Detection of human papillomavirus in the saliva of women with concurrent human papillomavirus related genital lesions

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ABSTRACT

الأهداف: تحديد العلاقة بين فيروس الورم الحليمي البشري والآفات التناسلية المرتبطة وذلك من ناحية الأنواع الفرعية لهذا الفيروس.

الطريقة: أُجريت هذه الدراسة المقطعية في عيادات النساء التابعة لمستشفى الزهراء التعليمي، تبريز، إيران وذلك خلال الفترة من يوليو 2006م إلى أغسطس 2009م. شملت الدراسة 104 امرأة مصابة بالآفات التناسلية المرتبطة بفيروس الورم الحليمي البشري، حيث تم التقصي عن الأنواع الفرعية لهذا الفيروس من خلال سحب العينات اللعابية. ولقد تم التعرف على التنوع الجيني لجزيء الحمض النووي من خلال تقنية التفاعل التسلسلي المبلمر، وكانت أنواع الفيروس كالتالي: 16، و18، و31، و33، و6، و11.

النتائج: أشارت النتائج إلى أن فيروس الورم الحليمي 16 كان من أكثر أنواع الفيروس انتشاراً في اللعاب (29.8%)، وفي عنق الرحم (24%). ولقد أظهرت النتائج أن أنواع الفيروس في العينات اللعابية وعينات عنق الرحم كانت مرتبطة إلى حد كبير (p=0.009)، كما وكان هناك علاقة كبيرة بين أنواع الفيروس في كل من العينات اللعابية وعينات عنق الرحم والشرج (p=0.00)، وكانت هناك علاقة بين العينات اللعابية وعينات الشرج (p=0.001). وعلى خلاف ذلك كان هناك اختلافاً واضحاً بين الأنواع الفرعية في كل من عينات عنق الرحم وعينات الشرج (p=0.000).

خاتمة: أثبتت الدراسة أن فيروس الورم الحليمي من النوع 16 يعد من أكثر أنواع الفيروس ظهوراً في عينات اللعاب وعنق الرحم، ومعرفة الأنواع الجينية لهذا الفيروس قد يسهل تحديد أسباب الآفات التناسلية المتكررة والمرتبطة بهذا الفيروس.

Objectives: To determine the association of the human papillomavirus (HPV) subtypes in the saliva of women and HPV-related genital lesions.

Methods: In a cross-sectional study, 104 women with documented genital HPV-related lesions and known HPV status were selected. These cases were examined for the HPV subtypes in their salivary specimens from July 2006 to August 2009 at the Gynecologic Clinics of Alzahra Teaching Hospital, Tabriz, Iran. To detect HPV DNA subtypes of 16, 18, 31, 33, 6, and 11, HPV was genotyped by polymerase chain reaction assay.

Results: Type 16 HPV was the most frequently detected subtype in the saliva (29.8%), and cervix (24%). In addition, there was a significant association between the saliva and cervix with co-infection (p=0.009), and between the viral types of salivary and cervical+vulvar samples (p=0.00), and salivary and vulvar samples (p=0.001). On the other hand, there was a significant difference between the cervical and vulvar samples for the viral subtypes (p=0.000).

Conclusion: The high risk HPV 16 is the most common simultaneous HPV subtype in the saliva and cervix of the cases. Identifying the HPV subtypes in saliva may facilitate recognizing persistent genital infections.

