Original

Asymptomatic oral human papillomavirus (HPV) infection in women with a histopathologic diagnosis of genital HPV

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Abstract: The oral route of human papillomavirus (HPV) transmission is not fully understood. It has been suggested that genital infection can act as a reservoir for oral HPV infection. We investigated the presence of oral HPV DNA and anti-HPV IgA in the buccal cavity of patients with a histopathologic diagnosis of cervical HPV infection. One hundred women underwent oral clinical examinations to detect HPV-DNA by polymerase chain reaction and salivary anti-HPV IgA by indirect immunofluorescence. Information on the personal habits of all the women was collected in personal interviews. Our results showed that 99% of the patients had no clinical manifestations of oral HPV. However, HPV DNA was detected in 81% of oral mucosa samples, and anti-HPV IgA was detected in the saliva of 44% of the patients. Consumption of alcoholic beverages was significantly associated with detection of oral HPV DNA and salivary anti-HPV IgA. Other behavioral risk factors associated with oral HPV and anti-HPV IgA are also discussed. In conclusion, patients with genital HPV infection are at risk for subclinical oral HPV infection. Thus, a molecular assay might be necessary to diagnose such infections. (J Oral Sci 53, 451-459, 2011)

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Introduction

The human papilloma viruses (HPVs) are DNA viruses that infect squamous epithelial cells (1). At least 150 genotypes of HPV have been identified, 24 of which are involved in the development of benign and malignant lesions of the oral cavity (2,3) specifically tonsillar lesions and carcinoma of the oropharynx (4,5).

The oral route of HPV transmission is not fully understood. It has been suggested that genital infection can act as a reservoir for oral HPV infection (2). The possibility of self-transmission has also been proposed, based on the detection of genetically similar HPV strains in the oral and genital mucosa and the histologic and histochemical similarities between oral and genital lesions (6,7). Women with a history of cervical cancer and partners of women with cervical cancer were reported to have an increased risk of oral cancer (8). Furthermore, it has been hypothesized that cell-associated HPV can circulate in blood, which could also facilitate self-transmission between genital and oral mucosa in an individual (9,10). To analyze the potential role of genital HPV as a route of self-transmission, we used clinical examinations and molecular detection of HPV-DNA to determine whether patients with genital HPV were at risk for HPV in their oral mucosa. We also attempted to identify synergisms between different behavioral factors that might aid in oral HPV transmission. Most of these factors are personal habits, including alcohol consumption, smoking, oral hygiene, sexual habits, and diet, and the results have been somewhat conflicting, mainly because of differences in study design (1,2,4,11-14).

This study also investigated the role of IgA response in the oral cavity among women with genital HPV infection.

Although the association between HPV and oral tumors is well established, the local immune response against HPV is not well understood (15,16). Some research has shown that persistence of the cervical HPV antigen is required to maintain an anti-HPV IgA response and that the presence of anti-HPV IgA in cervical mucosa is commonly associated with clinical lesions in the genital area (17-19). Most studies on HPV are based on assays that detect serum anti-HPV IgG; there are few studies of salivary IgA response (20-23). Thus, we analyzed salivary IgA immune response in patients and attempted to identify synergisms between different behavioral factors that might facilitate or interfere in the IgA response.

We investigated whether patients with a diagnosis of genital HPV are at risk of developing HPV at other anatomic sites, ie, oral mucosa, and to clarify the role of salivary IgA in the detection of oral HPV infection.

Methods

Patients

This study enrolled 100 women (mean age, 30 years; range, 20 to 40) from outpatient clinics in the Gynecology Department of the Santo Antonio Hospital, Salvador, Bahia, Brazil who had a clinical record of a histopathologic diagnosis of genital HPV with exophytic lesions, with or without recurrence of clinical lesions. Personal interviews were used to collect information from all the participants ($n = 100$) on their alcohol drinking habits, smoking habits, lifetime sexual activity, oral sex activities, and oral hygiene habits. This study protocol was approved by the Ethics Committee of Santo Antonio Hospital (Ethics Committee Number 06/04), and formal consent was obtained from each patient involved in this study.

