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Epstein-Barr virus in oral hairy leukoplakia scrapes: identification by PCR

Vírus Epstein-Barr em esfregaço de leucoplasia pilosa oral: identificação por PCR

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ABSTRACT: Oral hairy leukoplakia (OHL) is a lesion associated with a compromised immune system, and its diagnosis is determined by the demonstration of the presence of Epstein-Barr virus (EBV) in lesional tissue. The purpose of this article was to develop a simple technique to help the diagnosis of OHL, using PCR as an alternative technique to evidence EBV in scrapings. DNA samples were obtained by scraping the lateral border of the tongue of 38 adult patients: 29 HIV-positive patients (4 with clinical evidence of OHL; 4 with history of OHL, but without lesion at the moment the samples were collected; and 21 without clinical evidence of OHL), and 9 healthy volunteers for the control group. DNA was extracted from scrapes and amplified by PCR using specific primers for EBV. Of the 29 cases of HIV-positive patients, 22 (75.86%) were positive for EBV: 2 patients with clinical evidence of OHL, 4 patients with history of OHL, but without lesion at the moment the samples were collected, and 16 patients without clinical evidence of OHL. In the control group, samples of 5 (55.56%) healthy volunteers presented amplification for EBV. We concluded that the use of PCR in oral scrapes suggests a high sensitivity but low specificity for the diagnosis of OHL.

DESCRIPTORS: Epstein-Barr virus infections; Leukoplakia, hairy; Polymerase chain reaction; HIV.

RESUMO: A leucoplasia pilosa (LP) é uma lesão associada ao comprometimento do sistema imune e seu diagnóstico é determinado pela demonstração da presença do vírus Epstein-Barr (EBV) no tecido lesionado. O objetivo deste trabalho foi desenvolver uma metodologia simples para auxiliar no diagnóstico da LP, utilizando-se a técnica da PCR como uma alternativa para evidenciar o EBV em esfregaços da lesão. Amostras de DNA foram obtidas por meio de raspado de borda lateral de língua, de 38 pacientes adultos, sendo estes: 29 pacientes HIV positivos (4 com evidência clínica de leucoplasia pilosa, 4 que haviam apresentado LP previamente, mas não no momento da coleta, e 21 sem evidência clínica de leucoplasia pilosa) e 9 voluntários sadios para o grupo controle. O DNA foi extraído do material obtido por raspagem e amplificado pela PCR utilizando-se iniciadores específicos para o EBV. Dos 29 casos de pacientes portadores do vírus HIV, 22 (75,86%) foram positivos para o EBV, sendo: 2 de pacientes com evidência clínica de LP, 4 de pacientes que haviam apresentado LP previamente e 16 de pacientes sem sinais clínicos de LP. No grupo controle, as amostras de 5 (55,56%) indivíduos clinicamente sadios mostraram amplificação para o EBV. Concluímos que o uso da PCR em esfregaços sugere uma alta sensibilidade e baixa especificidade no diagnóstico da leucoplasia pilosa.

DESCRITORES: Infecções por vírus Epstein-Barr; Leucoplasia pilosa; Reação em cadeia da polimerase; HIV.

INTRODUCTION

Oral hairy leukoplakia (OHL) is a white patch usually ocurring on the lateral margins of the tongue, often bilaterally. Tipically, vertical corrugations occur bilaterally on the lateral borders or ventral surfaces of the tongue, but if present on the dorsum of the tongue the appearance tends to be more homogeneous[9]. OHL is related to immunosuppression in general. It has been described in HIV-positive patients, patients receiving immunosuppressive therapy for acute leukemia and after solid organ transplantation[5,11]. OHL may be confused with other white lesions seen in the mouth, such as lichen planus, white sponge nevus, idiopathic and tobacco-associated leukoplakia, galvanic lesions and frictional keratosis or hyperplastic candidiasis[8,9]. It is seen less frequently on the

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buccal mucosa and oropharynx[9]. There are some reports in patients with no evidence of clinical immunodeficiency[6].