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Human papillomavirus (HPV) infection is the most common sexually transmitted disease.[1] More than 100 HPV subtypes have been identified, approximately 40 of which infect the squamous epithelium of the lower anogenital tract.[2-4] Although it is very common in young sexually active women, the infection is usually transient.[5] Approximately 10% of individuals develop a persistent infection.[6] As already studied, persistent HPV infection is a critical step in cervical carcinogenesis,[7-9] anal, and other anogenital cancers.[10] The HPV is also implicated in mouth, respiratory, head, and neck cancers.[11-15] Furthermore, HPV has been accepted as an independent risk factor for oral squamous cell carcinoma.[16,17] Scardina et al[18] reported that HPV infection is transmitted by oral sex in the partners carrying the virus. Unprotected orogenital contact, especially receptive oral intercourse, is associated with greater risk of viral transmission than previously thought.[18] Syrjanen[19] also reported oral viral infections caused by high risk (hr) HPVs among the family members of the patients with genital HPV infections. However, D'Souza et al[20] reported a different natural history for the HPV infection in the oral rinse samples compared with the concurrent cervical vaginal lavage samples, and found no association. Thus, the true relationship between genital and oral HPVs is far from being completely elucidated.[21,22] Establishing a place for hrHPV testing in oral rinse in clinical practice seems necessary,[19] since early detection and subsequent early treatment of HPV in precancerous lesions, like the uterine cervical screening programs, can prevent progression to cancer.[20,23,24] As mentioned, the importance of persistent infection in the mouth has not been diagnosed as a cause for genital cancers. In the literature, there are not enough data proving that oral-genital contact can really be a vehicle for HPV transmission.[19] Quantitative polymerase chain reaction (PCR) has been used to detect HPV type 16 in the salivary rinses, and a screening method for head and neck squamous carcinoma (HNSC). However, the presence of specific limitations prohibited the application of this method as a screening technique for a broad population.[25] Hence, it would be logical to find out another strategy of screening and prophylaxis that is cost-effective. In a pilot study, we found that the saliva of patients with positive genital HPVs, in some cases was positive for the same genotype. We designed this study, first to determine the type-specific frequency of HPV subtypes in the saliva of women with concurrent HPV genital lesions, second to determine the co-contamination of HPV subtypes in both regions, third, to find out the diagnostic value of salivary samples and association between the viral types of salivary, cervical, and vulvar samples, and finally, to obtain an alternate source for HPV testing to diagnose infected individuals.

Methods. In a cross-sectional study that was carried out in the Gynecologic Clinics of the Alzahra Teaching Hospital of Tabriz University of Medical Sciences, Tabriz, Iran, from July 2006 to August 2009, patients with genital HPV related lesions were selected and sampled for histologic examination. One hundred and four participants were eligible. After histologic confirmation, other samples were obtained from the genital lesions and saliva for HPV genotypes 16, 18, 31, 33, 6, and 11 with PCR assay, a method used by Esmaeili et al.[26] The HPV genotyping was performed through restriction fragment mass polymorphism (RFMP) sequencing, and hybrid capture (HC) assays. In this process, disposable blades were used to cut serial sections of 20 µm thickness from each formalin-fixed paraffin embedded (FFPE) block, and placed in a sterile 1.5-ml micro tubes afterwards for extracting DNA. In order to confirm that there was no cross-contamination among samples, HPV-negative control tissues were also used. Next, DNA was extracted from the sections for amplification of a broad spectrum of HPV subtypes. Consensus PCR primers GP5+/6+ were used with slight modifications for lowering stringency of the reaction. The samples were genotyped using 2 type-specific multiplex PCRs (TSM-PCR). In the first TSM-PCR, HPV-16, and HPV-18 were detected. The amplified fragments were resolved by electrophoresis on 2.5% agarose gel and ethidium bromide staining. The 6% polyacrylamide gel electrophoresis, however, was used for better resolution of the bands. In the second TSM-PCR, HPV-31, and HPV-33 were identified according to the new type-specific primers and PCR conditions as described by Baay et al[27] with slight modifications in the post-PCR detection. In order to separate the PCR product of type 31 (110 bp) from that of 33 (117), 7 microliters of the products were run on 15% polyacrylamide gel electrophoresis (100 V, 5 hours). Negative controls and distilled water were also used to confirm that there was no cross-contamination during PCR runs. In order to assess the quality of the DNA samples in the samples with negative results, connexin 26 gene was amplified using 35delG normal set primers spanning 202 bp (**Figure 1**).[26]

The study was approved by the Ethical Committee, affiliated to Tabriz University of Medical Sciences Research Affairs, and all participants were given adequate information to participate and informed consent was obtained for the study.

Descriptive statistics and Fisher's exact tests were used for the assessment of association. To compare the groups, Chi square and independent samples t-test were performed. The data was expressed as mean ± standard deviation, and number (%). A p-value ≤ 0.05 was considered significant. All statistical procedures

were performed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) version 15 for Windows.

