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Biopsy vs. superficial scraping: detection of human papillomavirus 6, 11, 16, and 18 in potentially malignant and malignant oral lesions

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BACKGROUND: Several epidemiologic studies have shown a broad variation in the prevalence of human papillomavirus (HPV) in oral precancerous tissues and oral carcinomas.

METHODS: Biopsies and superficial scrapes of lesions, clinically suspected of HPV infection, were taken from patients with potentially malignant and malignant oral lesions, and subject to HPV DNA detection by PCR–Southern blot analysis.

RESULTS: From 22 patients with potentially malignant and malignant lesions analyzed, 41% of the biopsies were HPV DNA positive, whereas 95–100% of the superficial scrapes were positive (McNemar, $P < 0.0001$). Clinical presumption of HPV infection detected 67% ($P < 0.0001$) of the HPV DNA positive cases compared with 48% ($P < 0.0001$) determined by cytology and histopathology. The prevalence of HPV 6, 11, 16 and 18 in the oral mucosa was studied in 59 individuals. While 9% of normal controls were HPV DNA positive, 100% of the patients with potentially malignant and malignant lesions were HPV DNA positive, and the prevailing genotype was HPV 16 followed by HPV 18.

CONCLUSIONS: The higher HPV DNA detection rate in superficial oral scrapes than in biopsies suggests that accurate epidemiological information on oral HPV infection/oral carcinogenesis depends not only on the DNA detection technique, but also on the tissue/cell sampling procedure.

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Keywords: papillomavirus; oral squamous cell carcinoma; human papillomavirus clinical presumption; superficial scrapes; biopsy

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Introduction

Human papillomaviruses (HPVs) may cause papillomas (benign epithelial growths), or skin and genital warts. At least 100 genotypes of HPVs have been identified. Although most are found in benign proliferations, some are associated with malignant tumours of cervical, anogenital, skin, oral and nasal epithelia (1).

Epidemiological studies have identified HPV infection as an important risk factor for precancerous lesions and cancer of the cervix uteri (2). Furthermore, the oncogenic potential of the HPV E6 and E7 gene products in the cervix has been demonstrated (3). High-risk HPV-16 and HPV-18 were found to be associated with the development of genital cancer (4). Conversely, HPV-6 and HPV-11 were rarely found in genital cancer (5).

At least 16 HPV DNA genotypes (1, 2, 3, 4, 6, 7, 10, 11, 13, 16, 18, 31, 32, 33, 35, 57) have been isolated from oral lesions (6, 7). Most of them are low-risk HPVs (i.e. 6, 11, 13, 32) and are associated with benign proliferative epithelial lesions of the oral cavity (e.g. squamous papilloma, condyloma acuminatum, verruca vulgaris and focal epithelial hyperplasia). In addition, high-risk HPV genotypes (i.e. 16, 18, 31, 33, 35) have been reported to be associated with epithelial dysplasia and oral squamous cell carcinoma (OSCC) (8–11). A meta-analysis that included data from 94 reports analyzing 4680 samples was used to estimate the risk of HPV infection in normal oral mucosa, pre-cancerous oral tissue and OSCC. The study showed that the probability of detecting HPV was two- to threefold higher in precancerous oral mucosa, and four to sevenfold higher in oral carcinoma, when compared with normal oral mucosa. The probability of detecting high-risk HPVs in OSCC was 2.8-fold higher than that of low-risk HPVs (12).

The aim of the present study was to evaluate a non-invasive technique for HPV genotyping in potentially malignant and malignant oral lesions which, once clinical features of the lesions were determined and

confirmed by histology and HPV genotyping, would allow a future follow- up of oral cancer patients. Cervical superficial scraping is the standard technique for HPV DNA detection in the cervix (13), oral scrapes were previously tested to detect HPV in a northern European population (14). Although comparative analysis of cervical scrapes and biopsy specimens have shown no significant difference in the detection rate of HPV DNA, cervical scrapes provided more accurate information about the spectrum of HPV genotypes present in the cervix (15). Therefore, we performed a comparison between biopsies and superficial scrapes as sampling methods for the detection of HPV DNA and determination of the HPV genotype spectrum in potentially malignant and malignant oral lesions.

