Potential Impact

This investigation hopes to provide insight and shed light on the prevalence and incidence of these other oncogenic strains that are often omitted from HPV research. The research performed can have major implications, such as demonstrating “at risk” groups of individuals. Additionally, this research project will provide information to determine which strains are present and prevalent, which will help others to evaluate if there may be cross protective antibodies against these strains with the current HPV vaccines available. Lastly, the researchers of this investigation hope that their findings are able to provide an update recommendation for HPV vaccination.

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Chapter 2

Screening for High-Risk Oral Human Papillomavirus (HPV31, HPV33, HPV35) in a Multi-Racial Pediatric and Adult Clinic Patient Population

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Role of Authors:
KK and KMH were responsible for the overall project design. HH, SC, and JRS were responsible for data generation and analysis. KK, KMH and HH contributed to the writing and editing of this manuscript. All authors have read and agreed to the published version of the manuscript.

Abstract

Many human papillomavirus (HPV) strains induce cancer in the cervix and the oral cavity. Although high-risk strains including HPV16 and HPV18 are common - additional high-risk strains including HPV31, HPV33 and HPV35 may induce carcinogenesis although much less is known about their prevalence. Using an approved protocol, samples from a salivary biorepository were screened to find pediatric and adult samples from a university-based patient clinic population. A total of N=86 samples from the saliva biorepository met the quality and concentration standards and were screened for high-risk HPV. qPCR screening of adult samples revealed n=10/45 or 22.2% were HPV31- or HPV33-positive In addition, a total of n=9/41 or 21.9% of pediatric samples were either HPV31- or HPV33-positive (or both). No samples harbored HPV35. The majority of these samples were derived from patients within the recommended vaccination age range (age 9 - 26 years) or within the catch up age range (27 - 45

years). These results clearly demonstrate that a significant percentage of patients harbor additional high-risk HPV strains within the oral cavity, including HPV31 and HPV33. Although many studies have promoted the quadrivalent HPV vaccine (covering HPV6, 11, 16, and 18), these results suggest that oral healthcare providers may need to promote the newer nine-valent vaccine, which includes HPV31 and HPV33.

Key words: Oral screening, saliva, high-risk human papillomavirus (HPV), qPCR

Introduction

Human Papillomavirus (HPV) is a non-enveloped, double stranded DNA virus that is epitheliotropic in nature and is known to cause disease in a variety of tissues [1,2].There are between 150-200 different strains of HPV some of which are known to cause human disease, including cancer [3,4]. Approximately 40 types of HPV are known to cause cutaneous or mucosal infections within human hosts [5,6].

HPV infections can spread through skin-to-skin contact through both sexual and non-sexual transmission pathways [7,8]. Epidemiologic prevalence estimates suggest that more than 40 million people in the United States are currently infected with some form of HPV, with an incidence of more than 10 million new cases per year [9-11]. Although most HPV infections may be cleared by the immune system (90-95% by some estimates), some infections result in long-term infections that cause precancerous abnormalities and malignancies if left untreated [12-14]. The main clinical distinction between the many human papillomavirus (HPV) strains lies in their ability to mediate cellular transformation into cancer within various tissues, which are broadly

categorized into low-risk (LR) or high risk (HR) depending upon their most frequently associated clinical outcomes [15,16]. The low-risk HPV strains 6 and 11 are responsible for >90% of anogenital warts and lesions, but also include additional commonly identified strains such as 40, 42, 43, and 44 [17,18]. In addition, high-risk strains HPV 16 and 18 are responsible for the vast majority of cervical as well as the majority of HPV-associated oropharyngeal cancers, although there are many additional high-risk HPV strains commonly identified including HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 [19,20].