Oral examination and biopsy

A trained dentist performed an oral examination of all patients in the following order: lips (skin, mucosa, and semi-mucosa), lower alveolus, gingival area, upper alveolus, tongue, floor of the mouth, palate, retromolar area, and oropharyngeal area. All clinical findings were recorded in a chart report. Two patients with HPV-like lesions underwent biopsies, during which the tissue was removed surgically, fixed in 4% buffered formalin, and embedded in paraffin. The paraffin-embedded sections were stained with hematoxylin and eosin for histopathologic investigation of HPV by Santo Antonio Hospital, Department of Histopathology.

Buccal sample collection and DNA isolation

All patients ($n = 100$) rinsed their buccal cavity with mineral water before the buccal swab, which was performed using a Digene cervical sampler kit (Digene, Gaithersburg, MD, USA) throughout the oral mucosa in the same order as the clinical examination, with the exception of the oropharynx. The cells of the oral mucosa were scraped using a soft brush, and the collected material was placed in a tube containing 2 ml of a 1 M Tris, 0.5 M EDTA solution at pH 8. The tubes were centrifuged at 8000 rpm for 15 min at 4°C, and DNA was extracted from the cellular pellet using a QIAmp DNA mini kit (Quiagen Co., São Paulo, Brazil).

Detection of HPV in the oral cavity: polymerase chain reaction (PCR) amplification

Purified DNA from each buccal smear was amplified by PCR for the HPV L1 region and for the internal reference gene ß-globin. Oligonucleotide primers were MY11 5´-GCMCAGGGWCATAAYAATGG-3´ (upstream HPV L1) and MY9 5´-GCTCCMAARGGA-WACTGATC-3´ (downstream HPV L1) for the L1 region (450 bp) and GH20 (upstream ß-globin) 5´-GAAGAGC-CAAGGACAGGTAC-3´, PCO4 (downstream ß-globin) 5´-CAACTTCATCCACGTTACC-3´ (265 bp). Amplification was carried out as previously described (24). Briefly, each PCR reaction was performed under standard conditions in a 50-μl volume containing purified genomic DNA (20 μl), 50 pmol of each primer MY09/MY011, 2.5 IU Taq DNA polymerase, 50 μm of dNTP, and 5 μl of 10 x PCR Taq Buffer (Invitrogen Co., USA). Each PCR reaction include the following controls: specimen DNA integrity control (β-globin) amplification, positive control HeLa cells, and negative control (sterile water substituted for DNA). The PCR products were visualized in 1.2% agarose gel by staining with ethidium bromide.

Detection of anti-HPV IgA in the oral cavity: indirect immunofluorescence (IFI) using HeLa (HPV 18+) cells

Before saliva collection, all ($n = 100$) patients rinsed their buccal cavity with mineral water to remove any food remnants. A saliva sample (5 ml) was collected in sterile containers, without saliva-stimulating agents, and stored at -70°C until the IFI test.