As HPV genotyping is not easily accessible in developing countries and in underserved communities, a second goal of this work was to define the clinical features of HPV infected oral mucosa in potentially malignant and malignant lesions, in order to assist the clinician in the diagnosis of potentially malignant lesions that could evolve into OSCC or verrucous carcinoma (VC). After studying in detail, the clinical features of uterine lesions with confirmed HPV diagnosis (16, 17), we used this experience to support the clinicians in the detection of HPV infections in potentially malignant oral lesions, which presented similar clinical features to the ones we had seen in cervical lesions.

Materials and methods

Study subjects

Cytological scrapes and biopsy specimens from 59 consenting individuals either with oral lesions or healthy, apparently normal mucosa were analyzed. The case group included 33 subjects (21 men and 12 women) with a mean age of 58.7 years (range 27–83 years) with potentially malignant or malignant lesions. Potentially malignant lesions studied included: leukoplakias (18–19), as well as plaque and atrophic lichen planus (20–23), while malignant lesions included: OSCC and VC (24–26). Before being considered for the study, the lesions from the case group were diagnosed clinically and confirmed by histopathology. The normal control group consisted of 23 subjects (six men and 17 women) with a mean age of 38.1 years (range 8–72 years) with clinically normal mucosa. Three subjects with oral condyloma acuminata were selected as the positive HPV control group.

Clinical presumption of HPV infection

The clinical features considered as signs of putative HPV infection in oral mucosa were: white, bright, flat lesion with slightly elevated patches or plaques with erithematous background (red inflammatory halo directly associated with the lesion) and frankly verrucous (cauliflower-like) lesions.

Sampling of cytological scrapes and tissue biopsies

The sampling technique chosen for this study was with sterile metallic spatula, which allows a more precise selection of the sample sites than the technique with cyto-brush. The scrapings were performed in the involved mucosa and in the contra lateral normal non-keratinizant oral mucosa. Each site was scraped five times and care was taken to avoid cross contamination between sample sites. The tip of the spatula was immersed in 2.5 ml of phosphate buffer saline solution pH 7.5, and subsequently frozen at −80°C. The scrapings were done for each patient, for PCR–Southern blot genotyping and for cytological diagnosis. Tissue samples were collected through surgical biopsy from lesions of the oral mucosa. All biopsy samples were obtained from lesions clinically suspected of HPV infection systematically taking the full extension of the lesion, which assures clinically that the material being studied is from the lesion under examination; the obtained specimens were bisected for PCR–Southern blot analysis and for examination by histopathology.

Cytological and histopathological analysis

The material for histopathological studies was fixed in 10% formaldehyde, paraffin-embedded and stained with hematoxylin/eosin. The smears for cytological evaluation were fixed in 96% ethanol and stained with Papanicolau technique. The evaluation of the results of both procedures was performed as a double blinded study. The presence of several gathered koilocytos (27) in the intermediate and superficial cells of stratified squamous epithelia was considered as the possible indicators of HPV presence (28).

DNA extraction

Cytological scrapes and disaggregated biopsy specimens were centrifuged at 3000 rpm for 5 min, the supernatant was discarded and the pellet was washed twice with sterile TE buffer (10 mM Tris pH 8.3, 1 mM EDTA). Subsequently the pellet was dissolved in 150 μl of 10 mM Tris pH 8.3, 0.45% Tween-20; 0.45% Nonidet P-40, and 200 μg/ml of Proteinase K, and incubated at 55°C for 1 h for cytological scrapes and overnight for biopsy specimens. Proteinase K was deactivated at 95°C for 15 min. The DNA was further purified by phenol/chloroform extraction and precipitated with ethanol and NaCl (29). The purified DNA was dissolved in 50 μl of TE buffer and stored at −20°C.

PCR of HPV genome

Different volumes of each specimen were used as template with L1 consensus primers in a final volume of 50 μl at the following conditions: buffer 10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 3 mM $MgCl_2$, 300 μM dNTPs, 1 μM of primer $GP5^+$ (5′-TTTGTTACTGTGGTAGATACTAC-3′), 1 μM of primer $GP6^+$ (5′-GAAAAATAAACTGTAAATCA-TATTC-3′) (30) and two units of Taq polymerase. The PCR was done with the following amplification profile: a hot start at 94°C, denaturation at 95°C for 45 s, annealing at 55°C for 45 s, extension at 72°C for 45 s, followed by 5 min of final extension at 72°C. As positive controls for HPV 6 and 11, 100 μg of plasmid DNA containing either HPV6 or HPV11 genome were used

