LOW AND HIGH RISK HUMAN PAPILLOMAVIRUS IN THE ORAL MUCOSA OF MEXICAN WOMEN WITH GENITAL HUMAN PAPILLOMAVIRUS.

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Key words: HPV, oral cavity infection, cervical cancer, HPV and risk factors.

Running title: HPV infection in oral cavity and cervix.
Abstract.

Background: Human papillomaviruses are DNA viruses that infect the epithelium of the skin and mucous membranes. They are classified in low and high risk, based on their oncogenic potential. High risk HPV are implicated in cervical cancer and, nowadays, their role in oral cancer is being assessed, as well as the existing relation between the two sites of infection. In Mexico, there are few studies on oral cancer, therefore the interest in identifying the HPV frequency of low and high risk in samples of the oral and cervical cavities, as well as in determining some risk factors. The purpose on this pilot study was to determine the frequency of high and low risk HPV infection human papillomavirus in the oral cavity of women with cervical (HPV), and to correlate the infection site and its possible risk factors.

Methods: Eighteen female patients between 24 and 53 years, with antecedents of genital HPV infection were analyzed. DNA extraction from the epithelial cells was performed using the Qiagen kit. PCR was performed and the amplicon was observed in 2% agarose gels stained with ethidium bromide. Positive and negative controls were included in the reactions.

Results: HPV-DNA was detected in the 67% of both samples analyzed; cervix and oral cavity. The frequency of oral and cervix low risk HPV-DNA was 50%) in both samples, while high risk HPV-DNA in oral cavity was detected in 17%, and 39% in the cervix. The 100% of oldest group age participants (42-53) was infected with HPV. The study of the risk factors involved in HPV infection showed that the participants had the habits of smoking 39%; alcohol drinking 28%; and 78% oral sex.
**Conclusion:** The results of the present investigation showed a high frequency of HPV (67%) infection in the oral and genital mucosas, suggesting that patient’s habits could contribute to the infection; however, the smaller sample size don’t let us to reach a conclusion whether the HPV transmission goes from the oral cavity to the genital region or vice versa.
Background

The human papillomavirus (VPH) belongs to the *Papillomaviridae* family, it is a DNA virus, can be broadly grouped into cutaneous and mucosotropic types. The mucosotropic HPVs are typically found in the anogenital mucosa and oral mucosa. Genital infection with HPV can be transmitted to oral mucosa through autoinoculation, oral sex, or oral contact (kissing) [1, 2]. While the HPV related genital lesions are more frequently seen in adults, lesions like skin warts, oral and laryngeal papillomas are more frequently seen in children [3]. Further, the mode of viral transmission in the oral cavity could be by auto-and hetero-inoculation, indirect transmission via fomites, and by oral sex. In women, benign lesions are produced known as condylomas mainly in the vagina and the neck of the uterus; these lesions can progress to cervical-uterine cancer [4, 5]. Epidemiological studies correlate the presence of HPV to the development of cervical-uterine cancer [6-10]. In Mexico, cervical-uterine cancer is the most frequent sexually transmitted viral disease and constitutes the main cause of death in women older than 25 years [11]. Over the years, more than 130 types of HPV have been identified according to the nucleotide sequence alignment of its open reading frames [12]. Based on their association to cervical cancer and the lesions produced, HPV have been grouped in low (serotypes 6, 11, 42-44) and high (serotypes 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 70) risk viruses [4, 13]. The high risk HPV serotypes 16 and 18 are the most common, and have been detected in 95% of women with cervical cancer [14]. Recent studies indicate that these two serotypes are also related with oral cancer or with neck and head cancer [3, 15-17]. Oral cancer has been scarcely studied as compared to genital cancer; however some studies have revealed
that the variations in HPV frequency in this type of cancer are due to the used
detection methods, the size and type of the samples [18]. Besides, some risk
factors such as smoking, alcohol consumption and sexual habits have also
been associated to oral cancer [19-22]. Some authors consider that the oral
cavity can act as a reservoir for HPV and modify transmission in different
populations [23]. Various studies have been conducted to study the role of HPV
in oral lesions and malignancies. However, the association of HPV between
cervical and oral cavity remains unclear. Considering the fact that almost all the
cervical cancers are caused by HPV [24], this study was conducted to evaluate
the prevalence of HPV in the oral cavity of women with cervical HPV.
In Mexico, the presence and frequency of HPV in the oral cavity and if there is a
possible relation with the genital infection are unknown. Therefore, the purpose
of this study was to detect the prevalence of high and low risk HPV in the oral
cavity of women with cervical HPV, and to evaluate the risk factors which
contribute to its occurrence.
METHODS