The IFI test to detect salivary anti-HPV IgA was performed using HeLa (HPV18+cells) (CCL-2, ATCC) (25). The HeLa cells were cultured on slides in Dulbecco's modified Eagle's Medium supplemented with 10% fetal bovine serum (Invitrogen Co., Brazil). The cells were fixed in cold acetone for 10 min at -20°C, air-dried, and incubated overnight with each saliva sample (1:2 and 1:4 dilutions) in a humidified chamber. The slides
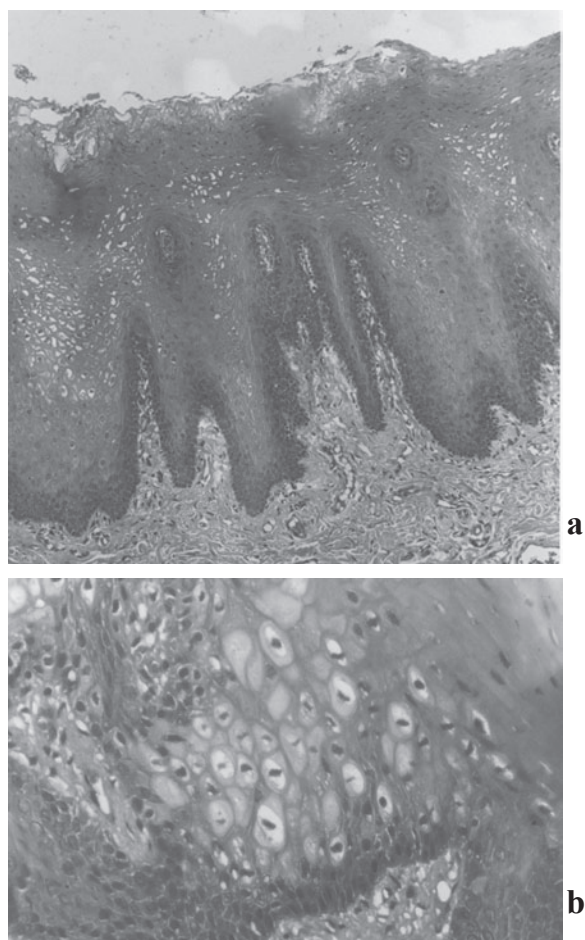
Fig. 1 Histologic findings from a biopsy of an HPV lesion. Papilloma characterized by (a) a papillary growth pattern in squamous epithelium; (b) clear cytologic evidence of typical koilocytic cells and perinuclear halo. Hematoxylin and eosin staining. Original magnification: ×100 and ×400, respectively.

Table 1 Clinical manifestations of HPV identified during examination of the oral cavity

Site	No.	(%)
Lips	18	18
Herpes-like	4	4
Tongue	8	8
Gingiva	6	6
Buccal mucosa	4	4
Vesicular-like lesion	21	21
Papilloma-like lesion	2	2
Palate	2	2
Floor of mouth	1	1
Others	17	17
None	17	17
Total	100	100

were rinsed with PBS (pH 7.4) three times for 10 min each, incubated with anti-human IgA FITC-conjugated antibodies (1:50; Sigma Chemicals Co., USA) for 1 h at 37°C, and rinsed with PBS. The final rinse was done with Evans blue (0.01%), and the slides were examined with a fluorescence microscope for the presence of apple-green fluorescence cells indicative of positive reaction.

The controls for IFI were saliva samples from a woman with a confirmed HPV oral lesion (biopsy) as positive control and saliva from an HPV-DNA-negative patient as negative control.

Statistical analysis

Data analysis was performed using the SPSS 12.0 software package for Windows (SPSS Inc.; Chicago, IL, USA). Bivariate analysis was performed to identify risk factors associated with HPV infection and anti-HPV IgA. Prevalence ratio (PR) with 95% confidence interval (CI) was used as the measure of association. The Mantel-Haenszel chi-square test was used to determine if differences between groups were statistically significant (i.e, $P \leq 0.05$). The results of PCR and IFI tests were compared using the criteria for sensitivity and specificity cited by Thrusfield (26).

Results

Detection of HPV DNA in the oral cavity

The results of clinical examination of the oral cavities of patients are shown in Table 1, including minor injuries or abrasions to the oral mucosa, tongue, and gingiva; however, the lesions resembled those produced by HPV in only two women (Table 1). The tissue was removed in both cases, and routine histologic analysis by the Department of Pathology of the Santo Antonio Hospital revealed a pattern indicative of HPV lesion in only one of the samples, namely, papillary growth (Fig. 1a) and koilocytosis (Fig. 1b) (combination of nuclear atypia and formation of a perinuclear halo).

