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Identification of Human Papilloma Virus (HPV) in the Oral Cavity of Asymptomatic Colombian Men

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Abstract

HPV infection is currently associated with the risk of lesions in the oral cavity and the oropharynx and has been recognized as a possible etiologic agent of a set of head and neck cancers. However, the detection of HPV in healthy individuals is rare, and little is known about the HPV types present in the oral cavity. A variety of sampling methods has been proposed to increase the sensitivity of viral detection, mostly intended to detect HPV in the oropharynx. Here we estimated the prevalence of HPV in 66 samples of healthy mucosa from the oral cavity of Colombian sexually active men using a liquid-based cytology strategy for sample collection. Generic HPV detection was performed by PCR using a fragment of the β-globin gene as a quality control for the isolated DNA. Type-specific detection was performed with the commercial linear array technique (Roche, USA). All oral samples were positive for β-globin gene. Overall HPV prevalence was 4.5% (3/66); one sample corresponded to HPV16, another was genotyped as HPV11 and the third could not be genotyped and was classified as undetermined. The prevalence of HPV in the oral cavity of asymptomatic men is consistent with that reported worldwide for oropharyngeal cancer. Despite the small sample size, this is the first study that detected HPV in the healthy oral cavity of Colombian men.

Keywords: HPV; Oral mucosa; Oral cancer; Male oral mucosa; HPV16; HPV11

Introduction

Human papillomaviruses (HPV) are a diverse group of epitheliotropic, naked, double-stranded DNA viruses that have been recognized and isolated from different locations of the human body. At this time, HPV are considered as the most common sexually transmitted infection (STI) around the world, with almost 6 million people diagnosed each year and more than 600 million people already infected [1].

According to their oncogenic potential, HPV are further categorized as high-risk types, mainly associated with malignancies such as cervical cancer, and low-risk genotypes, tightly associated with benign diseases [2,3]. Although several oncogenic HPV types could be detected in the oral cavity, most oral infections do not produce lesions or do not progress to the neoplastic stages of the disease [4]. However, recent estimations showed that viral presence confers an approximate 50-fold increase in the risk of HPV-positive oropharyngeal squamous cell carcinomas (OSCC) [5].

HPV infection accounts for approximately 5.2% of the worldwide human cancer burden, including cancers from the anus, the genital tract and the oropharynx [6]. It is currently known that oral HPV infection is the cause of a subset of OSCC that disproportionately affect men. While HPV-positive OSCC are associated with sexual behavior, HPV-negative OSCC are associated with chronic tobacco use and alcohol consumption [7]. Survival rates are higher in individuals with HPV-associated oral cancers compared with HPV-negative ones [8]. Today, the majority of positive oropharyngeal cancers are associated with HPV in the USA [9]; if their incidence continues to rise, it is expected that the total number of OSCC will exceed the number of cervical cancers by the year 2020 [10]. Unfortunately, the proportion of HPV-associated OSCC in most South American countries is practically unknown.

Despite the established link between HPV and oropharyngeal lesions, the natural history of HPV infections in the oral cavity remains unclear. Actual data suggest that HPV preferentially infect the base of the tong, tonsils and the wall of the pharynx [10]. In this sense, different sample collection methods have been proposed, finding different HPV prevalence rates [11]. In fact, literature search revealed that there were only a few published data about the effectiveness of an oral brush cytology method applied in normal volunteers.

The purpose of this study was to estimate the prevalence of HPV DNA in the oral mucosa of a group of asymptomatic men and to evaluate the feasibility of using liquid-based cytology (LBC) with brush scraping to provide an adequate and standardized sample of the oral cavity epithelium. This is the first study of its kind to determine the prevalence of HPV infection in the oral cavity of asymptomatic Colombian men.

Material and Methods

Study population

We carried out a cross-sectional study in men belonging to the Military Training Battalion in a city Colombian. A total of 66 men (age range, 18-58 years) were voluntarily enrolled. Participants signed an informed consent and filled out a questionnaire approved by the Ethics Committees from the Hospital Federico Lleras Acosta ESE, Ibague, and the Cancer Research Center of the San Diego Clinic, Bogotá, Colombia.

Oral specimen collection

Biological material was collected using a sterile cytobrush, scraping on the side walls of the oral cavity and the base of the tongue. Cells

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were resuspended in 5 ml of phosphate-buffered saline (1X PBS), pH 8.3, and stored at 4 °C until further processing. The cytopathological examination of all samples enabled us to analyze whether there was any abnormality in the oral mucosa. DNA extraction was performed using the QIAamp® Viral RNA Mini Kit (Qiagen, Germany) following the manufacturer's instructions. DNA concentration was quantified using a NanodropTM spectrophotometer and adjusted to 20 ng/µl.