Although high-risk strains including HPV16 and HPV18 are the most frequently identified - additional high-risk strains found in the cervix and oral cavity including HPV31, HPV33 and HPV35 may induce carcinogenesis although much less is known about their prevalence [21-23]. Based upon this understanding of additional clinically-relevant strains of HPV, the new nine-valent HPV vaccine incorporates not only the most common high-risk (16 and 18) and low-risk (6 and 11) HPV strains, but also includes additional high-risk strains such as HPV 31, 33, 45, 52, and 58 [24-26]. Although several studies from this institution have evaluated the prevalence of high-risk oral HPV strains 16 and 18 among both adult and pediatric patient populations, no study to date has evaluated these additional high-risk strains, such as HPV31, 33 and 35 [27-30]. Based upon lack of evidence regarding these high risk strains, the primary objective of this project is to evaluate the prevalence of these strains among pediatric and adult patients through screening of clinical saliva samples.

Materials and Methods

Study approval

This study was reviewed and approved by the University of Nevada, Las Vegas (UNLV) Institutional Review Board (IRB) under protocol [1717625-1] Retrospective analysis of microbial prevalence from DNA isolated from saliva samples originally obtained from the University of Nevada, Las Vegas (UNLV) School of Dental Medicine (SDM) pediatric and clinical population on March 3, 2021.

Human Subjects and Informed Consent

The original collection protocol was approved by the UNLV IRB under protocol OPRS#1305-4466M "The Prevalence of Oral Microbes in Saliva from the UNLV School of Dental Medicine Pediatric and Adult Clinical Population". Inclusion criteria included patients between the ages of 5 to 45 years of age that agreed to provide Informed Consent (adult over 18 years of age) or Pediatric Assent with Informed Consent (children under 18 years of age with guardian or parental permission and consent). Exclusion criteria included any patients (or parents/guardians that refused to provide informed consent or pediatric assent) and any samples from patients outside the UNLV School of Dental Medicine.

Original sample collection protocol

Original sample collection study patients were voluntary participants. Following informed consent and/or pediatric assent, patients were provided a sterile 50 mL saliva collection tube.

Patients were asked to provide up to 5.0 mL of unstimulated saliva. Each tube was labeled with a randomly-generated, non-duplicated number to prevent the collection or subsequent disclosure of any patient-specific identifying information. Demographic information including the patient sex, age, race or ethnicity and orthodontic status was noted. Samples were then transferred to a biomedical laboratory for storage at -80C.

DNA isolation and analysis

A total of N=253 samples from the biomedical sample repository were identified for potential inclusion in the current retrospective analysis. DNA was isolated from each sample using the phenol:chloroform extraction method. In brief, samples were thawed, vortexed, and then 500 uL was transferred to a sterile microcentrifuge tube and mixed with 500 uL of TRIzol DNA isolation reagent from Invitrogen (Waltham, Massachusetts, USA). To each sample 200 uL of molecular grade Chloroform from Invitrogen (Waltham, Massachusetts, USA) was added prior to incubation on ice for 15 minutes. Samples were then centrifuged at 12,000X relative centrifugal force (RCF) for 15 minutes at 4C in a refrigerated microcentrifuge (Model 5425) from Eppendorf (Hamburg, Germany).

The upper aqueous phase (approximately 400 - 500 uL) was transferred to a new, sterile microcentrifuge tube and mixed with molecular grade Isopropanol from Invitrogen (Waltham, Massachusetts, USA) to precipitate the DNA. Each sample was then centrifuged using the settings described above. The isopropanol was removed and the DNA-containing pellet was washed with molecular grade Ethanol from Invitrogen (Waltham, Massachusetts, USA) and centrifuged for an additional 10 minutes. The ethanol was removed and the DNA was

resuspended using 100 uL of nuclease-free water from Fisher Scientific (Waltham, Massachusetts, USA). Each sample was analyzed for DNA quantity and DNA quality using a NanoDrop 2000 spectrophotometer from Fisher Scientific (Waltham, Massachusetts, USA) at absorbances of A260 and A280 nm. Samples with sufficient quantity (> 10 ng/uL) and sufficient quality (A260:A280 ratio > 1.65) were subsequently screened using qPCR.