Study participants. We studied 18 women, aged between 24 and 52 years, who came to the Gynecology and Obstetrics Services of the Hospital Español with antecedents of genital infection by HVP. The protocol was approved by the institutional Research and Ethics Committee.

Smears of three out of eighteen patients with clinical and histopathology confirmation of high-grade squamous intraepithelial lesion were taken before and after three months of the treatment with cryotherapy. Cryotherapy was performed using a single freeze technique with nitrous oxide as refrigerant, and it was applied to the cervix during three minutes. The details of the study were explained to all participants and written informed consents were obtained before entering into the study. After informed consent was granted, information on age, education, tobacco use, and sexual behavior was collected. A questionnaire was applied to gather information on personal and clinical data to determine some risk factors.

Sampling. Two samples were taken per patient. Cervical cells were obtained by using Ayre’s spatula and an endocervical cytobrush, oral samples were collected by brushing (Oral CDX; CDx Labs) and performing ten complete backward and forward brushes at each oral site (upper and lower gum, cheeks, orders and dorsum of tongue), the brush was squeezed immediately into the side of a tube containing 1 ml of cold phosphate-buffered saline solution, After collection, both cervical and oral specimens were centrifuged at 2,000g x 10 min, washed with saline solution, centrifuged at 12,000g x 10min, and the pellet stored at -20ºC [25].
**DNA extraction.** For DNA extraction, samples were centrifuged at 2 000 x $g$ for 10 min and DNA was extracted from the pellets using the QIAamp DNA Mini kit (Qiagen Maryland, USA), according to the manufacturer’s instructions. The samples were resuspended in 500 µl of a digestion buffer containing [1M Tris HCl (Invitrogen, Carisbad,CA) pH 8.3; 10 % SDS; 0.5 M EDTA (Sigma-Aldrich) pH 8.0; 10 mg/ml proteinase K (Sigma-Aldrich, St.Louis, MO), and 10 mg/ml RNase (Sigma-Aldrich). The samples were digested overnight followed by incubation at 48 ºC, for proteinase K (Sigma-Aldrich) inactivation. In order to adsorb the nucleic acids of DNA a silica-gel column containing buffer of (10 mM Tri-HCl; 0.5 mM EDTA), and stored at -70º C until used.

**Detection of HPV by polymerase chain reaction (PCR).** In order to identify HPV DNA of high or low oncogenic potential, we utilized two consensus sequence primer pairs within the E6 and E7 open reading frames (ORF) to amplify HPV DNA.[21]. Each 100 µl polymerase chain reaction (PCR) contained 10x PCR buffer with KCl, (NH$_4$)$_2$SO$_4$, 25 mM MgCl$_2$, 10mM dNTP, 2.5 U Taq DNA Polymerase (Advance Biotechnologies Ltd), 0.100 mM of the primers and 5 µl of the DNA sample (1-5 µg/µl). Amplifications were carried out in a DNA thermal cycler (Perkin Elmer, Norwalk,USA), using the following conditions: One cycle of initial denaturation at 95º for 5 min; 35 amplification cycles; 94 ºC (40 s), 55 ºC (40 s), 72 ºC (40 s), and polymerisation at 72ºC for 7 min. DNA from HeLa and CaSki cells was used as positive controls of high risk, and DNA serotype 6 as low risk control. And as negative control DNA from HEp-2 cells or distilled water instead of DNA was used. All controls were obtained from the American Type Culture Collection (ATCC) and glyceraldehyde-3-phosphate dehydrogenase; (GAPDH) was used as internal control. PCR samples was
separated by electrophoresis in a 2% agarose gel (Sigma-Aldrich) containing ethidium bromide (Sigma), and the gels were visualized under UV light with a transiluminator (UltraLum, California, USA).