HPV DNA was detected by PCR in 81 of the 100 oral mucosa samples (Table 2). The presence of a 450-bp fragment corresponding to the L1 gene (Fig. 2), a highly conserved region in many types of HPV, confirmed the presence of the virus in cells of the oral mucosa. Amplification of the human ß-globin gene in all DNA samples indicated that the amount of DNA used was sufficient to amplify the HPV L1 region.

Table 2 shows the associations of behavioral factors with oral HPV infection in this group of patients. In bivariate chi-square analysis, consumption of alcoholic beverages was significantly associated with oral HPV (PR: 1.3, 95% CI: 1.02 − 1.7, $P \leq 0.01$) 88.1% of

Table 2 Associations of clinical and behavioral factors with HPV in the oral cavity of patients with genital HPV infection

Variable	HPV DNA[a] in oral cavity		Bivariate analysis		
	Positive n (%)	Negative n (%)	PR[1]	95% CI[2]	P value[3]
Alcohol consumption					
Yes	59 (88.1)	8 (11.9)			
No	22 (66.7)	11 (33.3)	1.32	1.02-1.70	0.01*
Oral sex habit					
Yes	51 (79.7)	13 (20.3)			
No	30 (83.3)	6 (16.7)	0.96	0.79-1.1	0.38
Age at first sexual intercourse					
≤ 16	51 (78.5)	14 (21.5)			
> 16	30 (85.0)	5 (14.3)	1.09	0.91-1.3	0.38
Frequency of teeth brushing (daily)					
3 times	33 (75.0)	11 (25.0)			
Up to 2 times	48 (85.7)	8 (14.5)	1.14	0.93-1.4	0.18
Use of mouthwash					
Yes	12 (66.7)	6 (33.3)			
No	69 (84.2)	13 (15.8)	1.26	0.90-1.7	0.09
Clinical recurrences of HPV lesions					
Yes	18 (78.3)	5 (21.7)			
No	63 (81.8)	14 (18.2)	0.96	0.75-1.2	0.70

a: Detection of HPV DNA in oral mucosa by polymerase chain reaction, 1: Prevalence ratio, 2: 95% confidence interval, 3: x^2 Mantel-Haenszel Test, *: Significant P value
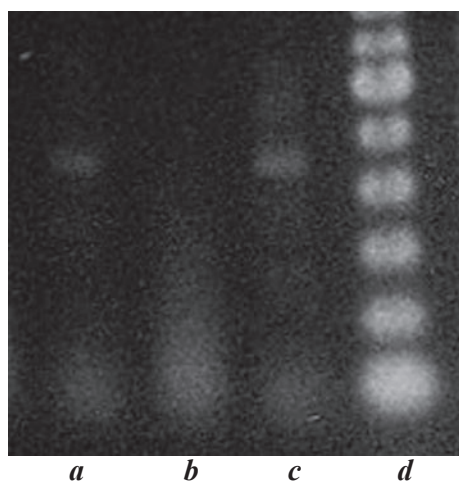

Fig. 2 PCR products for primers targeted against L1 of HPV after 35 cycles of amplification (see Methods) and analyzed in 1.2% agarose gel. Lanes (*a, c*): Oral mucosa-positive samples containing a 450-bp amplified product from the L1 region of the HPV genome; Lane (*b*): Oral mucosa-negative sample; Lane (*d*): DNA ladder (100 bp; Invitrogen Co., USA).

women who reported consuming alcoholic beverages were positive for HPV in oral mucosal cells. Other social and sexual behaviors were not significantly associated with the detection of HPV DNA in oral mucosa, even though some had a PR > 1. Detection of HPV DNA in oral mucosa was not associated with clinical viral recurrence in the genital area, as HPV in the oral cavity was found in 78% of samples with viral recurrence and 80% of samples without viral recurrence.