qPCR screening

The study samples that met the minimum criteria for DNA quantity and DNA purity (N=86) were screened for high-risk HPV strains 31, 33 and 35 using quantitative polymerase chain reaction (qPCR). Each reaction consisted of 15 uL Fast SYBR Green Master Mix from Applied Biosystems (Waltham, Massachusetts, USA), 1.5 uL of forward primer, 1.5 uL of reverse primer, 2.0 uL of sample DNA and 5.0 uL of nuclease-free water. Reactions were performed using the QuantStudio 3 from ThermoFisher Scientific (Waltham, Massachusetts, USA) and the following validated primers:

HPV31 forward: ATTCCACAACATAGGAGGAAGGTG; 24 nt, 45.8% GC, Tm=62.9C

HPV31 reverse: CACTTGGGTTTCAGTACGAGGTCT; 24 nt, 50.0% GC, Tm=64.6C

HPV33 forward: ATATTTCGGGGTCGTTGGGCA; 20 nt, 50.0% GC, Tm=60.4C

HPV33 reverse: ACGTCACAGTGCAGTTTCTCTACGT; 25 nt, 48.0% GC, Tm=64.6C

HPV35 forward: TCGGTGTATGTCTGTTGGAAAC; 23nt, 47.8% GC, Tm=62.8C

HPV35 reverse: CATAGTCTTGCAATGTAGTTATTTCTCCA; 29 nt, 34.5% GC, Tm=61.8C

Statistical analysis

Demographic variables for the study sample were compiled and presented as simple, descriptive statistics. Analysis of differences between the study sample and the overall clinic population with respect to categorical variables, such as sex and race or ethnicity, were done using Chi square statistics which is appropriate for non-parametric data analysis. Analysis of qPCR screening results were also presented as simple descriptive statistics, such as percentages, and the differences between HPV-positive and HPV-negative samples were also analyzed using Chi square statistics and the GraphPad Prism software, Version 8 (San Diego, CA, USA). Comparisons for parametric data, such as age, were completed using two-tailed Student's t-tests, using an alpha level of 0.05 for statistical significance.

Results

A total of N=86 samples from an existing biorepository were identified for inclusion in this retrospective study, which were nearly equally divided between adults (52.3% or n=45/86, Table 1) and pediatric (47.7% or n=41/86) patients (Table 2). Analysis of the adults in the study sample revealed approximately half were derived from females 55.6% or n=25/45), which closely approximates the percentage of females in the overall clinic population (49.1%), p=0.1614 (Table 1). In addition, analysis of the demographic characteristics demonstrated that the majority of the adult study samples were derived from racial or ethnic minorities (55.6% or n=25/45), which was slightly lower but not significantly different from the percentages observed within the overall main clinic population (65.4%), p=.0592. Finally, the average age of the adult samples was found to be 41.5 years (range: 18 to 73 years), which closely

approximates the average age of the main patient clinic population of 42.3 years (range 18 to 89 years), p=0.7738.

Table 1. Demographic analysis of adult study samples.

Demographics	**Study sample Adults (n=45)**	**UNLV-SDM Clinic (Adult patient)**	**Statistical analysis**
Sex			
Adult - Females	(n=25) 55.6%	49.1%	X^2=1.961, d.f.=1
Adult - Males	(n=20) 44.4%	50.9%	p=0.1614
Race			
White	(n=20) 44.4%	34.6%	X^2=3.560, d.f.=1
Minority	(n=25) 55.6%	65.4%	p=0.0592
Hispanic	(n=13) 28.8%	58.6%	
Black	(n=6) 13.3%	10.2%	
Asian/Other	(n=6) 13.3%	6.6	
Age			
Mean	41.5 years	42.3 years	Two-tailed T-test
Range	18 - 73 years	18 - 89 years	p=0.7738