**Statistical analysis of risk factors.** The association of HPV infection with risk factors (smoking and/or oral sex habit) was analyzed with the non parametric Spearman's rank correlation coefficient. The predetermined level of significance was $p<0.05$. Statistical analysis was performed with the STATISTICA v 4.5 (StatSoft Inc., USA)
RESULTS

The study group comprised 18 women (mean age: 36 years; range: 20-53 years) with cervical HPV infection.

HPV-DNA was detected in 12/18 (67%) of the participants, in both oral and cervical cavities. HPV-DNA was not detected, neither oral cavity nor cervix in 3/18 (17%) in patients that were treated with cryotherapy.

The frequency of oral and cervical low and high risk HPV-DNA in the eighteen participants shows that 9/18 (50%) had low risk HPV-DNA in oral and cervical cavities, while high risk HPV-DNA was detected in 3/18 (17%) and 7/18 (39%), respectively (Figure 1).

In the oral and cervical cavities of the eighteen patients low HPV-DNA was detected in 8/18 (44%); high risk HPV in both cavities 2/18 (11%); in the cervix of one patient 1/18 (6%) was detected either low and high risk HPV-DNA, whereas, one patient exhibited high risk HPV-DNA in the oral cavity (6%) but not in cervix; and in 3/18 (17%) was detected only high risk HPV-DNA in the cervix not in the oral cavity (Figure 2).

The analysis of smoking/alcohol drinking/sex habits showed that 7/18 (39%) presented smoke habit; 5/18 (28%) alcohol drinking habit; 14/18 (78%) practiced oral sex (p<0.02). The results of the analysis of the oral sex habit showed that 10/18 (56%) had HPV-DNA in oral cavity and in cervix.

Regarding the participants age we observed that the most affected group with HPV-infection was the age group between (42-53). Table 1.
DISCUSSION

Molecular, clinical and epidemiological studies have verified that the HPV is the main etiological agent of cervical cancer [6, 8, 13]. HPV infections represent one of the most common sexually transmitted viral diseases with frequencies going from 30% to 50% in sexually active women [26]. Mexico has one of the highest mortality rates; 4270 deaths were recorded in 2005, with a rate of 10.2 per 100,000 women [27]. HPV appears to play an etiologic role in many cancers of the oral cavity [16-19, 21, 22, 26]. A link between human papillomavirus and squamous cell carcinoma of the head and neck was suggested more than 20 years ago [28]. Investigators showed a strong association between high-risk HPV infection and oral squamous cell carcinoma in Mexican, Sweden Japanese, and Chinese populations [29-31]. The role of high risk oncogenic HPV in premalignat and malignant oral lesions has been an issue of extreme controversy with conflicting data reported by numerous studies (3, 9, 15-18). So HPV infection is not limited to the genital tract; it can extend to extragenital sites and particularly to the oral cavity, but the mechanism of infection of HPV into the oral mucosa remains unknown. Possibilities include self inoculation and/or through the practice of fellatio on HPV-positive male partners [32]. Genital HPV is the commonest sexually transmitted infection worldwide, detectable in approximately 40% of women under age of 25 years, and frequently in the sub-clinical form [33, 34]. As a consequence, there are high rates of HPV infected, healthy and sexually active women, who will contribute significantly to horizontal transmission of the virus, also including transmission via the oral cavity [35-37].
The prevalence of HPV in oral cavity of women with cervical HPV was high 67%, we detected both low and high risk HPV-DNA virus. All the HPV positive subjects had clinically normal oral mucosa.

