

## Epidemiology of oral HPV in the oral mucosa in women without signs of oral disease from Yucatan, Mexico

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### Abstract

High-risk human papillomaviruses (HR-HPV) are considered necessary for the development of cervical cancer. Furthermore, there is no doubt that some types of oral squamous cell carcinoma are associated with HR-HPV. The epidemiology of oral HPV infections in healthy subjects remains unclear due to a lack of knowledge. The objective of this study was to investigate the epidemiology of human papillomavirus infections of the oral mucosa without pathology. A cross-sectional study was performed; samples from 390 women seeking prenatal care, Pap smears, family planning or gynecological diseases were studied. Oral cells were collected by direct swab sampling. Information regarding sociodemographic status, sexual behavior, infectious diseases, contraceptive history and tobacco and alcohol consumption were obtained through direct interviews. HPV and genotypes were detected by type-specific polymerase chain reaction. Our results revealed that 14% of the women studied had an oral HPV infection. Women  $\leq 20$  years of age had the highest HPV prevalence (24.5%). In total, seven genotypes were identified, including the high-risk genotypes 16, 18, 58 and 59 and the low-risk genotypes 6, 81 and 13, the latter of which is a type exclusive to oral mucosa. Sexual behavior was not associated with the presence of genital HPV types in the oral mucosa. Genital HPV types were present in the oral mucosa of women without associated clinical manifestations; however, sexual behavior was not associated with infection, and therefore others routes of transmission should be explored.

**Key words:** Papillomavirus, oral mucosa, HPV13, Mexico.

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### Introduction

Human papillomavirus (HPV) belongs to a large family of small non-enveloped double stranded DNA viruses with an 8 Kb genome. These viruses infect basal cells of the squamous epithelium of the skin or mucosa (López-Saavedra, and Lizano-Soberón., 2006).

More than 100 HPV types have been described, 35% of which are generally found in the genitals. Genital HPV has been classified into high risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82); probable high-risk types (26, 53, and 66) and low-risk types (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108) according to their associ-

ation with neoplasias (De Villers *et al.*, 2004; Lizano *et al.*, 2009). Today, there is no doubt that oncogenic HPV infection is necessary for the development cervical cancer (Walboomers *et al.*, 1999).

During the past two decades, numerous studies have associated HPV infection with other epithelial neoplasias, such as head and neck squamous cell carcinoma (HNSCC) (D' Souza *et al.*, 2011).

Oral cancer is a subtype of HNSCC constituting 3% and 2% of all malignant neoplasms of males and females, respectively. During the past decade, the incidence has increased nearly five-fold in subjects younger than 40 years old (Khalili, 2008).

Although oral cancer has been associated with tobacco and alcohol consumption, a fraction of these neoplasias are associated with HPV infection, primarily among individuals without other risk factors (D'Souza *et al.*, 2011; Mao *et al.*, 2004; Smith *et al.*, 2004).

The presence of HPV DNA in HNSCC has motivated the study of HPV in the oral mucosa in different groups. The prevalence in subjects without oral pathology is highly variable and depend on the methodology used, the populations studied and sampling methods (Galvão and Bussolati, 2006). To understand the natural history of HPV infection in the oral mucosa, epidemiologic studies are required. The aim of this study was to describe the epidemiology of HPV in normal oral mucosa of women without oral lesions.

## Materials and Methods

### Study population

This report is a cross-sectional study describing the epidemiology of oral HPV infections in a group of women seen for Pap smears, family planning, pregnancy or gynecological diseases at the public hospital Centro Materno Infantil, Secretaria de Salud, in Merida, Yucatan, Mexico during 2009. The women who utilize this facility are low income native Mexicans of urban, suburban or rural origin without social security.

### Ethical considerations

This project was approved by the scientific and bioethical committee of Centro de Investigaciones Regionales "Dr. Hideyo Noguchi", Universidad Autónoma de Yucatan. All participants signed an informed consent.

### Specimen and data collection

After oral clinical evaluation, all women without lesions were sampled. Buccal mucosa cells were collected by direct swab sampling and then agitated in 5 mL of cold PBS containing antibiotics (500 U/mL penicillin, 500 µg/mL streptomycin and 4 mg/mL gentamicin).

Information about sociodemographic status, sexual behavior, infectious diseases, contraceptive history and tobacco and alcohol consumption were obtained through a direct interview with patients conducted by an experienced social worker.