The Epstein-Barr virus is present in a productive, infective state in epithelial cells of the superficial layers of the lingual epithelium[11]. Demonstration of the presence of Epstein-Barr virus DNA in the epithelial cells of OHL is the definitive criterion in the diagnosis of this lesion[9]. The Epstein-Barr virus is a herpesvirus group member and is not only associated with OHL but also with Burkitt's lymphoma, nasopharyngeal carcinoma, infectious mononucleosis and Hodgkin's disease[11]. It is a matter of discussion if the EBV is associated with squamous cell carcinoma of the oral cavity[7]. The lesion must be accurately diagnosed because a diagnosis of OHL has serious implications as to the immune status and even HIV serostatus of the patient[9]. In HIV-seropositive patients OHL is considered a predictor for progression to AIDS[11]. The histopathological diagnosis exhibits characteristic features, but it is not pathognomonic. This may pose a difficult diagnostic dilemma, because of the serious implications in suspecting of an HIV-infection[11].

The definitive diagnosis of OHL currently relies upon such evidence of EBV in lesional tissue[14]. Although this can be achieved by immunohistochemistry, the most accurate and sensitive method is *in situ* hybridization. However, these techniques require a biopsy which may not be readily obtainable, either due to patient refusal or because of coexisting bleeding diatheses due to conditions such as chronic liver disease, haemophilia, or thrombocytopenia, commonly encountered in HIV-seropositive patients[11]. The Polymerase Chain Reaction (PCR) is a sensitive technique that can be used on very small tissue samples, but only detects the presence or absence of the target DNA sequence in the sample, and not its location[11].

The purpose of this study was to investigate the potential role of PCR as a minimally-invasive and simple technique for detection of EBV in tongue scrapings from OHL lesions.

MATERIALS AND METHODS

Patients

Tongue scrapings for PCR analysis were obtained from 29 HIV-seropositive adult patients: 21 without OHL, 4 with clinical evidence of OHL, and 4 with a history of OHL but no lesion at the moment samples were collected. These patients were attending the Special Care Dentistry Center (SCDC), School of Dentistry, University of São Paulo. Control specimens were obtained from 9 healthy volunteers with clinically normal oral mucosa. For ethical reasons the HIV-serostatus of these patients was not determined. This study was approved by the Ethics Committee, School of Dentistry, University of São Paulo.

Samples for PCR analysis were obtained by scraping the lateral border of the tongue after an oral PBS 1 x rinse (0.01 M KH_2PO_4, 0.1 M Na_2HPO_4, 1.37 M NaCl and 0.027 M KCl – Invitrogen, Carlsbad, USA) for 2 minutes, in order to reduce possible salivary contamination[1]. Smears were collected using a cytobrush (Kolplast CI Ltda., SP, Brazil). The brush was held against the lateral border of the tongue and rotated for ten full turns. Samples were placed in 1.5 ml tubes with 1 ml of PBS and stored at –80°C.

DNA extraction and PCR

Before DNA extraction, samples were centrifuged at 1,000 x g for 10 min at 4°C; pellets were resuspended in 1 ml of PBS and centrifuged one more time at 1,000 x g for 1 minute.

Tubes containing the pellets received 300 µl of sterile digestion buffer (1 M NaCl; 1 M Tris-HCl, pH 8; 0.5 M EDTA pH 8; 10% SDS – Invitrogen, Carlsbad, USA) and 100 µg/ml of proteinase K (Invitrogen, Carlsbad, USA). The tubes were placed in a water bath at 55°C for 5 h. Proteinase K was inactivated at 95°C for 10 minutes. Two hundred microliters of 1 N ammonium acetate (Invitrogen, Carlsbad, USA) were added to the tube containing the cell lysate. Tubes were vortexed vigorously at high speed for 20 seconds, incubated on ice for 5 minutes and centrifuged at 13,000 x g for 3 minutes. DNA was precipitated with isopropanol (Sigma Chemical Co., St. Louis, MO, USA) and centrifuged at 16,000 x g for 5 minutes. Thirty microliters of TE (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA, pH 8 – Invitrogen, Carlsbad, USA) were added to the pellet. DNA was stored at –20°C until use.