The results of our study showed that high risk HPV-DNA was more prevalent in the cervix of women 39%, whereas oral high risk HPV-DNA was detected only in 17%. A predominance of low risk HPV-DNA in oral cavity and cervix (50%) was observed (Figure 1).

A study was conducted to determine the HPV prevalence and concurrent infection in the cervix and oral cavity of 577 pregnant women, found 29% positivity in the cervix and 2.4% positivity in the oral cavity. No association was found between HPV positivity and its types detected in the cervix and oral cavity of these women, suggesting the author that the inoculation was rare [41].

The finding that the high risk HPV-DNA in oral cavity was found in less proportion than in cervix could have the follow explanation (Figure 2): Firstly, although oral cavity is in direct contact with carcinogens present in tobacco and alcohol, making them the primary cause of oral carcinogenesis, this is not the case with cervical region, where there is no direct contact with this carcinogens but the cervical region does not have the immune innate response that oral cavity does. Secondly, the low prevalence of oral HPV infection might be due to the body’s immune response, like immunoglobulin IgA and proteolytic enzymes in the saliva that protect the oral mucosa from viral infections like cystatins, mucins serin-proteases inhibitors, and Von-Ebner proteins[42]. Thirdly, antibodies produced in response to initial infection, in this case cervical infection, might as well protect the body against further infections by the same virus on other sites. Fourthly, although the oral mucosa epithelium resembles the
epithelium of the genital tract [43], antimicrobial action of saliva, along with its cleansing and lubricating properties, may reduce the possibility of virus entry into the oral epithelial cells by reducing the contact period of the virus with the oral mucosa [44]. These important factors could contribute to prevent the implantation of a high risk HPV in the oral mucosa.

To determine if HPV could be eliminated with cryosurgery, we included three patients in these conditions. HPV-DNA was not detected in these patients, neither oral cavity nor cervix, this could be because cryotherapy eliminated damage tissue on the cervix, and HPV could be eliminated during cryosurgery. Our results showed that the higher of HPV positivity was in the oldest group age participants (43 to 53 years) was infected with low risk and high risk HPV, this could be explained partially because the infection with high risk serotypes (16 and 18) involves factors that influence the acquisition and progression of the injury degrees caused by HPV infection, the most common ones are: early start of sexual activity, the number of sexual encounters (in women with only one partner, HPV is observed in 17 to 21% and increases to 69-83% in those with more than five sexual partners), the immune compromise, hormonal action (including the prolonged use of oral anticonceptives associated with a higher susceptibility to malignant progression), nutritional deficiencies, other sexually transmitted infections, and genetic predisposition (haplotypes of the major histocompatibility system associated to a greater susceptibility to malignant progression) [38-40]. Other possibility is that this finding could be the result of an accumulated lifetime exposure to the viruses, with the added risk of sexual activity. In this work statistical analysis of risk factors showed that oral sex and
smoking habits (p<0.02) are dependent variable that could promote the HPV establishment in the oral cavity.

Studies have shown that smoking has potential to alter oral epithelium, thus it has and influence on HPV expression in oral cavity [45]. The analysis of the risk factors as cigarette smoking, in combination with infection with HPV oncogenic types, is strongly associated with cervical dysplasia [46, 47]. Smoking also increases the risk of oral and pharyngeal cancer [48-50]. Given the apparent association between oral cancer and HPV infection and given the association between smoking and oral cancer, we thought it of interest to examine the association between HPV infection and smoking. We were able to detect HPV-DNA infection from 39% of the participants that had the smoking habit.

On the other hand, it is well known that alcohol increases the permeability of the mucous membranes inducing local immunosuppression and thereby facilitating HPV infection [51]. In our study the 28% of the participants had the alcohol drinking habit.