Analysis of the pediatric patients in the study sample revealed approximately half were derived from females 56.1% or n=23/41), which closely approximates the percentage of females represented in the overall pediatric clinic population (52.8%), p=0.5478 (Table 2). Analysis of the racial and ethnic distribution of these patients found that the overwhelming majority of the pediatric study samples were derived from racial or ethnic minorities (82.96% or n=34/41), which was higher but not significantly different from the overall percentages of minorities observed within the pediatric clinic population (75.34%), p=0.0647. Finally, the average age of the pediatric samples was 12.7 years (range 5 to 17 years), which closely approximated the average age of the overall pediatric patient clinic population of 10.4 years (range 0 to 17 years), p=0.2531.

Table 2. Demographic analysis of pediatric study samples.

Demographics	**Study sample Pediatrics (n=41)**	**UNLV-SDM Clinic (Pediatric clinic)**	**Statistical analysis**
Sex			
Pediatric - Females	(n=23) 56.1%	52.8%	X^2=0.361, d.f.=1
Pediatric - Males	(n=18) 43.9%	47.2%	p=0.5478
Race			
White	(n=7) 17.1%	24.7%	X^2=3.413, d.f.=1
Minority	(n=34) 82.9%	75.3%	p=0.0647
Hispanic	(n=23) 56.1%	52.1%	
Black	(n=4) 9.8%	11.8%	
Asian/Other	(n=5) 12.2%	11.4%	
Age			
Mean	12.7 years	10.4 years	Two-tailed T-test
Range	5 - 17 years	0 - 17 years	p=0.2531

Screening of the adult patients revealed that n=10/45 or 22.2% different samples harbored one

or more of the three high-risk strains of HPV analyzed, including 31, 33, and 35 (Figure 1). More specifically, n=4/10 or 40% of the HPV-positive samples harbored HPV31 while n=7/10 or 70% harbored HPV33 - including one that was also positive for HPV31. However, none of the samples evaluated harbored HPV35.