For DNA quality control, exon 7 (190 pb) of the human cytokeratin 14 (CK14) gene was amplified by PCR. The sequences of CK primers were as follows:

Forward (K7F3): 5'-GTT CGA ACC AAG AAC TGA GGG-3'.

Reverse (K7F3N): 5'-CCA GAG AGA GGC GAG AAT TA-3'.

Polymerase chain reactions were performed in 0.5 ml microcentrifuge tubes in a final mixture of 25 µl containing 1% formamide, PCR buffer 1 x (200 mM Tris-HCl, pH 8,4; 500 mM KCl), 0.3 mM dNTP, 2 mM $MgCl_2$, 100 pM of each primer, for-

ward and reverse, 2 U of Taq DNA polymerase, 3 µl of DNA, and sterile H_2O, in a total volume of 25 µl (all reagents from Invitrogen, Carlsbad, USA). PCR cycling conditions (PTC-100 Programmable Thermal Controller, MJ Research, Inc., Watertown, USA) were: denaturation: 3 min/95°C and 35 cycles of denaturation: 1 min/94°C, annealing: 1 min/56°C, extension: 72°C/50 seconds and a final extension: 4 min/72°C. A case of fibrous hyperplasia was included.

A case of lymphoma with the EBV virus demonstrated by *in situ* hybridization was used as positive control. EBV-specific oligonucleotide primers, shown below, were directed towards the Bam-HI-K conserved region of the EBV genome encoding EBNA (269 bp – Ammatuna *et al.*[2], 1998). In each reaction a tube with no DNA was included.

Forward: K1: 5'-GTC ATC ATC ATC CGG GTC TC-3'.

Reverse: K2: 5'-TTC GGG TTG GAA CCT CCT TG-3'

PCR reagents were described previously with a primer concentration of 250 pM and 6 µl of target DNA. The PCR cycling conditions were: denaturation: 3 min/95°C and 40 cycles of denaturation: 1 min/94°C, annealing: 50 seconds/56°C, extension: 1 min/72°C and a final extension: 7 min/72°C.

The PCR product was submitted to electrophoresis (EasyCast™ OSP-105, Owl Separation Systems, Portsmouth, USA) in a 2% agarose gel containing ethidium bromide (Invitrogen, Carlsbad, USA) (0.5 g/ml) and visualized under ultraviolet illumination (Fotodyne, Hartland, USA). Low DNA mass ladder (Invitrogen, Carlsbad, USA) was used as base pair molecular weight pattern.

RESULTS

Twenty-two (75.86%) of twenty-nine cases were positive for EBV. Two out of four patients with clinical evidence of OHL, 4 out of 4 patients who have had OHL previously but not at the moment samples were collected, and 16 out of 21 patients with no clinical evidence of OHL (Table 1).

Figure 1 shows a 2% agarose gel with EBV DNA amplification by PCR of some studied cases. In the control group, samples of 5 volunteers showed amplification for EBV (55.56%), and 4 (44.44%) did not (Figure 2). In all reactions the lymphoma case used as positive control of the reaction was amplified. The cytokeratin-14 gene was amplified in all samples (data not shown).

DISCUSSION

EBV virus DNA was amplified by PCR in tongue scrapes from HIV-seropositive patients with or without lesions clinically suggestive of OHL, and from healthy volunteers. This study's proposition was to verify if PCR could help improve OHL diagnosis since it has serious implications as to

TABLE 1 - Number (percentage) of studied cases according to EBV amplification by PCR in HIV-seropositive patients and healthy volunteers, with or without oral hairy leukoplakia (OHL).