D´Souza et al.,[52] demonstrate that oral sexual behaviours are associated with the detection of prevalent oral human papillomavirus, with this study the investigators have taken an additional step toward improving our understanding of the epidemiological profile of oral HPV transmission. In our study we found that the smoking and the oral sex habit was present in the oral cavity of women with cervical HPV (p<0.02), if the tobacco could alter the oral epithelium and subjects with cervical HPV practice oral sex, then HPV could be established in the oral cavity with any problem.
Our results showed a high prevalence from 78% (14/18) of oral sex habits, so probably this risk factor could be relevant in the Mexican population, but it has to be analysed a bigger sample size in order to conclude the importance of the oral sex habit in the HPV transmission. It has been theorized that oral infections are likely the result of oral-genital contact. The present study suggests an could expands on that theory by demonstrating that not only are oral-sexual behaviours important, but more common habits, such as smoking or drinking alcohol, may also contribute to the HPV infection to the oral region.

Interestingly, studies conducted to detect the presence of *Helicobacter pylori* in oral cavity and the association with gastroesophageal disease, has shown that there is a strong and clear association between *H. pylori* presence in the oral cavity and gastroesophageal disease ($P<0.0001$) associated with this microorganism, while healthy subjects and those with other non-gastric disease do not present *H. pylori* in their oral cavity [53]. It could be interesting to evaluate whether the oral cavity could be not only the reservoir for HPV, but for other pathogens. However, the small number of samples we can not give a conclusion.

**Conclusions**
The results of this study suggest that the mode of viral transmission the oral cavity could be auto-and hetero-inoculation, indirect transmission via fomites and the oral sex habit, as relevant risk factor that contributed to the develop of this infection. Additional studies are necessary to clarify the physiopathological mechanisms for the installation of HPV in the oral mucosa. The presence of HPV in the oral mucosa may act as reservoirs for new HPV infections and/or as sources of recurring HPV lesions, as has been observed with other microorganisms. It could be interesting to evaluate whether the oral cavity could be not only the reservoir for HPV, but for other pathogens.

The results of the present investigation showed a high frequency of HPV (67%) infection in the oral and genital mucosas, suggesting that patient’s habits could contribute to the infection; however, the smaller sample size don’t let us to reach a conclusion whether the HPV transmission goes from the oral cavity to the genital region or vice versa.

**Competing interests**

The authors declare that they have no competing interests.

**Author’s contributions.**

DR. Carried out the molecular studies.

SP. Participated in the clinic studies and patient data case studies.

FM. Obtained the samples and performed the data analysis.

JD. Performed the statistical analysis

ME. Participated in the study design, coordination and helped to draft the manuscript.

AM. Conceived of the study, participated in its design, and drafted the manuscript.
All authors read and approved the final manuscript.

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patients with metastatic squamous cell carcinoma of the head and neck.


**Legends from figures:**

Figure 1. Frequency of low and high HPV-DNA in oral cavity and in cervix
HR, high risk; LR low risk. (oc= oral cavity; cx= cervix)

Figure 2. Presence of the HPV-DNA low and high risk binomial (oral cavity-cervix), or in a single cavity.

Table 1 Distribution of low and high risk HPV-DNA in oral cavity and cervix stratified by group age, sex habits, smoking and alcohol drinking.
Figure 1

The bar chart shows the percentage distribution of patients in low and high risk groups.

- **Low Risk**
  - Oral cavity: 50%
  - Cervix: 50%

- **High Risk**
  - Oral cavity: 17%
  - Cervix: 39%
Figure 2

- oc: oral cavity
- cx: cervix
- LR: Low Risk
- HR: High Risk

Patients

<table>
<thead>
<tr>
<th>Group</th>
<th>Percentage</th>
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<td>OC + CX LR</td>
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<tr>
<td>OC CX HR</td>
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<tr>
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<td>CX LR+HR</td>
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<tr>
<td>OC HR</td>
<td>6%</td>
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<tr>
<td>CX HR</td>
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Additional files provided with this submission:

Additional file 1: Table 1.doc, 53K
http://www.biomedcentral.com/imedia/1385397292677651/supp1.doc