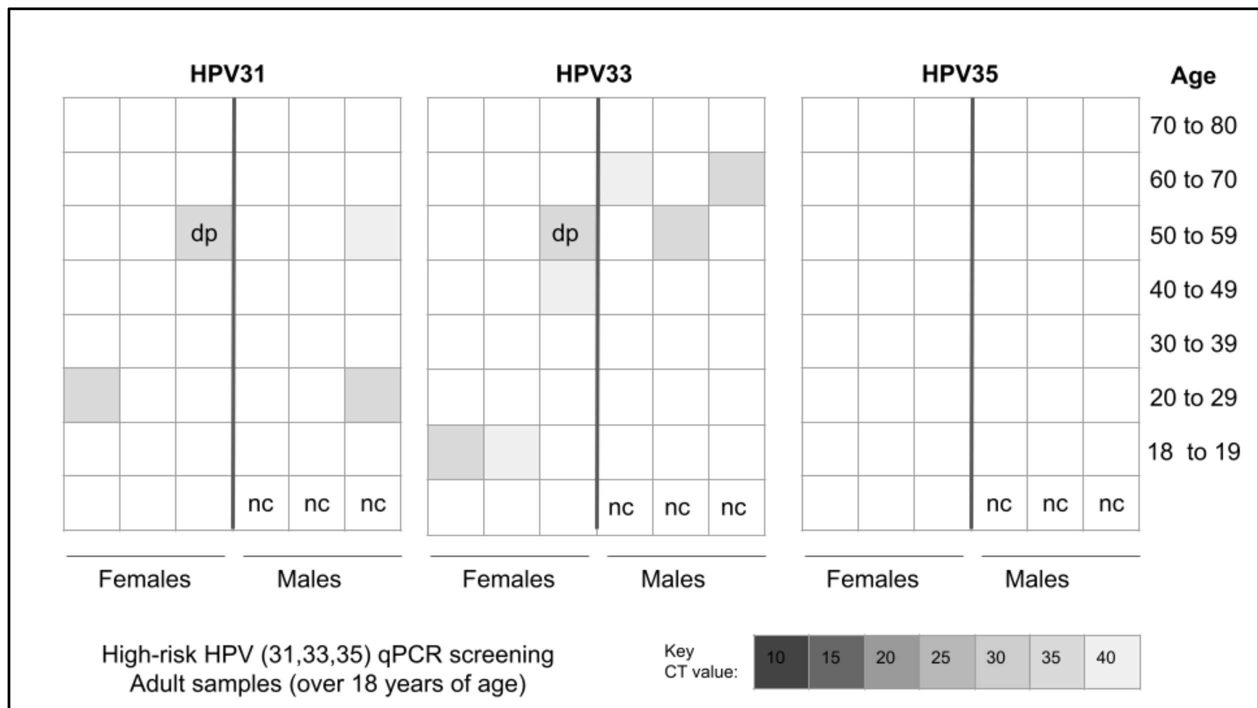

Figure 1. Heatmap analysis for qPCR screening of adult samples for high-risk HPV. A total of n=10 samples tested positive for HPV with n=4/10 or 40% testing positive for HPV31, n=6/10 testing positive for HPV33 and one sample tested positive for both HPV31 and HPV33. No samples tested positive for HPV35. (nc = negative control, dp=double positive)

More detailed analysis of the adult samples revealed an equal distribution of HPV-positive samples between males (n=5/10 or 50%) and females (n=5/10 or 50%), which closely matched the distribution of HPV-negative samples from males and females, p=0.5478 (Table 3). In addition, the analysis of race and ethnicity revealed the majority of HPV-positive samples were derived from minority patients (60%), which closely matched the proportion of HPV-negative samples from minority patients (54.3%), p=0.2286. Finally, the proportion of HPV-positive samples and HPV-negative samples from patients within the catch up range (under 45 years) was nearly equal and not significantly different (40%, 42.9%, respectively), p=0.5445.

Table 3. Demographic analysis of adult HPV-positive and HPV-negative samples.

Demographics	**HPV-positive**	**HPV-negative**	**Statistical analysis**
Adult - Males	50% (n=5/10)	42.9% (n=15/35)	X^2=0.361, d.f.=1
Adult - Females	50% (n=5/10)	57.1% (n=20/35)	p=0.5478
Total	22.2% (n=10/45)	77.8% (n=35/45)	
Adult - Non-Minority	40% (n=4/10)	45.7% (n=16/35)	X^2=1.449, d.f.=1
Adult - Minority	60% (n=6/10)	54.3% (n=19/35)	p=0.2286
Total	22.2% (n=10/45)	77.8% (n=35/45)	
Below Catch-up Age (Under 45 years)	40% (n=4/10)	42.9% (n=15/35)	X^2=0.367, d.f.=1
Above Catch-up Age (Over 45 years)	60% (n=6/10)	57.1% (n=20/35)	p=0.5445
Average Age	44.8 years	46.1 years	

Screening of the pediatric patient samples revealed that n=9/41 or 21.9% samples harbored one

or more of the three high-risk strains of HPV, such as 31, 33, and 35 (Figure 2). More specifically, n=6/9 or 66.7% of the HPV-positive samples harbored HPV31 while n=7/9 or 77.8% harbored HPV33 - including four that were double positive for both HPV31 and HPV33. However, none of the samples evaluated harbored HPV35.