### HPV DNA detection

DNA was extracted using the DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's instructions by experienced personnel.  $\beta$  globin was amplified as a control for DNA quality using the primers PC04 and GH20 (260 bp amplicon) (Saiki *et al.*, 1985).

To confirm HPV infection, a nested PCR assay was performed using MY09 and MY11 as outer primers and GP5/GP6 as inner primers. A 3 µL aliquot of the amplicon product from the first round of amplification was used as the

template for a second round of PCR using the primers GP5/GP6 (150 bp) (Qu *et al.*, 1997).

### HPV typing

High-risk genotypes (16, 18, 31, 59, 45, 33, 35, 58, 52, and 56) were detected using a nested multiplex PCR assay that amplifies the E6 and E7 genes. Three degenerate consensus primers were used for the first round of amplification. Type specific primers were used for multiplex nested PCR according to a previously published method (Sotlar *et al.*, 2004). Previously typed samples and a PCR mix without DNA were used as positive and negative controls respectively. All PCR reactions were performed on a DNA Engine thermal cycler (DYAD), and amplicons were visualized on 8% polyacrylamide gels using a 100 bp molecular weight marker (Invitrogen).

### Sequencing

Samples that did not amplify with any type specific primers were typed by direct sequencing. GP5/GP6 amplicons were gel purified using the Wizard SV Gel and PCR Clean-Up system (Promega) and sequenced using the dideoxyterminal method on 3700 DNA Analysis equipment (Applied Biosystems) at the Laboratorio Nacional de Genómica CINVESTAV Irapuato, Mexico. The sequences were analyzed using Chromas bioinformatics software and were Blasted against NCBI nucleotide databases.

### Database analyses

The variables studied were input into an SPSS database. The frequencies of variables and chi squares were calculated. Chi squares were calculated to determine association between HPV prevalence and age, age at first intercourse, number of sexual partners, condom use, oral sex, anal sex, the presence of genital warts in women and/or partners, smoking, passive tobacco exposure and alcohol consumption.

For the analysis, the following variables were categorized as follows: condom use: always or occasionally *vs.* never; age of first intercourse: < 18 years *vs.*  $\geq$  18 years; number of sexual partners:  $\leq$  2 *vs.*  $\geq$  3. P-values  $\leq$  0.05 were considered statistically significant.

## Results

### Characteristics of the study population

In total, 390 women were included in this study. The mean age of the study population was 31 years (15-71), 39% were  $\leq$  25 years old, and 79% lived in urban areas. At the time of the study 73% of participants were housewives, 3% were sex workers, and 24.3% were working in various fields. Among the study participants, 15% had no formal education. A total of 19% of the participants' sexual partners lived in another town for work, 6.4% did not have a sexual partner at the time of the study, and four women had never had sex.

With respect to sexual behavior, 62% of participants had their first intercourse before the age of 18, 97% had  $\leq 3$  sexual partners in their lifetimes and 63% engaged in both oral and vaginal sex, 38% in both anal and vaginal sex and 31% in oral, anal and vaginal sex.

A total of 5% of the women had a history of sexually transmitted diseases and 2.8% and 5% of the women and their sexual partners, respectively, had genital warts at the time of the study. Information regarding genital warts was reported by the women, and no clinical evaluation was performed. Finally, 10% of participants smoked, 48% reported passive tobacco exposure, and 64% reported alcohol consumption (Table 1).

## HPV infections

Seven samples were excluded because  $\beta$  globin could not be amplified. Therefore, 383 samples underwent HPV detection.

HPV was successfully amplified from 14.8% of the samples (57/383); 36.8% (21/57) were positive using the MY09 and MY11 primers, and 63.1% (36/57) were positive after a second round of amplification with the GP5/GP6 primers.

Seven genotypes were identified among 47 HPV positive samples: the high-risk 16, 18, 58 and 59 genotypes and the low-risk 6, 81 and 13 genotypes, the latter of which only infects the oral mucosa. Two dual infections were identified: 13/16 and 58/59. Ten samples were not tested for genotypes due to technical issues.

**Table 1** - Characteristics of 390 Mexican women seen for Pap smears, family planning, pregnancy or gynecological diseases.