(provided by Dr E.M. De Villiers, Heidelberg, Germany). Moreover, for HPV 16 and 18, 500 pg of CaSki or Hela cell DNA were used as positive controls; both human cervical carcinoma cell lines were derived from HPV-infected tumours and known to carry HPV 16 and 18 sequences, respectively (31, 32). The PCR reaction was performed in a Perkin Elmer GeneAmp PCR System 9600 (Perkin Elmer Corporation, Norwalk, CT, USA), and the PCR products were analyzed by electrophoresis in 6.5% polyacrylamide native gels (29). To rule out contamination on the PCR reaction, three negative controls were included containing all reaction components, except DNA template.

Southern blot

The PCR products were analyzed by electrophoresis in 0.8% agarose gel. The gel was then denatured in 0.5 M NaOH, 1.5 M NaCl for 15 min, neutralized twice in 1.5 M NaCl, 0.5 M Tris–HCl pH 8, and blotted overnight onto a nylon membrane (Biodyne type A, Gibco BRL, Gaithersburg, MD, USA) in 10× SSC (1.5 M NaCl, 0.15 M sodium citrate dihydrate). The membrane was saturated twice for 15 min in 2× SSC in 1% SDS, UV cross-linked for 1 min, baked at 80°C for 30 min, and pre-hybridized at 55°C for 45 min in a solution containing: 6× SSC, 5× Denhardt's solution, 0.1% SDS and 100 μg/ml of Herring Sperm (Calbiochem, La Jolla, CA, USA). The membrane was subsequently hybridized at 55°C for 1 h in the same solution supplemented with biotinylated HPV genotype-specific oligonucleotide probes: MY12: 5′-CATCCGTAACTACATCTTCCA-3′, MY13: 5′-TCTGTGTCTAAATCTGCTACA-3′, Probe 16: 5′-GTCATTATGTGCTGCCATATCTACTTCAGA-3′, Probe 18: 5′-TGCTTCTACACAGTCTCCTGTACCTGGGCA-3′ (33, 34). After hybridization, the membrane was washed and the probe detection with streptavidin-alkaline phosphatase was done using the Photogene Nucleic Acid Detection System Version 2.0 (Gibco BRL), according to the manufacturer's instructions.

The positive controls of Southern blot were the PCR positive controls assayed with the corresponding probe (i.e., HPV 11 DNA was assayed with HPV 11 type probe). Instead the negative controls were assayed with a different HPV genotype probe (i.e., HPV 11 DNA was assayed with HPV 6, 16 and 18 type probe).

PCR of β-globin gene

The following β-globin primers were used to confirm the presence of amplifiable DNA in the extracted specimens: PCO3 (5′-ACACAACTGTGTTCACTAGC-3′) and PCO4 (5′-CAACTTCATCCACGTTCACC-3′) (35). PCR conditions were the same as described for the general primers $GP5^+/GP6^+$ except that 0.5 μM of the PCO3 and PCO4 primers were used.

DNA quantitation

To quantify DNA, the ACES 2.0 Human DNA Quantitation System was followed (Gibco BRL). DNA from 0.5, 1, 5, 10, 100, and 1000 of either CaSki or Hela cells were quantified to use as standards. Biopsy and cytological scraping specimens that were PCR–Southern blot negative were also subject to DNA quantitation.

Statistical analysis

McNemar test was applied to test homogeneity of proportion in cases where the samples were not independent. A *P*-value of 0.05 was considered significant (36). Sensitivity and specificity of clinical presumption and histopathology (Table 3) were calculated in reference to PCR.