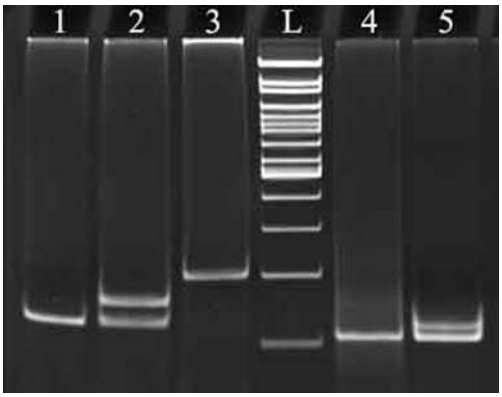

Figure 1 - Electrophoresis of polymerase chain reaction (PCR) products on 10% polyacrylamide gel. 1 - positive for genotype 16; 2 - positive for genotype 16 and 18; 3 - PCR product for Connexin 26 (internal control of products); 4 - positive for genotype 31; 5 - positive for genotype 31 and 33; L - marker of molecular weight for 100 pairs of alkaline.

Results. Some characteristics of the participants are shown in **Table 1**. Vulvar condylomatous lesions were found in 22.1% of patients. History of smoking and drug abuse was found in 22.1% of the male partners. There was no immunosuppression in the studied group. There was no statistically significant difference in basic characteristics such as age, age of marriage, occupation, and place of birth between salivary HPV positive and negative patients. All FFPE genital specimens were analyzed. A total of 115 (8.8%) out of 1303 amplifiable samples were positive for 6, 11, 16, 18, 31, and 33 HPV DNA. The distributions of different HPV genotypes in both genital and salivary specimens are shown in **Table 2**. The results of simultaneous genital and salivary HPV subtypes are shown in **Table 3**. **Table 4** depicts the results of salivary HPV co-infection with vulva, cervix, and cervix+vulva.

The HPV viral types in the saliva and vulva are reported in the simple percentage form, and Fisher's exact test showed a significant association (odds ratio [OR] = 4.68, 95% confidence interval [CI]: 1.93-11.35),(χ^2=5.07, degrees of freedom [df]=1, p=0.02). In

Table 1 - Basic characteristics of the study group (N=104).

Variable	Mean ± SD	Minimum-Maximum
Age, years	36.81 ± 10.09	18 - 65
Marital age, years	21.722 ± 5.06	14 - 44
Gravidity	2.60 ± 1.99	0 - 12
Parity	2.12 ± 1.77	0 - 10
Frequency of sexual contact, per week	2.28 ± 1.05	0 - 4
Duration of present illness, months	15.65 ± 2.35	1 - 156
Number of life-time partners of spouse, n (%)		
1	99 (95.2)	
≤2	5 (4.8)	
Number of life-time partners of husband, n (%)		
1	94 (90.4)	
2	9 (8.7)	
4	1 (1.0)	
Route of sexual contact		
Vaginal, n (%)	81 (77.9)	
Mixed (genital-oral, genital-anal)	22 (21.1)	
No sexual contact	1 (1.0)	

SD - standard deviation

Table 2 - The frequency of genital and salivary human papillomavirus (HPV) subtypes (N=104).

HPV subtypes	Vulvar positive	Cervical positive	Salivary positive
	n (%)		
6	20 (19.2)	4 (3.8)	11 (10.6)
11	18 (17.3)	4 (3.8)	10 (9.6)
16	15 (14.4)	25 (24.0)	31 (29.8)
18	0 (0.0)	2 (1.9)	2 (1.9)
31	10 (9.6)	11 (10.6)	12 (11.5)
33	2 (1.9)	0 (0.0)	1 (1.0)

Table 3 - The frequency of simultaneous human papillomavirus (HPV) subtypes infection in the genital area and saliva (N=104).

Number of types	Vulva	Cervix	Saliva
	n (%)		
No viral	69 (66.3)	73 (70.2)	55 (52.9)
1	10 (9.6)	23 (22.1)	33 (31.7)
2	20 (19.2)	4 (3.8)	14 (13.5)
3	5 (4.8)	1 (1.0)	2 (1.9)
4	0 (0.0)	3 (2.9)	0 (0.0)

Table 4 - The results of salivary human papillomavirus co-infection with vulva, cervix, and cervix+vulva (N=104).