Characteristic	Number	%	Characteristic	Number	%
Age			NO	138	36
< 25	141	36	Data lost	1	
25-34	102	26	*Anal and vaginal intercourse		
35-44	87	22	YES	147	38
45-54	43	11	NO	239	62
55-64	15	4	*Condom use		
65 +	2	1	Never	211	55
Residence			Ever	154	40
Rural	79	20	Always	21	5
Urban	311	80	*Sexual partner with genital warts		
Education			YES	21	5
None	60	15	NO	365	95
Elementary	111	29	Women with genital warts		
Middle-school	148	38	YES	15	4
High-school	31	8	NO	371	96
College, University, Technical	40	10	Sexual transmitted disease history		
Occupation			YES	21	5
Student	14	4	NO	365	95
Housewife	286	73	Alcohol consumption		
Employee	78	20	YES	257	66
Sex worker	12	3	NO	133	35
*Age of first intercourse (years)			Smoking		
< 18	242	63	YES	39	10
$\geq 18$ years	144	37	NO	351	90
Lifetime number of sexual partners			Passive tobacco exposure		
0	4	1	YES	188	48
1	173	44	NO	202	52
2	93	24			
$\geq 3$	120	31			
*Oral and vaginal sex					
YES	247	64			

None: analphabets; elementary: 6 years after preschool; middle-school: 3 years after elementary; high-school: 3 years after high-school; college/university/technical: includes studies after high school and/or professional training after middle-school. \* 4 were excluded due they never have had intercourse.

Considering only typed samples, the frequency of each genotype was as follows:

HPV13, 70% (33/47); HPV6, 13% (6/47); HPV58 and 16, 6.3% (3/47) each; HPV81, 59 and 18, 2% (1/47) each. Altogether, 17% of the HPV-positive women were infected with high risk HPV.

The overall distribution of HPV according to age was as follows: ≤ 20 years, 24.5%; 21-25, 21%; 26-30, 17.5%; 31-35, 12.3%; 36-40, 8.7%; 41-45, 7%; 46-50, 7%; and ≥ 51, 1.7% (Table 1).

The epidemiological data for the HPV positive women revealed an age range of 16-57 years, with a mean of 29 years. The level of education for 85% of study participants was lower than high school, 46 were housewives, 2 were students, 8 worked in several fields and one was a sex worker.

Data regarding sexual behavior revealed the following: the age of first intercourse was 9-34 years, with a mean of 18.8 years; 80.7% of study participants had fewer than 3 sexual partners; 68% never use condoms, 47% practiced oral sex, 38% practiced anal sex, and 24.5% practiced both anal and oral sex; and among the 27 women who practiced oral sex, 15% always used condoms, 63% never did, and 22% occasionally used condoms. We did not determine whether condoms were used for vaginal, oral or anal sex. Two (3.5%) women had genital warts, and 10% reported genital warts in their sexual partners.

With respect to habits, 5% smoked, 38% reported passive tobacco exposure, and 57% drink alcohol.

To identify the factors associated with genital HPV, chi square analysis was performed excluding women infected with HPV type 13 (which is not a genital type) or non-typed HPV and women who had never had intercourse. None of the variables studied were associated with HPV infection (Table 2).

## Discussion

The prevalence of oral HPV infection among women aged 14 to 69 years in Southeast Mexico is 14.8%, a higher prevalence than has been reported by other (Smith *et al.*, 2004; Kurose *et al.*, 2004; Gillison *et al.*, 2012). In this study, HPV13 was identified, which is a genotype considered to be exclusively oral; but was recently reported in the cervix (Illades-Aguilar *et al.*, 2010; Tonani de Mattos *et al.*, 2011). HPV13 was the most frequent genotype found in the study population (59.6% of all positive samples). If HPV13 positive samples are excluded, the global prevalence of HPV was 6.1%.

It bears mentioning that the discovery of HPV13 was fortuitous; the samples that could not be typed using oligonucleotides specific to genital types were sequenced using purified MY or GP amplicons. The sequencing results were further verified using primers specific to HPV13 (Cuberos *et al.*, 2006).

**Table 2** - Bivariate analysis of factors associated to genital HPV types infecting oral mucosa.

Variable	p
*Age of first intercourse	0.194
**Total number of sexual partner	0.295
***Oral sex	0.151
Anal intercourse	0.162
Intercourse without protection	0.164
Sexual partner with genital warts	0.984
Woman with genital warts	0.984
Smoking	0.659
Passive tobacco exposure	0.399
Alcohol consumption	0.734

Only women with genital types were included. N = 24.

\*18 years vs. ≥ 18 years, \*\*number of sexual partners ≤ 2 vs. ≥ 3, \*\*\*active and passive.