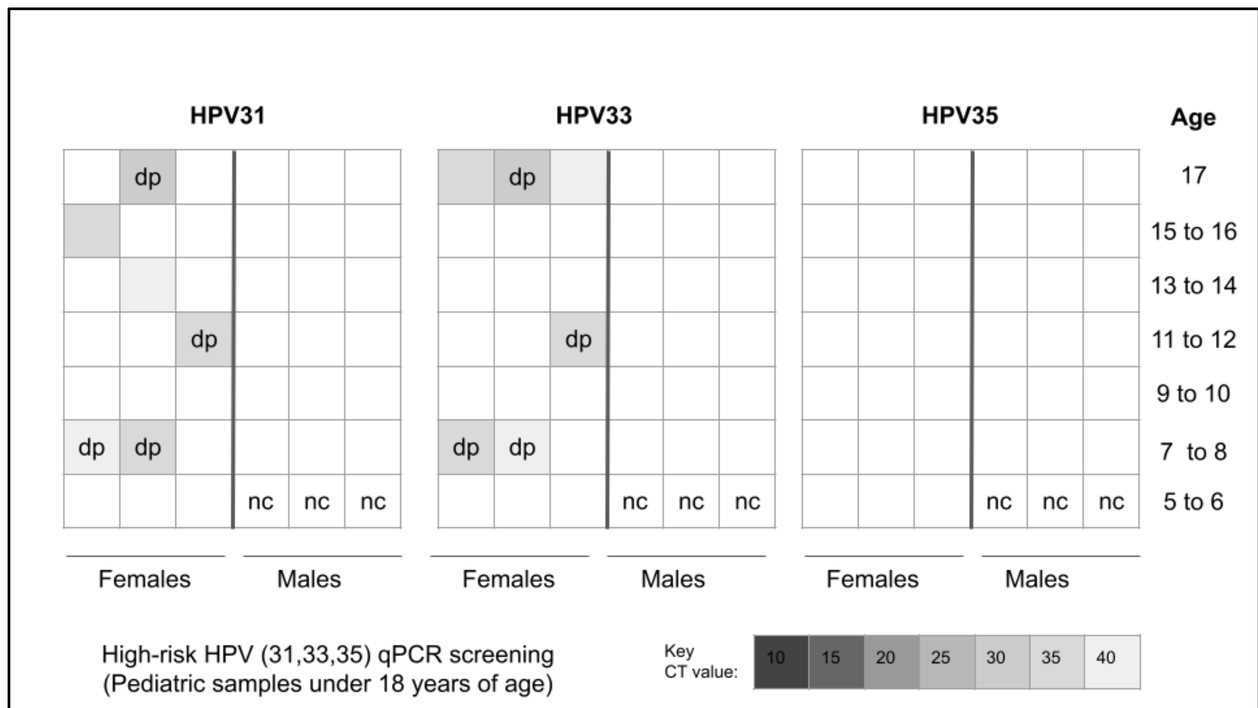

Figure 2. Analysis of heatmap for qPCR screening of pediatric samples for high-risk HPV. A total of n=9/41 or 21.9% of samples tested positive for HPV with n=6/9 or 66.7% testing positive for HPV31, n=7/9 or 77.8% testing positive for HPV33 and four samples testing positive for both HPV31 and HPV33. No samples tested positive for HPV35. (nc = negative control, dp=double positive)

More detailed analysis of the pediatric samples revealed an unequal distribution of HPV-positive samples between males (n=3/9 or 33.3%) and females (n=6/9 or 66.7%), which was significantly different from the distribution of HPV-negative samples from males and females, p=0.005 (Table 4). In addition, the analysis of race and ethnicity revealed the majority of HPV-positive samples were derived from minority patients (n=7/9 or 77.8%), which did not differ significantly from the proportion of HPV-negative samples from minority patients (n=27/32 or 84.4%), p=0.1017. Finally, the proportion of HPV-positive samples and HPV-negative samples from patients within the HPV vaccination age range (11 to 17 years) was significantly different (77.8%, 83.7%, respectively), p=0.0001.

Table 4. Demographic analysis of pediatric HPV-positive and HPV-negative samples.