	Total number	EBV amplification n (%)
Total HIV-seropositive	29	22 (75.86%)
HIV-seropositive with OHL	4	2 (50%)
HIV-seropositive without OHL	21	16 (76.19%)
HIV-seropositive without OHL*	4	4 (100%)
Volunteers	9	5 (55.56%)

*HIV-seropositive with a history of OHL, but without lesion at the moment the samples were collected.

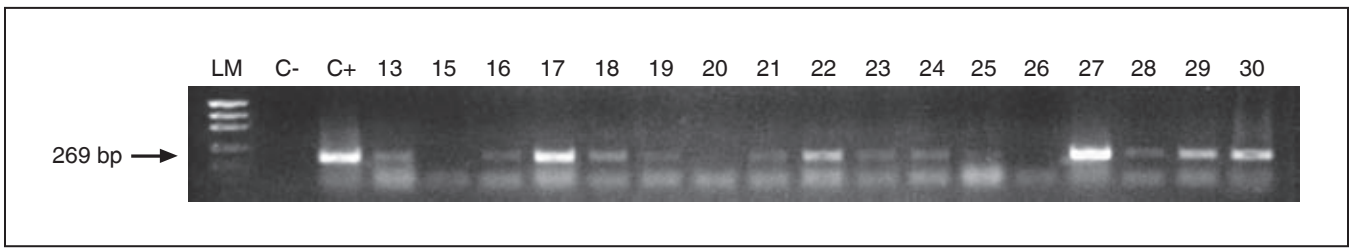

FIGURE 1 - 2% agarose gel showing EBV DNA amplification by PCR (269 bp) in some HIV patients. LM: base pair molecular weight pattern (Low Mass), C–: negative control, C+: positive control. HIV-seropositive without OHL: cases 13, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 26, 28, 29. HIV-seropositive with OHL: 27. HIV-seropositive with a history of OHL, but without lesion at the moment the samples were collected: 25, 30. No amplification: cases 15, 20, 26.

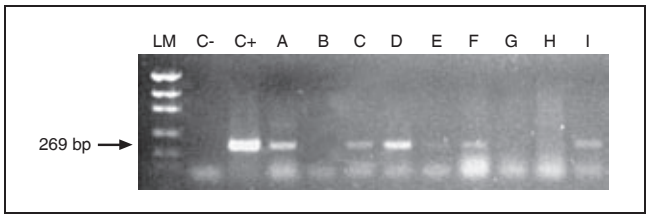

FIGURE 2 - 2% agarose gel showing EBV DNA amplification by PCR in healthy volunteers. LM: base pair molecular weight pattern (Low Mass), C–: negative control, C+: positive control. No amplification: B, E, G, H.

the immune status and even HIV serostatus of the patient. Our results showed that EBV DNA can be found not only in tongue scrapes of HIV-seropositive patients with or without OHL but also in healthy volunteers, as demonstrated by Scully *et al.*[14] (1998) and Mabruk *et al.*[11] (1994). This can be explained by the fact that PCR has the inconvenient of not determining if the amplified EBV was present in the cell scrapes or if it was present in saliva. To reduce the possibility of EBV contamination from saliva, an oral rinse with PBS[1] was performed in this study.