Results

In order to genotype HPV 6, 11, 16, and 18 in patients with potentially malignant and malignant oral lesions, PCR HPV genome amplification followed by Southern blot analysis were implemented using the general primers $GP5^+/GP6^+$ (30), with the specific probes for genotypes 6, 11, 16 and 18 as described in Materials and methods (Fig. 1). With the purpose of evaluating a non-invasive technique for HPV genotyping to follow-up oral cancer patients, superficial scrapes vs. biopsy tissues were studied in patients with potentially malignant and malignant lesions. Biopsies from nine patients (41%) with potentially malignant and malignant lesions were HPV positive by PCR-Southern blot analysis in the biopsy tissues (Table 1). On the other hand, 21 (95%) and 22 (100%) of these lesions were HPV positive when superficial scrapes of the lesion's mucosa and the contralateral normal mucosa were, respectively, analyzed. There was no significant difference in HPV detection between lesion and contralateral normal mucosa scrapes (McNemar, $P = 0.3938$), but there was a significant difference between biopsies and superficial scrapes either from the lesion or the contralateral normal mucosa (McNemar, $P < 0.0001$). It is important to point out that β globin gene was normally amplified from all negative biopsy samples. Biopsy DNA (median 14 ng) was between four and 2000 times higher than scraping DNA (median 0.27 ng). In addition, the sensitivity of the PCR–Southern blot assay established for Hela cells was around 0.155 pg of Hela DNA. Hence, the lower HPV detection in biopsy tissues was not due to insufficient amount of DNA.

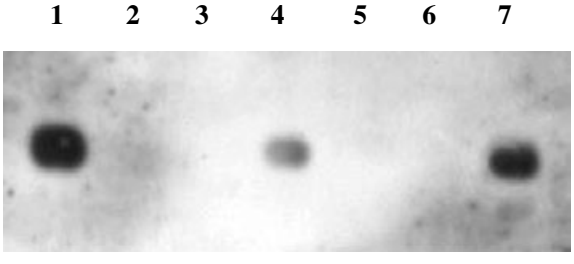

Figure 1 Human papillomavirus 18 genome detection by PCR–Southern blot assay. The PCR products from cytological scrapes were subjected to a Southern blot analysis using an HPV 18 probe. Lanes 1, 2, 3, 4, and 5 correspond to patients 3, 6, 10, 19, and 20, respectively. Lane 6 is a negative control, and lane 7 corresponds to Hela DNA (HPV 18 positive).

Table 1 Detection of HPV 6, 11, 16 and 18 in biopsy tissues and cytological scrapes of the lesions and contralateral mucosa

Patient	*Histopathological diagnosis*	*HPV genotypes*		
		Biopsy	*Cytological scrape (lesion mucosa)*	*Cytological scrape (contralateral mucosa)*
1	Malignant lesion	16	16	16
2	Malignant lesion	16–18	16–18	16–18
3	Malignant lesion	16–18	16–18	16–18
4	Malignant lesion	Negative	6-16-18	[a]
5	Potentially malignant lesion	11	11–16	11–16
6	Potentially malignant lesion	16	16	16
7	Potentially malignant lesion	Negative	16–18	16–18
8	Malignant lesion	16	16	16
9	Malignant lesion	Negative	11–16	11–16
10	Malignant lesion	Negative	16	16
11	Malignant lesion	[a]	[a]	16
12	Malignant lesion	Negative	Negative	18
13	Potentially malignant lesion	Negative	[a]	[a]
14	Malignant lesion	Negative	11	[a]
15	Potentially malignant lesion	Negative	[a]	[a]
16	Malignant lesion	Negative	16	16
17	Potentially malignant lesion	Negative	[a]	[a]
18	Malignant lesion	Negative	[a]	[a]
19	Malignant lesion	11	18	18
20	Malignant lesion	11	11	11
21	Potentially malignant lesion	Negative	11	[a]
22	Potentially malignant lesion	Negative	11	[a]

HPV, human papillomavirus.
[a]Other HPV than 6, 11, 16 and 18 genotypes.

Another purpose of this study was to investigate if similarity with clinical features of uterine lesions caused by HPV infection could be used to infer HPV infection in the equivalent oral lesions. The diagnostic criteria for clinical presumption of HPV infection were white, bright, flat lesions with slightly elevated patches or plaques with erithematous background and frankly verrucous (cauliflower-like) lesions (Fig. 2). Clinical presumption of HPV infection was correlated to the presence of koilocytosis determined by histopathology or cytopathology, and HPV genome detection by PCR–Southern blot, in normal control individuals and patients (Table 2). Sixty-seven percent of potentially malignant and malignant lesions were HPV positive by clinical criteria, 48% were koilocytosis positive, and 100% HPV genome positive. As expected, compared with PCR–Southern blot genome detection, both clinical HPV presumption and histopathological diagnosis underestimated the incidence of HPV infection. Therefore, a McNemar test was performed to compare PCR vs. clinical presumption and PCR vs. histopathological results. Significant differences were found between PCR and clinical presumption results as well as between PCR and histopathological results (Table 3).