Demographics	**HPV-positive**	**HPV-negative**	**Statistical analysis**
Pediatric - Males	33.3% (n=3/9)	56.9% (n=15/32)	X^2=7.868, d.f.=1
Pediatric - Females	66.7% (n=6/9)	53.1% (n=17/32)	p=0.005
Total	21.9% (n=9/41)	78.1% (n=32/41)	
Pediatric - Non-Minority	22.2% (n=2/9)	15.6% (n=5/32)	X^2=2.679, d.f.=1
Pediatric - Minority	77.8% (n=7/9)	84.4% (n=27/32)	p=0.1017
Total	21.9% (n=9/41)	78.1% (n=32/41)	
Within Vaccination Age Range (11 to 17 years)	77.8% (n=7/9)	93.7% (n=30/32)	X^2=45.390, d.f.=1
Below Vaccination Age Range (Under 11 years)	22.2% (n=2/9)	6.3% (n=2/32)	p=0.0001
Average Age	13.4 years	12.7 years	

Discussion

The primary goal of this study was to assess the prevalence of high-risk HPV strains 31, 33 and 35 using an existing biorepository including both pediatric and adult clinical saliva samples. The results of this study successfully demonstrated that HPV31 and HPV33 were found among both pediatric and adult samples in similar proportions (21.9%, 22.2% respectively), although no samples tested positive for HPV35. These data represent the first clinical descriptions of non-HPV16 and non-HPV18 high-risk HPV prevalence within this patient population [27-30]. However, some notable differences were found regarding the prevalence of these high-risk HPV strains compared with other previous studies of HPV strains HPV16 and HPV18. For example, this study found nearly one-quarter of adults (22.2%) harbored either HPV31, HPV33 or both. This is somewhat lower than the most recent study of HPV16 and HPV18 prevalence among adults within this patient population, which found an overall prevalence of 30.2% [27]. However, it is also much higher than the first description of HPV16 and HPV18 prevalence among adults from nearly a decade earlier that found an overall prevalence of only 2.6% within the same clinical patient population [30]. Moreover, these data also confirm other recent observations of HPV31 and HPV33 oral prevalence within other patient populations, which ranged between 5.7% and 14.3% [31-33].

In addition, these data also demonstrated that HPV31 and HPV35 were found in approximately one-fifth of pediatric patient samples (21.9%), which corresponds to similar prevalence levels of HPV16 and HPV18 found within these patients as recently as last year (19.5%) [27]. Although this represents the first non-HPV16 non-HPV18 screening within this patient population, the more aspect is the rise in pediatric oral HPV prevalence observed with HPV16 and HPV18, which was 2.5% in 2012, 9.2% in 2016, and 19.5% in 2022 [27-29]. As more studies confirm

oral prevalence levels of high-risk HPV among pediatric populations at similar levels, the case for screening and evaluating these additional HPV strains becomes more critical [34,35]. Despite the significance of epidemiological data regarding high-risk HPV prevalence as more and more studies are now screening for HPV strains other than HPV16 and HPV18, there are some limitations associated with this type of study that should also be considered [36,37]. More specifically, this was a retrospective study of previously collected saliva samples from an existing biorepository and may not reflect the most current oral prevalence, which may have shifted due to behavioral and vaccination practice changes following the onset of SARS-CoV-2 (COVID-19) pandemic [38,39]. In addition, due to the retrospective nature of this study - no other health information (oral or systemic) was available to determine if other factors, such as smoking, vaping or oral microbiota might have influenced the outcomes of this study [40-42]. Finally, due to the parameters of the original protocol these samples were part of cross-sectional studies involving one-time saliva collections and therefore have no information regarding the temporal nature of the HPV detected and whether this was a short- or long-term infection.

Conclusions

The importance of these findings, including the high prevalence of high-risk strains HPV31 and HPV33 detected in this study, combined with previous data about the increasing prevalence of HPV16 and HPV18 within this patient population may suggest a more robust and focused effort on HPV vaccination and awareness of oral HPV infection may be warranted [27-30]. However, recent evidence regarding increasing levels of vaccine hesitancy also suggest that more evidence

may be needed to demonstrate the relevance of HPV prevention - particularly among this patient population [43,44]. This study may be among the first to provide this type of evidence through the assessment and evaluation of oral HPV infection outside of the conventional HPV strains of HPV16 and HPV18.