All patients, except for the healthy volunteers, were under antiretroviral therapy when the samples were colected. This could explain the absence of amplification of the two DNA samples from patients with clinical evidence of oral hairy leukoplakia. It is known that this therapy increases CD4 cells, which are important in the host defenses against oportunistic organisms like EBV[13]. Thus, this therapy would have an indirect effect in OHL regression by improving the host's defense. Scully *et al.*[14] (1998) also did not observe EBV amplification in all samples of OHL. The possibility of inaccurate diagnosis must be considered because of other white oral lesions that can mimic OHL such as lichen planus, white sponge nevus, idiopathic and tobacco-associated leukoplakia, galvanic lesions and frictional keratosis or hyperplastic candidiasis. In this study, 5 out of 9 cases showed amplification of EBV in healthy volunteers. For ethical reasons, the sorologic conditions of these individuals were not determined, but there are some reports of OHL in HIV seronegative and immunocompetent persons. In these reports, it is questioned if OHL might represent a transient and isolated EBV infection of surface epithelium without serious or profound prognosis implications[4]. Considering the fact that the differential diagnosis of a white patch lesion on the lateral margin of the tongue is extensive, atypical forms of OHL may mimic some of theses conditions, and this inaccurate diagnosis may contribute to the underreporting of this lesion[6]. According to Scully *et al.*[14] (1998) the detection of EBV in oral scrapes cannot be regarded as reliable or specific for OHL. It is known that EBV is not only associated with this lesion, but also with nasopharyngeal carcinoma, Burkitt's lymphoma, Hodgkin's disease, mononucleosis and recently with oral squamous cell carcinoma. Thus, the detection of EBV could represent other infections associated with this virus previously presented by the individuals.

In the present study the presence of EBV in (80%) seropositive patients without clinical evidence of OHL was observed, which is suggestive of a subclinical lesion. It is known that PCR is highly sensitive, amplifying small amounts of the target sequence. The existence of a subclinical fase[3] is thus considered possible, caused by reinfection of the ephitelium by virus in a quantity detectable by PCR. This reinfection, however, would not necessarily evolve to a clinical manifestation, liable to happen only if the immune response of the host in controlling the viral replication is suppressed[2]. Recently, more studies[4,6,12,14,15] about EBV in healthy individuals have been made and now it is accepted that infection by the Epstein-Barr virus (EBV) is often subclinical in the presence of a healthy immune response; thus, asymptomatic infection is largely uncharacterized[15].

The presence of the virus in the saliva is another important fact to be considered, even with the oral rinse with PBS. According to Wolf *et al.*[16] (1984) the virus tropism for ephitelial cells of the nasopharynx, and also for salivary glands, suggests that these could be persistant sites of the virus, with viral particles of theses places liberated to be shed by the saliva. After the primary EBV infection, the virus is episodically recoverable from the saliva, suggesting that healthy, normal individuals regularly reactivate and shed the virus[11]. This can explain the amplification of the virus in the DNA obtained from HIV seropositive patients that have previously had OHL. In the two negative samples from patients with clinical evidence of OHL, the absence of amplification could be explained by the reduction or absence of EBV in the saliva. *In situ* hybridization studies demonstrated that EBV can be found only in the upper and intermediate cell layers of oral epithelium, and not in the basal cell layer, as it would be expected in case of latent infection. This suggests that the lingual infection of EBV by the saliva would be

more probable than the reactivation of a lingual latent infection[11].

In accordance with previous studies[1,2], the percentage of amplification for EBV in seropositive patients without lesion (80%) is higher than that found in healthy volunteers (55.56%). This could be related to the higher capacity of immunocompetent patients in controlling and limiting viral replication[2]. In HIV seropositive patients the rate of EBV shed in the saliva may be raised[1]. Factors that can contribute to the success of the establishment of EBV in the oral ephitelium are the immune system dysfunction of the host and the absence of Langerhans' cells[10] observed in OHL lesions. EBV has been demonstrated in the clinically normal oral ephitelium of HIV-seropositive individuals, suggesting that the expression of this virus can precede the appearance of OHL[2].

CONCLUSIONS

Our results suggest that EBV detection is not specific for OHL since it was detected in HIV-seropositive patients without OHL and in healthy volunteers, as demonstrated by Scully *et al.*[14] (1998) and Mabruk *et al.*[11] (1994). Thus, we concluded that the use of PCR in oral scrapes suggests a high sensitivity but low specificity for the diagnosis of OHL. Therefore, PCR can be useful in the detection of EBV in oral scrapes, and as an adjunct in the diagnosis of OHL.

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