HPV detection and typing

A fragment of the β-globin gene was amplified by PCR to establish the quality of the genetic material extracted from the oral cavity before performing HPV DNA detection. β-globin positive samples were later studied for HPV presence, amplifying a 450 bp fragment from the L1 region of the HPV genome, using the My09 and My11 primers previously described [12]. Reactions were performed in medium containing Flexi 1X PCR buffer (BiolineTM, Maryland, USA), 3 mM MgCl2, 0.1mM of each dNTP, 2 µM of each of the My09/My11 primers, 2 units of high fidelity Taq polymerase (BiolineTM) and 50 ng genomic DNA (final volume, 50 µl). The amplification was carried out for 35 cycles, as follows: 94°C for 2 min, 46°C for 1 min and 72°C for 1 min. The first denaturation step was performed at 94°C for 2 min and the final elongation at 72°C for 5 min. The DNA obtained from HeLa cells (kindly provided by the Institute of Immunology, Colombia) was used as positive control, whereas molecular biology quality degree water (mixture without DNA) was used as a negative reaction control. PCR product detection was performed by electrophoresis in 2% agarose gels containing 0.5 mg/ml ethidium bromide, in 1X TBE, pH=8 at 170 V for 45 min. A 320 nm ultraviolet transilluminator was used to identify the expected 450 bp HPV fragment (Figure 1). All samples positive for the generic HPV were further analyzed with the Linear Array HPV Genotyping test (Roche®), following the manufacturer´s instructions.

Results

The cytological analysis showed that samples were negative for neoplastic cells and that sample cellularity was acceptable for DNA analysis. All samples (n=66) were positive for the β-globin gene fragment and therefore suitable for generic and type-specific HPV detection. Generic HPV amplification showed that 3 out of 66 samples (4.5%) from the oral cavity of asymptomatic men were positive for HPV-DNA.

Type specific HPV detection was performed by the Linear Array method (®Roche), since this methodology allows the simultaneous identification of 37 viral genotypes, including genotypes of HPV considered high risk types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 69, 73 and 82), those considered probably high risk types (HPV 26, 53, 66), those considered of undetermined risk (HPV 62, 71, 83 and 84) and those classified as low risk types (HPV 6, 11, 40, 42, 54, 55, 61, 64, 67, 68, 70, 72, 81, IS39, CP6108), being the technique of choice to be implemented in epidemiological studies [13].

After generic HPV detection, the three positive samples were genotyped, obtaining one HPV16-positive sample, one HPV11-positive sample and a third sample that could not be genotyped with this method and was therefore classified as undetermined.

The median age of participants was 27 years (range 18-58), 34.85% (23/66) were 18-24 years, 31.82% (21/66) were 25-31 years old, and 31.82% (21/66) 32-58 years. Concerning marital status, 43.94% (29/66) were married or living with a partner and 54.66% (36/66) were single or divorced. Five out of 66 men (7.58%) had undergraduate or graduate university education and/or technological training, 56% (37/66) had secondary education level (finished or unfinished), and 31.8% (21/66) had primary school (complete or incomplete) (Table 1).

Around 30% (20/66) of participants reported that they did not use tobacco, while 69.69% (46/66) reported that they used it at some point in their lives (average age of smoking initiation, 14 years). Also, 62.12% (41/66) reported drinking alcohol in the past month, while 37.88% (25/66) said they did not do so (Table 2).

Regarding STIs, 81.82% (54/66) of the participants reported not to have suffered any, 13.63% (9/66) claimed to have had any at some point in their lives, and 4.55% (3/66) were not aware of having had any STIs (Table 3A and 3B).

Concerning sexual behavior, 84.85% (56/66) were heterosexual, 4.55% (3/66) bisexual and 1.52% (1/66) homosexual. Only 71.21% (47/66) of participants claimed to have performed oral sex in their lives, 84.85% (56/66) reported vaginal intercourse and 60.61% (40/66) reported having had anal sex (Table 4).

Regarding the possible levels of association, no relationship was found between HPV positivity and the variables of interest.