This genotype was excluded from the bivariate analysis because as a non-genital type the risk factors associated to its acquisition and transmission are different to genital types, are not related to sexual behaviors, and is not considered oncogenic (Bascones-Martínez *et al.*, 2012; Hans-Ulrich *et al.*, 2010)

HPV13 is a genotype associated with Multifocal Epithelial Hyperplasia (MEH), which is a rare pathology of the oral mucosa that has been described in some ethnic groups, principally in Americas (Cuberos *et al.*, 2006). Our group has reported this pathology in Mayan locations in the Mexican southeast (Gonzalez-Losa *et al.*, 2011; Lopez-Villanueva *et al.*, 2011). In the present study, we found that 8.6% of the included participants were asymptotically infected with this HPV type. The presence of HPV13 in subjects without lesions has been previously described (Cuberos *et al.*, 2006; Lopez-Villanueva *et al.*, 2011), although in closed communities and not open populations. Because evaluating this genotype was not one of our original objectives and was an unexpected finding, questionnaire did not include questions about a family or personal history of MEH, and therefore we cannot determine whether these are cases of clinical remission of the disease or cases that have always been asymptomatic. Studies of the natural evolution of this infection are not available.

MEH typically presents during the first decade of life. Asymptomatic infection among women 16-40 years of age could play a role in the transmission and maintenance of this virus in the community and within families; indeed, our group has identified HPV13 in asymptomatic relatives in families with MEH (Lopez-Villanueva *et al.*, 2011). These findings suggest the need for longitudinal studies to increase our knowledge of the dynamics of HPV13 infection.

With respect to the other genotypes, the most frequent was the low risk HPV6, identified by sequencing, which was identified in 6 women. High risk genotypes (16, 58, 59

and/or 18) were found in 1.8% of the study population which is lower than what has been reported by other authors (Smith *et al.*, 2004; Turner *et al.*, 2011) but similar to our previous study of a group of odontology patients using High Risk Hybrid Capture 2 (Gonzalez-Losa *et al.*, 2008) and higher than a large study conducted in the USA by Gillison (2012).

Data on oral HPV infections in healthy subjects in Mexico are limited; Kreimer (2010) conducted study that included 591 healthy Mexican men as part of a multinational investigation. The prevalence of HPV DNA was 5.9%, and 1% of the study population was infected with high-risk HPV, with 16 being the most frequent. These data are similar to those found in this study.

Analyzing the distribution of genital HPV by age range, the group with highest prevalence of HPV ranged from 21 to 25 years (18.2%), results similar to those reported for HPV infections of the cervix. Gillison (2012) performed a large study in North America and reported two peaks in the prevalence of cervical infection: the first at age 25 years and the second at age 55. This second increase was not found in our study. Overall, we found that women younger than 25 years old had the highest prevalence of HPV infection of the oral mucosa, which is similar to what is reported worldwide for cervical infection.

Finding HPV genital types in the oral cavity raised questions about possible routes of transmission, oral sex being one of the most likely. Therefore, sexual behaviors as risks factors have been the subject of research by several groups, although the evidence is limited. Smith *et al.* found that young persons with sexual behaviors clearly associated with sexual transmission of HPV have a higher prevalence of oral HPV than those who do not engage in risky sexual practices (Smith *et al.*, 2004). Anaya-Saavedra (2008) conducted a case-control study in which age at the onset of sexual activity and the number of sexual partners was associated with oral infection with high-risk HPV types.

In this study, a statistical association between sexual behavior and oral infection with genital types of HPV was not found; similar results were reported in a large study of men (Kreimer *et al.*, 2011).

In this study, oral HPV infection was detected during the first of round PCR for 36.8% of positive samples, whereas 63.2% required nested PCR. We assume that this indirectly reflects viral load. Interestingly, when investigating genotypes, 57% of HPV13 positive samples were detected by MYs PCR (first round); in contrast, only 8% of samples infected with the other genotypes could be amplified using this PCR and required a nested reaction to detect the infection. Nested PCR is a much more sensitive technique, and therefore, samples that only amplify using this technique likely have a lower viral load. Quantitative PCR is required to confirm this hypothesis.

Current information about oral HPV infection is limited and incomplete. There are many questions to address,

such as the following: what is the natural history of infection?; what factors are associated with oral HPV infection?; which factors are involved in the persistence of oral HPV infection?; what is responsible for the development of the oral neoplastic lesions associated with HPV? and what is the role of oral sex in the transmission of HPV?

Therefore, it is important to perform longitudinal studies in different populations as well as case-control studies.

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