Informed Consent Statement: The original protocol for the collection of saliva samples from the UNLV SDM clinic was approved under OPRS#1305-4466M titled “The Prevalence of Oral Microbes in Saliva from the UNLV School of Dental Medicine Pediatric and Adult Clinical Population”. Under this protocol, saliva samples were collected from volunteer patients at the beginning of their clinic appointment. Informed Consent was collected from adult patients who chose to participate, while Pediatric Patients above the age of seven were also required to provide Pediatric Assent in addition to the Informed Consent and approval of the accompanying guardian or parent.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 3

Summary and Conclusions

This study was the first to investigate and screen for the presence of high-risk HPV strains that are not HPV16 or HPV18 within this patient population. This study is also one of the only projects to date that has evaluated HPV31, HPV33 and HPV35 among oral samples from any patient population [1]. These findings therefore represent some of the earliest and most important indicators of high-risk HPV prevalence when combined with the numerous other studies from this institution and other groups that have found increasing HPV prevalence among both pediatric and adult populations over time [2].

In addition, the fact that the majority of the HPV-positive samples were derived from patient samples that were also within the recommended vaccination age range (9 - 26 years) or the updated catch up range (27 - 45 years) strongly suggests that these data may be extremely valuable to epidemiologists and public health professionals. Evidence-based research that can demonstrate the risk of HPV infection among patients within the recommended vaccination ranges could be an important tool to help combat the rising incidence of HPV hesitancy among young adults and their parents or guardians [3,4]. These data are part of a growing body of evidence that confirms the importance of prevention efforts, including the nine-valent HPV vaccine, which are demonstrated to reduce HPV-related diseases, including oral cancers [5].

Furthermore, this group has investigated other high-risk HPV strains, such as HPV52 and HPV58 and found that a total of n=4/45 or 8.9% of adult saliva samples tested positive for high-risk HPV52 and n=2/45 or 4.4% tested positive for high-risk HPV58. In addition, a total of n=6/42 or 14.3% of the pediatric saliva samples tested positive for high-risk HPV, including n=5/42 or 11.9% with HPV52 and n=3/42 or 7.1% for HPV58.. These data demonstrated the presence of the high-risk oncogenic HPV52 and HPV58 strains among both adult and pediatric clinical patient samples.

Research question: What is the incidence and prevalence of various “overlooked “ high risk oral strains of human papilloma virus?

- Null Hypothesis: Oral high risk HPV strains are uncommon and their prevalence are not statistically significant
- Alternative Hypothesis: Oral high risk HPV strains are common and more prevalent than previously thought and are statistically significant

Based upon this research, the null hypothesis must be rejected and the alternative hypothesis should be accepted.

Is there a relationship between age, race, and gender with these specific HPV strains?

- Null Hypothesis: There is no relationship amongst the given criteria and the HPV strains being investigated

● Alternative Hypothesis: There is a relationship/correlation with the given criteria and the HPV strains being investigated

Based upon this research, the null hypothesis must be rejected and the alternative hypothesis should be accepted.

Limitations and Recommendations

Although many limitations exist for any retrospective study, the primary limitation for this study was the sample size. In future studies, a larger sample size would be recommended to increase the ability to extrapolate results to a larger population - although the financial and other limitations would still likely present obstacles and challenges to overcome.

In addition, any prospective studies on these high-risk HPV strains may also want to record and evaluate whether the patients were already vaccinated for HPV. Almost half of younger adults are now receiving HPV vaccinations, although this number is highly variable and may be difficult to collect. If possible those studies may also include an evaluation of whether these patients already possess anti-HPV antibodies in the collected saliva, which is another variable that might provide more insight into the prevalence information already collected.

Appendix A

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