Detection of anti-HPV IgA in oral mucosa

Anti-HPV IgA was detected in the saliva of 44 of 100 patients on IFI. Figure 3 shows cytoplasmic fluorescence detection of HeLa cells HPV 18 (+) when anti-HPV IgA was present in saliva. The saliva was considered positive if both the 1:2 and 1:4 dilutions had positive reactions in HeLa cells. In four saliva samples, the reaction was weak in both dilutions; however, these were still considered positive.

Table 3 shows the frequencies of variables and their associations with anti-HPV IgA in saliva. Consumption of alcoholic beverages was marginally significant associated with anti-HPV IgA in saliva (PR: 1.6, 95% CI: 0.95 – 2.9; P = 0.05). However, smoking was not associated with anti-HPV IgA in saliva, possibly due to the low number of smokers among the participants. Although recurrent HPV infection, age at first sexual intercourse, and oral sex behaviors were positively associated (PR > 1) with anti-HPV IgA in saliva, the differences between groups were not statistically significant. An oral hygiene variable, frequency of teeth brushing, was the only one not associated (PR < 1) with anti-HPV IgA in saliva in these patients.
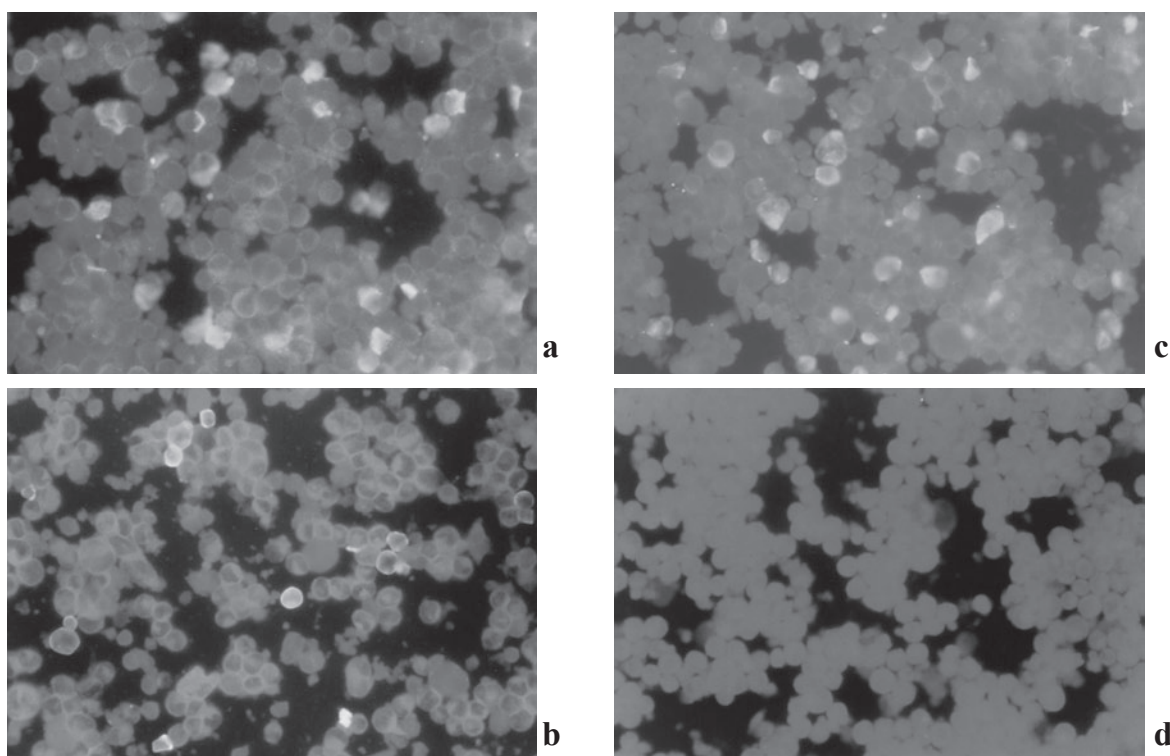
Fig. 3 Indirect immunofluorescence assay: detection of anti-HPV IgA in saliva. HeLa cells HPV 18(+) were incubated with saliva from patients to detect anti-HPV IgA and then incubated with a fluorescein-conjugated secondary anti-human IgA antibody. (a,b): Positive samples at 1:2 and 1:4 dilutions, respectively. (c): Positive control (saliva from woman with biopsy-confirmed HPV oral lesion). (d): Negative control (saliva from an HPV DNA-negative patient).