Discussion

Oral brush cytology is undoubtedly well-tolerated by the majority of people as well as a simple, rapid and non-aggressive sampling technique. Our results demonstrated a uniform distribution of cells, mainly represented by epithelial cell types and only a few basal and/or parabasal cells. Probably, this situation was seen due to the minimally invasive properties of the cytobrush used. Moreover, the present findings showed that liquid-based oral cytology could be useful to obtain cell samples to be analyzed by classical and modern diagnostic molecular techniques. Even further, the high percentage of β-globin positive samples (100%) highlights the efficiency of this technique as a sampling procedure for HPV detection in the oral cavity.

This method has potential application for screening programs and for the surveillance of patients with confirmed cancerous and precancerous lesions. In this study, HPV DNA was easily detected in the healthy population in cells obtained from apparently normal oral mucosa using an average of 50 ng genomic DNA, as suggested by [14]. Moreover, according to these results, specimens collected using brush

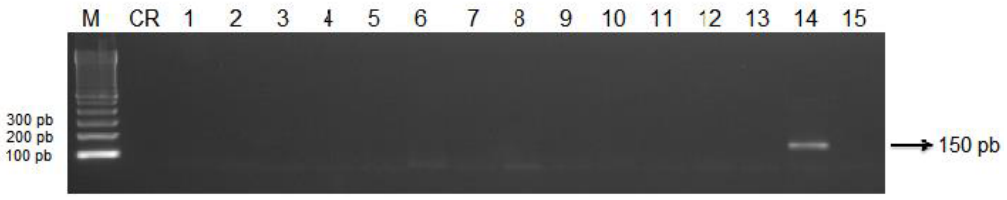

Figure 1: Amplification of HPV gene L1 in samples (n=15) from the oral cavity of healthy men. Agarose gel (2%) stained with ethidium bromure showing the 150 pb amplification product with GP5+/GP6+ primers. M, marker of molecular weight 100 b; RC, reactive control.

Characteristics		
Age (y)	**n = 66**	**%**
18 - 24	23	34.8
25 – 31	22	33.3
32 - 58	21	31.9
Etnnicity		
Afro-Colombian	5	7.6
Caucasian	44	66.7
Indigenous	6	9.1
Mixed blood	4	6.1
No data	7	10.6
Marital status		
Married and/or consensual union	29	43.9
Single and/or divorced	36	54.6
No data	1	1.5
Education		
University	5	7.6
Secondary school	37	56
Primary school	21	31.8
None	1	1.5
No data	2	3

Table 1: Socio-demographic characteristics of the study sample.

History		
	n = 66	**%**
Alcohol use		
No	25	37.9
Yes	41	62.1
Tobacco use		
No	20	30.3
Yes	46	69.7
Age at first use		
9 - 15	21	31.8
16 - 20	22	33.3
21 - 32	4	6
No data	19	28.8

Table 2: History of alcohol consumption and tobacco use.

cytology resulted as good as those obtained using oral gargle, since the prevalence of oral HPV infection remained almost identical to that reported in other studies [11,15-17]. Any oral exfoliative cytological procedure capable of reducing false negative or false positive results would support the method as a more effective tool to screen for oral pre-cancer or cancer lesion, and for other specific viral diseases; the finding that recent tooth-brushing increases HPV detection also suggests that the current sampling techniques may be improved by epithelial scraping [18]

Recent studies suggest that oral HPV prevalence is substantially lower than genital HPV infection (D'Souza et al. and Read et al.) and greatly fluctuating in normal oral mucosa because of differences in sample types, sample collection methods, sample quantitation, level of sensitivity, PCR primer specificity and/or presence of PCR inhibitors [14,19]. In this sense, the HPV prevalence obtained in this study (4.5%) is in agreement with other reports and systematic reviews about the oral cavity and oropharynx of healthy men and women [11,19].

Although the relatively small proportion of HPV-positive samples does not allow for broader inferences, the descriptive analysis of their demographic information showed that the three HPV-positive samples belonged to men between 18 and 24 years. This is not in line with other reports, where HPV positivity increased with increasing age, with the highest prevalence appearing in men over 55 years [11]. However, sample size and the number of HPV-positive samples were not enough to make statistically significant statements.

The three participant's positive for oral HPV declared the current

Record									
Any STI	**n = 66**	**%**	**Genital warts**	**Genital herpes**	**Chlamydia**	**Gonorrhea**	**Syphilis**	**Hepatitis B**	**HIV**
Yes	1	1.5	1 (1.5%)	2 (3%)	1 (1.5%)	9 (13.6%)	3 (4.5%)	5 (7.6%)	1 (1.5%)
No	54	81.8	44 (66.7%)	42 (63.6%)	41 (62.1%)	40 (60.6%)	42 (63.6%)	41 (62.1%)	41 (62.1%)
Does not know	2	3	10 (15.2%)	9 (13.6%)	9 (13.6%)	9 (13.6%)	9 (13.6%)	9 (13.6%)	10 (15.2%)
No data	9	13.6	11 (16.7%)	13 (19.7%)	15 (22.7%)	8 (12.1%)	12 (18.2%)	11 (16.7%)	14 (21.2%)

Table 3A: History of sexually transmitted infections.