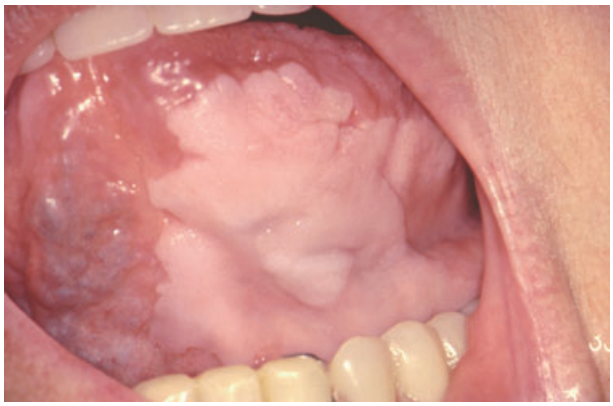

Figure 2 Oral lesion presumptively infected with human papillomavirus. A 62-year-old female patient presenting a like thick white bright plaque lesion. It is noteworthy that the patient did not smoke nor consumed alcoholic beverages.

Table 2 Clinical presumption of human papillomavirus (HPV) infection, koilocytosis, and PCR–Southern blot analysis of HPV in oral mucosa

Diagnosis	*HPV presumption*	*Koilocytosis*	*HPV genome*
Normal controls ($n = 23$)	0	0	2
Potentially malignant and malignant lesions ($n = 33$)	22	17	33

Table 3 McNemar test results, sensitivity and specificity of PCR vs. clinical presumption, PCR vs. histopathology and clinical presumption vs. histopathology

	McNemar P-value	*Sensitivity*	*Specificity*
PCR vs. clinical presumption	< 0.0001	0.63	1.0
PCR vs. histopathology	< 0.0001	0.49	1.0
Clinical presumption vs. histopathology	0.1797		

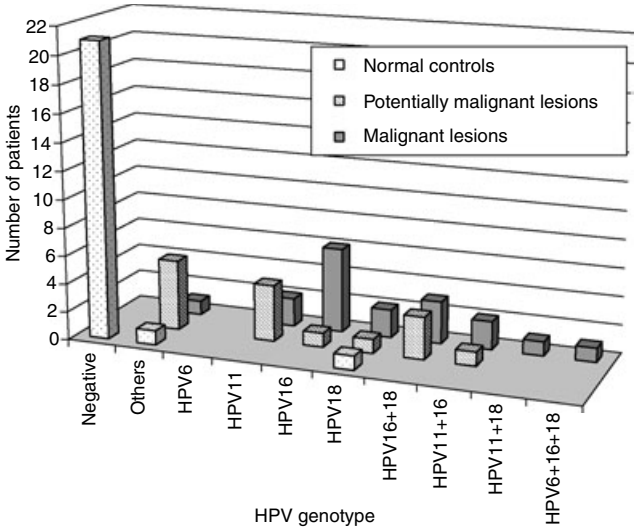

Figure 3 Prevalence of human papillomavirus (HPV) 6, 11, 16 and 18 in normal controls and patients with potentially malignant and malignant lesions. DNA from cytological scrapes were subjected to HPV genome detection by PCR–Southern blot analysis.

Considering that superficial scrapes resulted in higher HPV detection rates than biopsies, the prevalence of HPV 6, 11, 16 and 18 in potentially malignant and malignant oral lesions was subsequently analyzed using the scraping collection technique. As shown in Fig. 3, from 23 normal controls, 91.3% were HPV negative, 4.3% were HPV 18 positive and 4.3% presented HPV genotypes other than 6, 11, 16 and 18. From 15 patients with potentially malignant lesions, none were negative or HPV 6 positive, 34% presented HPV 11, 34% were positive for HPV 16, and 27% for HPV 18. Remarkably, 27% of the cases with potentially malignant lesions had two HPV genomes (16/18 or 11/16). From the 18 patients with malignant lesions, all were HPV positive, 6% presented HPV 6, 28% HPV 11, 67% HPV 16, and 39% HPV 18. The total percentage is over 100% of the casuistic, because 33% of the patients with malignant lesions were positive for two HPV genomes (16/18, 11/16, or 11/18), and 6% had three HPV genomes, i.e. HPV 6/16/18.