Table 3 Associations of anti-HPV IgA frequency in the oral cavity with clinical and behavioral factors in patients with genital HPV infection

| Variables | Anti-HPV IgA(IFI)[a] $n = 100$ | | Bivariate analysis | | |
	Positive n (%)	Negative n (%)	PR[1]	95% CI[2]	P value[3]
Smoking					
Yes	8 (53.7)	7 (46.7)	1.26	0.74-1.2	0.43
No	36 (42.4)	49 (57.6)			
Alcohol consumption					
Yes	59 (88.1)	8 (11.9)	1.67	0.95-2.9	0.05*
No	10 (30.3)	23 (69.7)			
Oral sex habit					
Yes	32 (50.0)	32 (50.0)	1.50	0.89-2.5	0.11
No	12 (33.3)	24 (66.7)			
Age at first sexual intercourse					
≥ 16	25 (38.5)	40 (61.5)	1.41	0.92-2.1	0.13
< 16	19 (54.3)	16 (45.7)			
Frequency of teeth brushing (daily)					
3 times	21 (47.7)	23 (69.7)	0.86	0.55-1.3	0.51
2 times or few	23 (52.7)	33 (47.7)			
Use of mouthwash					
Yes	7 (38.9)	11 (61.1)	1.16	0.62-2.7	0.63
No	37 (45.1)	45 (54.9)			
Clinical recurrences of HPV lesions					
Yes	12 (52.2)	11 (47.8)	1.26	0.78-2.0	0.37
No	32 (41.6)	45 (58.4)			

a: Indirect immunofluorescence assay, 1: Prevalence ratio, 2: 95% confidence interval, 3: x^2 Mantel-Haenszel Test, *: *borderline* significance

Table 4 Relationship between presence of HPV DNA and anti-HPV IgA in the oral cavity of patients with genital HPV infection

Anti-HPV IgA[b]	HPV DNA[a]		Total
	Positive (%)	Negative (%)	
Positive	35	9	44
Negative	46	10	56
Total	81	19	100

Sensitivity: 52.6% (95% CI: 42.9 – 62.42)
Specificity: 43.2% (95% CI: 33.50 – 52.9)
a: Detection of HPV DNA in oral mucosa by polymerase chain reaction, b: Detection of IgA in saliva by indirect immunofluorescence assay

Comparative detection of anti-HPV IgA in saliva and oral HPV DNA

The results of IFI testing for anti-HPV IgA in saliva differed from those of HPV DNA detection. The comparative results (Table 4) of the PCR assay and IFI test show that salivary anti-HPV Ig A by IFI was not reliable in detecting oral HPV infection: the sensitivity was 52.6% (95% CI: 42.9 – 62.42) and the specificity was 43.2% (95% CI: 33.5 – 52.9). As shown in Table 4, the detection of anti-HPV IgA in saliva was not associated with HPV DNA in oral mucosa. Salivary anti-HPV IgA was detected in patients who were negative for oral HPV DNA, and it was not detected in patients who were positive for oral HPV DNA.

Discussion

Our study revealed that women with a prior histopathologic diagnosis of cervical HPV—the usual method of diagnosis in public hospitals in Brazil—are at high risk for subclinical oral HPV, as indicated by the presence of the virus in the oral cavity of 81% of the present patients. A higher frequency was reported in the oral mucosa of patients with genital HPV than in those without genital infection in Brazil (27), although the authors of that study did not mention oral examination or the presence of buccal lesions. In the present study, we performed a careful oral clinical examination of all patients and found an oral HPV lesion in only one patient. This finding indicates that oral examination alone cannot exclude the possibility of HPV infection in these patients.