Sexual partner with genital warts	n = 66	%	Sexual partner with STI	Sexual partner with HPV vaccine
Yes	12	18.2%	5 (7.6%)	2 (3%)
No	51	77.3%	46 (69.7%)	36 (54.5%)
Does not know	1	1.5%	15 (22.7%)	27 (40.9%)
No data	2	3%	_	1 (1.5%)

Table 3B: History of sexually transmitted infections.

	n = 66	**%**
Age at first intercourse (years)		
8 - 12	17	25.6
13 – 15	32	48.8
16 - 20	13	19.7
Missing data	5	6
Sexual tendency		
Bisexual	3	4.5
Heterosexual	56	84.8
Homosexual	1	1.5
Missing data	6	9.1
Vaginal, oral and/or anal intercourse		
Yes	49	74.2
No	12	18.2
Missing data	4	7.5
Oral intercourse		
Yes	47	71.2
No	15	22.7
Missing data	4	6
Vaginal intercourse		
Yes	56	84.8
No	8	12.1
Missing data	2	3
Anal sex		
Yes	40	60.6
No	24	26.4
Missing data	2	3
Anal sex penetrative		
Yes	3	4.5
No	19	28.8

Missing data	4	6
Last sexual intercourse with a new partner		
Yes	27	40.9
No	30	45.5
Missing data	9	13.7
Stable sexual partner		
Yes	41	62.1
No	20	30.3
Missing data	5	7.5
Last sexual intercourse with a steady partner		
Yes	40	60.6
No	23	34.8
Missing data	3	4.5
Sex intercourse with a man		
Yes	5	7.6
No	51	77.3
Missing data	10	15.1
Oral sex to a man		
Yes	4	6.1
No	44	66.7
Missing data	18	27.3
Anal sex with a man		
Yes	5	7.6
No	44	66.7
Missing data	17	25.8
Pay for sex (men and women)		
Yes	32	48.5
No	28	42.4
Missing data	6	9
Oral		
Yes	6	9.1
No	2	3
Missing data	58	87.9
Vaginal		
Yes	22	33.3
No	25	37.9
Missing data	19	28.8
Anal		
Yes	5	7.6
No	1	1.5

Table 4: Sexual behavior of the study sample.

use of tobacco and alcohol. Tobacco habit alters a wide range of the immune functions in the oral cavity, including the adaptive and innate immunity response, generating direct genetic damage to epithelial cells [20]. Moreover, tobacco use is listed as a factor tightly associated with HPV infection in the oral cavity [21]. This situation could be more intense, since the oral mucosa is directly exposed to the carcinogens contained in tobacco smoke compared to the indirect pathway observed in cervical neoplasia [14]. Contrarily, little is known about alcohol consumption and risk of HPV infection. Thus far, although only one study has shown an association between alcohol consumption and high-risk HPV infection [22], the impact of alcohol on oral mucosa from healthy men is not completely clear.

Finally, differences in sexual behavior could be observed among participants positive for oral HPV, namely, two stated that they practiced oral sex, while the other denied it. In this sense, some studies recognize oral sex as a mechanism of viral spreading while others do not find any association [14,21,23,24]. In a recent work [18], informed that oral HPV infection could be difficult to acquire, but once infected it might persist for many years. The low prevalence of oral HPV infection may be due to i) the presence of highly keratinized tissue, characteristic of the oral cavity, which can act as a mechanic barrier against HPV preventing the invasion of the basal cell layer; and ii) the protective factors present in saliva, such as lysozyme, lactoferrin, IgA and cytokines.

Patients positive for HPV infection in the oral cavity were referred to the corresponding health service program in order to follow and control the progression of their oral health.

This is the first study reporting the prevalence of HPV in the oral cavity of healthy Colombian men. Such prevalence was 4.5% and mainly represented by HPV16 and HPV11. This study could complement the current Colombian oral health policies, promoting the prevention of HPV infection in the oral cavity. Understanding the epidemiology of oral HPV infection at population level can be useful to identify groups at risk of HPV infection and incorporate them in HPV vaccination programs, thus reducing the occurrence of future OSCCs.

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