Discussion

The present study demonstrates that in potentially malignant and malignant oral lesions, HPV DNA is more likely to be detected in superficial scrapes than in biopsy specimens. Similar results were obtained by Lawton *et al.* (14), who found that in normal oral mucosa, there was a higher detection rate of HPV DNA in mouthwashes and scrapings compared with biopsies. The difference between superficial scrapes and biopsy specimens was not due to PCR inhibition, as constitutive β- globin gene was normally amplified from all negative biopsy samples. Moreover, we ruled out that the lower HPV detection sensitivity in biopsies was because of an insufficient amount of DNA, as all samples were submitted to DNA quantification by slot blot assays using a specific human DNA probe as described in Materials and methods. The results are significant considering that the biopsy samples yield higher amounts of cellular DNA than superficial scrapings. Consequently, cytological scraping should be considered the ideal sampling technique for HPV detection in the oral cavity. This finding is in agreement with a study by Syrjänen and Syrjänen (13) on 102 cervical biopsies, which was known to contain HPV DNA. These investigators demonstrated that the presence of HPV in epithelium increased progressively from the basal cell layer (5.8%) to the superficial layers (100%). These results could be due to the two different cellular localizations of HPV DNA replication, i.e. in the germinative and superficial epithelial layers. The first type of replication is localized in the cells of the lower portion of the epithelium including the basal cells, where the viral DNA is apparently maintained as a stable multi-copy plasmid, and replicates at an average of once per cell cycle and is distributed to the daughter cells, ensuring a persistent and latent infection of the basal epithelial cells. The second type of DNA replication is the vegetative replication, which occurs in the differentiated cells of the epithelium where there is no cellular DNA synthesis, a burst of viral DNA synthesis occurs, generating sufficient viral DNA for packaging into virions (37).

In addition, our results show that HPV DNA was equally detected in superficial scrapes of the lesion and contralateral normal mucosa, as the whole oral mucosa is likely to be infected.

Oral mucosa lesions showing white and bright features, or thickened white patches, exophytic and wart-like lesions, erithematous areas within white patches could be considered as the clinical manifestations of oral HPV infection. Positive correlation was observed between clinical and histopathological manifestations of potentially malignant and malignant oral lesions, probably because the morphologic changes generated by viral infection produced pathognomonic gross manifestations that could help in some cases a qualified professional to infer HPV infection. The higher sensitivity and specificity of HPV detection using PCR–Southern blot analysis in comparison with the less precise evaluation of clinical manifestations of HPV infection and H&E histopathological diagnosis is supported by prior studies reported in HPV genital tract infection (38). This discrepancy is because of the fact that molecular biology techniques can detect latent infections without viral production. Despite this underestimation, the clinical parameters described above could be used for malignant lesions prevention and follow- up of patients with potentially malignant and malignant oral lesions in developing countries, where molecular biology techniques are not readily available.

The HPV epidemiology of the current study showed that 100% ($P < 0.001$) of the patients with potentially malignant and malignant lesions were HPV DNA positive. Thirty-four percent of the patients with potentially malignant lesions were positive for HPV 16, and 27% of them had HPV 18. In malignant lesions, 67% of the patients had HPV 16, and 39% were positive for

HPV 18, 27% of the patients with potentially malignant, and 39% with the malignant lesions exhibited more than one HPV genotype. A previous meta analysis done comprising studies from 1982 to 1997 showed that the likelihood of detecting HPV in OSCC was between 36.6% and 55.5% (95% CI), and the probability of detecting high risk HPV in OSCC was 2.8 times higher than that of low-risk HPVs (12). The difference in HPV incidence with our data is probably because of the fact that the meta-analysis was based on biopsy specimens. Other data showing differences between sampling procedures performed in the cervix showed that multiple genotypes were more frequently detected in cervical scrapes than in biopsy specimens (15).

As a whole, there are conflicting data on the potential role and incidence of HPV in oral carcinogenesis. It is essential to determine real HPV prevalence in order to infer the significance of HPV infection in oral carcinogenesis. Thus, any future effort to detect HPV DNA in the oral cavity has to take into consideration the type of specimen used (biopsy, swabs, scrapes, and lavages), the sensitivity of detection methods (*in situ* hybridization, single PCR, nested PCR, PCR-Southern blot, etc.), and the anatomical sites that are tested.

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