The prevalence of concomitant oral HPV/cervical HPV infection varies (30%–80%) (28) with the detection method used and the characteristics of the patients. We found a high prevalence of oral HPV, possibly due to the sampling method, the use of mouthwash before superficial scraping of the mucosa, (29) and the use of molecular detection based on a region that is highly conserved in many types of HPV (i.e., the L1 region)

(30). Many reports have found no significant association between cervical and oral HPV infection (6,22,31,32). We therefore concluded that a molecular test using a region common to all HPV types (L1 region) would be a good alternative to increase the sensitivity of viral detection.

Preliminary studies reported that alcohol consumption is a behavioral risk factor (13,14,33) for oral HPV infection, and we also observed a statistically significant association with oral HPV detection ($P < 0.01$), even in the absence of clinical manifestations. Alcoholic beverages might facilitate oral viral infection by interfering with the integrity of the oral mucosa or with homeostatic balance in the buccal cavity. Other risk factors analyzed in our study were associated with sexual behavior (e.g., oral sex habit), as noted previously (34,35), but there were insufficient data to prove that oral–genital contact is a mode of transmission for oral human papillomavirus.

We also examined IgA response against HPV in saliva and did not find anti-HPV IgA in all patients with oral HPV-DNA, which suggests that IFI is not sensitive enough to detect anti-HPV IgA in patients with subclinical oral HPV infection. In addition to this technical point, the absence of specific Ig A antibodies might be due to delayed immune recognition, as HPV did not result in clinical lesions in oral mucosa. Meanwhile, an association between anti-HPV IgA in saliva and clinical recurrence of HPV lesions in the genital area was observed. Over 50% of women with recurrent clinical genital HPV lesions were positive for anti-HPV IgA, whereas fewer than 50% of women who reported only one episode of lesions were positive for IgA. Although this difference was not statistically significant, prevalence ratios and measures of association indicate that recurrent clinical HPV lesions in the genital area might be associated with anti-HPV IgA in saliva. This finding reinforces the hypothesis that there is a mucosal immune system that connects different anatomic compartments. We surmised that anti-HPV Ig A was produced locally

in the genital mucosa by mucosal plasma cells and that, after this initial triggering, precursor cells disseminate to oral mucosa via regional lymph nodes, lymph, and blood (transudate IgA) (1,36-38).

Studies have identified factors and behavioral habits that might facilitate viral infection by interfering with the integrity of the oral mucosa or the homeostatic balance in the buccal cavity. We found a statistically significant positive association between alcohol use and anti-HPV-IgA in saliva. Such an association is plausible given that alcohol might alter the integrity of oral mucosa (39) and thus the local immune response; however, the present finding does not conform with those of other studies. Our data reinforce the concept of a mucosal immune system, as anti-HPV IgA in saliva might come from another anatomic site, as was previously suggested (22,36,40). Other factors, including oral–genital sex and age at first sexual contact, were positively associated with anti-HPV IgA in saliva, but the associations were not statistically significant.

This study shows that patients with genital HPV infection are at risk for oral infection without clinical manifestation and are very likely to have the virus in their oral mucosa. In addition, the behavioral risk factor of alcohol consumption was clearly associated with oral HPV and anti-HPV IgA detection. The absence of clinical symptoms in the oral cavities of these patients suggests subclinical infection, and a molecular assay might thus be necessary to diagnose this infection. It is not known whether this subclinical infection is as contagious as infections with exophytic lesions; however, despite the absence of lesions, anti-HPV IgA could be detected. We suspect that the IgA we detected in this group of patients is a response to prior sensitization at the initial anatomic site of infection, ie, the genital area.

Future long-term studies of patients with subclinical oral HPV infection will clarify the pathophysiologic development of this type of infection and the role of anti-HPV IgA (via transudation or local production) in protecting oral mucosa.

Acknowledgments

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