Salivary	Vulva		Cervix		Cervix+vulva	
	n (%)					
	Negative	Positive	Negative	Positive	Negative	Positive
Negative	45 (81.8)	10 (18.2)	45 (81.8)	10 (18.2)	35 (63.6)	20 (36.4)
Positive	24 (49.0)	25 (51.0)	28 (57.1)	21 (42.9)	4 (8.2)	45 (91.8)

addition, the results of the same test for the viral types of salivary and cervical samples showed a significant association (χ^2=7.54, df=1, p=0.009) (OR=3.37, 95% CI: 1.38-8.20). There was also a significant association between the viral types of salivary and cervical+vulvar samples (χ^2=34, df=1, p=0.000) (OR=19.68, 95% CI: 6.16 -62.85). There was a significant difference between the viral types of cervical and vulvar samples, and only one out of the total 35 patients with vulvar lesions were positive for cervical HPVs, and in one out of the total 31 patients with cervical positive HPVs, were the vulvar HPVs positive. In 39 (56.5%) cases, both the cervix and the vulva were HPV subtype negative.

Discussion. The present study reports on the frequency and distribution of 4 hrHPV, and 2 general HPV genotypes in the genital HPV related lesions and saliva of the same patients from the northwest of Iran. The results indicate that HPV 16 in the salivary secretion is by far the most common HPV type associated with the cervical HPV-related lesions (**Table 2**).

In the literature, the role of persistent hrHPV in cervical carcinogenesis, especially HPV 16, has been well-recognized.[7,8] The possibility of mixed infection with several HPV types as shown in our study (**Table 3**) has been well documented.[2,4,8] The findings also explain that there are significant positive associations between the salivary and cervical HPV subtypes, and viral types of salivary and cervical+vulvar samples (**Table 4**). In our study, no association was found between cervical and vulvar HPV subtypes. These findings are in contrast to the study of Sadan et al,[28] in which an association was found between the exophytic vulvar condyloma acuminata and abnormal Pap smear or positive cervical biopsy, in generally healthy women without HPV typing. Our results probably reflect the fact that the vulvar lesions are caused by the HPV subtypes other than the subtypes in cervical lesions. Similarly, Scardina et al[18] found a relation between vaginal, anal, or oral sexual contact with carrier partners and HPV infection. Syrjanen[19] confirmed the same result and showed that oral viral infections could be transmitted orogenitally. However, Castro et al[29] found a limited correlation between oral and genital HPV in the same patient. An association between HPV infections of oral and upper respiratory cancerous diseases has also been reported.[30,31] Routine application of molecular techniques such as PCR for detection, and analysis of HPVs in patients with respiratory papillomatosis (RRP) has been proven to have diagnostic and prognostic significance.[25] Whereas, in our study, there was only one case of laryngeal cancer in a patient who was HPV 16 positive in both cervix and saliva. Due to the small sample size, we were unable to show this relationship.

In the study of Draganov et al,[32] there was also an association between the genital HPV and RRP. In spite of different natural history of oral and cervical HPV, concordance of the same subtypes in genital organs and saliva seems to be of importance.[20] Therefore, positive saliva may have a significant meaning. As type-specific HPV infection is cleared within 2 years, meanwhile, a woman might be re-infected with a new HPV genotype,[33] or might infect her family members by the infected saliva.[19] In addition, the presence of certain HPV subtypes infection may be a risk factor for the presence of another HPV genotype. This could be a rationale for the assessment of HPV genotypes for women with genital HPV related lesions. In cervical cancer prevention programs, the role of HPV DNA testing is well-known.[9] Unlike the genital tract, natural history of oral HPV infection is studied poorly. In the study of Syrjanen,[19] the detection rate of hrHPVs in oral rinse varied from 15-27%. The author demonstrated that oral sex had no association with oral HPV infection, but a persistent oral HPV infection of the spouse increased the risk of persistent oral HPV infection 10-fold. The author has also confirmed HPV as an independent risk factor for oral SCC with OR of 3.7-5.4.[19] The role of sexual partner has also been investigated in the study of Fukuchi et al.[34] Having a high-risk partner is a potentially modifiable risk factor for persistent HPV infection.[34] As mentioned above, the main result of our study was the high co-existence of HPV-16 in cervical lesions and saliva (**Table 4**). According to the literature, HPV persistence is more likely with HPV 16 rather than with other oncogenic HPV types.[19,35] Screening is not possible before adolescence and during childhood in order to detect infection. Therefore, infection may have started long before adolescence, and the patient may have several, or continuous non-sexual exposure.[19] In addition, at the time of prophylactic vaccination, the patient may be positive, hence, the vaccine will not work, and she will be at risk for several cancers. Everybody might be susceptible to infection and prophylaxis at birth, and a more effective screening method may be used to identify the population at risk.

The role of oral or genital HPV infections in developing upper respiratory or cervical cancers has been studied well.[7,30] But the effect of one on another has not been elucidated yet. In this study, we found a positive association between salivary and genital infection, but the role of persistent salivary infection, or re-infection by the hrHPV in putting the patient at risk of genital cancer remains to be confirmed. Therefore, a follow-up on patients with positive HPV in their saliva may be useful in finding out the individuals at risk for genital and upper respiratory cancers.

Recent studies advocated vaccination prior to a female's first sexual experience.[36] On the other hand, according to Pichichero,[37] considering recommendation, HPV vaccine for 11- and 12-year-old girls before sexual activity may put them at risk for viral infection. Overall, the vaccine efficacy would be lower when administered to sexually active females, as sexual contact is not the only way for viral contamination.[19,20] Prophylactic vaccine should be administered prior to exposure, ideally during pre-adolescence. In addition, screening with an appropriate test may obtain approval for vaccination after birth in the future. Given the size of this study, there is a need to examine a larger cohort in order to understand the effect of HPV in the saliva, and genital carcinoma in more detail. Increasing public awareness, reduction of exposure by behavioral changing, withholding oral and hand contact as an environmental risk factor are the key points, and would prevent a substantial proportion of genital and oral lesions.[4] Protection against re-infection and protecting family members will reduce a persistent infection, and eventually, a reduction in cancer risks.

Although, it is not explicitly addressed in our study, HPV typing in saliva might identify the specific subgroup of women with undiagnosed genital lesions who might further benefit from colposcopy, in addition to Pap smear. However, it remains unclear whether behavioral intervention is useful.[38] Vaccination at a certain age before females are exposed to HPV, would have the greatest impact, but HPV vaccines do not eliminate the risk of cervical cancer.[39] Therefore, it seems that screening should be continued for the salivary detectable hrHPV similar to the high risk cervical HPV infections, because the HPV infections cannot be treated, but pre-cancerous changes can be detected by screening and surgically removed.[21,23,39] By understanding the known mechanisms of these individual viruses, there is a chance that these viruses could affect cell cycle control and inhibit apoptosis, thus potentially causing genetic instability and promote oncogenesis.[40] In addition, relevant HPV typing information in saliva samples is very important for planning a more efficient screening program, and further HPV vaccine design. It seems likely, if salivary HPV DNA testing is widely adopted, hr patients could be monitored closely for early detection of cervical and upper oral-respiratory cancers.

The limitations of this study were essentially related to the unavailability of patients to collect the salivary samples, and the small sample size. More prospective studies with large sample size are needed to show the relationship between oral and genital HPV infection.

In conclusion, the results of the present study indicates that the subtype HPV 16 is by far the most common HPV genotype associated with genital HPV-related lesions and salivary secretion. Finding HPV subtypes in saliva as a screening method may help to recognize the persistent infection. If the viral triage instead of cytological triage started at the early ages, the saliva could be a good source to recognize the high-risk population. This finding remains to be elucidated clinically, and it might be a study that turns out to be significant in the future. We conclude that salivary HPV typing, standardization of screening, and preventative methods against re-infection are essential to prevent precursor lesions. However, given the size of this study, further studies are required to examine a larger cohort in order to understand in more detail, the role of salivary HPV in genital carcinogenesis, in addition to implement HPV vaccination to decrease oral, as well as lower genital tract HPV infection.

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