Journal of Clinical Periodontology
Official Journal of The European Federation of Periodontology
Founded by the British, Dutch, French, German, Scandinavian, and Swiss Societies of Periodontology

J Clin Periodontol 2008; 35: 838–845 doi: 10.1111/j.1600-051X.2008.01307.x

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Herpes viruses in periodontal compromised sites: comparison between HIV-positive and -negative patients

Sabrina Rosa Grande[1,2], Ana Vitória Imbronito[1], Osmar Shizuo Okuda[1], Roberto Fraga Moreira Lotufo[1], Marina Helena Gallottini Magalhães[2] and Fabio Daumas Nunes[2]

[1]Department of Periodontology, School of Dentistry, University of São Paulo, São Paulo, Brazil; [2]Department of Oral Pathology, School of Dentistry, University of São Paulo, São Paulo, Brazil

Grande SR, Imbronito AV, Okuda OS, Lotufo RFM, Magalhães MHG, Nunes FD. Herpes viruses in periodontal compromised sites: comparison between HIV-positive and -negative patients. J Clin Periodontol 2008; 35: 838–845. doi: 10.1111/j.1600-051X.2008.01307.x.

Abstract

Aim: The objective of this study was to compare the frequency of herpes simplex virus type 1 (HSV-1), Epstein–Barr virus (EBV) and human cytomegalovirus (HCMV) in subgingival plaque, saliva and peripheral blood of HIV-positive and-negative patients with periodontal disease.

Material and Methods: Fifty HIV-positive subjects (23 with gingivitis, 27 with periodontitis) and 50 healthy HIV-negative patients with chronic periodontitis were included in the study. Parameters of probing depth (PD), clinical attachment level (CAL), gingival index and plaque index were recorded. The samples were processed for viral identification by the nested polymerase chain reaction technique.

Results: HCMV was the most prevalent virus in HIV-positive (82%) and-negative patients (84%), and the detection in the three samples was similar ($p > 0.05$). HSV-1 was the least prevalent virus in both groups, being detected in similar frequencies in oral sites and in peripheral blood. EBV-1 was found more frequently in saliva and subgingival plaque of HIV-positive patients than in HIV-negative patients ($p \leqslant 0.05$).

Conclusions: EBV-1 was more frequently recovered in oral sites of HIV-positive patients than in HIV-negative patients.

Key words: EBV-1; HCMV; HIV; HSV-1; periodontitis; saliva; subgingival plaque

Accepted for publication 10 July 2008

At the beginning of the AIDS epidemic, periodontal diseases were described as one of the oral manifestations of HIV infection (Reichart et al. 1987). Currently, it is accepted that periodontal diseases in HIV-positive patients are the same diseases presented in non HIV-infected patients (Armitage 1999). However, immunosuppressed patients can exhibit rapid onset and progression of the disease depending on the immunological status (Ndiaye et al. 1997).

Periodontitis is an infectious disease that involves specific bacteria and characteristic humoral and cellular host responses (Shapira et al. 2005). The connective tissue of inflamed periodontal sites reveals a dense infiltration of mononuclear cells, particularly T lymphocytes, B lymphocytes and macrophages (Berglundh & Donati 2005). Recent studies have implicated herpes simplex virus type 1 (HSV-1) (Nishiyama et al. 2008), human cytomegalovirus (HCMV) and Epstein–Barr virus type 1 (EBV-1) in the pathogenesis of human periodontitis, because viruses can infect a variety of inflammatory cells. In addition, HCMV and EBV-1 can reside in periodontal pockets, periodontal abscesses and saliva of immunocompetent patients (Greenberg 1996, Saygun et al. 2005). Further, HCMV and EBV type 1 virus had often been detected in chronic periodontitis patients (Contreras & Slots 1996, Wu et al. 2007), localized aggressive periodontitis (Ting et al. 2000) and in periodontitis in HIV-positive patients (Contreras et al. 2001).

HIV-induced immunosupression is known to facilitate herpes viruses' reactivation or re-infection (Fauci 1993). In HIV patients, concurrent infections by EBV/HCMV (Syrjanen et al. 1999) and HSV/HCMV (Griffthis 1996) also

Conflict of interest and source of funding statement

The authors state that there is no conflict of interest related to this study.
This study was supported by grants from FAPESP (04/07147-8).

increase the potential for herpes virus reactivation. Active periodontal herpes virus infection might be partly responsible for the increased level of gingival interleukin-1β and tumour necrosis factor α associated with HIV periodontitis (Lamster et al. 1998, Baqui et al. 2000). In addition, herpes viruses might interact with HIV at the cellular and molecular level to increase the rate of periodontal tissue destruction. (Ho et al. 1990, Lusso et al. 1991, Griffthis 1996, Miller 1996). HIV-related oral manifestations induced by herpes viruses are frequently reported (Ammatuna et al. 2001). In immunocompromised patients, EBV has been implicated in the aetiology of oral hairy leukoplakia, Hodgkin's disease, peripheral T-cell lymphoma and B-cell lympho-proliferative diseases (Ammatuna et al. 2001). HCMV infection in these patients may result in painful ulcers and erosions on the lips, tongue and buccal mucosa, usually co-infected with EBV (Doniger et al. 1999). The advent of highly active antiretroviral therapy (HAART) has decreased the prevalence of oral lesions (Revankar et al. 1998), especially HIV-associated periodontal disease (Patton et al. 2001), and the incidence of opportunistic HIV diseases (Clifford et al. 1999). But, according to Bower et al. (2006) and Miller et al. (2006), herpes viruses-related lesions and malignancies remain a problem for HIV-infected patients.

The aim of the present study was to identify and compare the presence of HCMV, EBV-1 and HSV-1 in periodontal pockets, peripheral blood and saliva of HIV-positive patients with gingivitis and periodontitis and HIV-negative patients with chronic periodontitis.

Material and Methods

Subject sample

The study was conducted from June 2005 through February 2006 under the approval of the Ethics Committee of Dental School of University of São Paulo. All patients provided written informed consent before enrolment in the study.

A total of 50 HIV-positive patients referred to the Special Care Dentistry Center at the School of Dentistry of University of São Paulo (HIV group) and 50 HIV-negative patients referred to the dental clinic for periodontal treatment and diagnosed with chronic periodontitis (CP group) were enrolled in this study. Patients scheduled for dental treatment for the first time were examined and the first 50 HIV-positive and the first 50 non-HIV-positive patients who fulfilled the inclusion criteria and signed the consent form were included in the study. All patients were at least 30 years old and had not received dental prophylaxis for at least 6 months. Patients who had diabetes mellitus, renal disorders or evidence of valve heart disease were excluded. Patients with <20 teeth and patients who had received periodontal treatment and/or antibiotics for at least 6 months were also excluded.

HIV-positive patients (HIV-group) were divided into two groups: a group of subjects with gingivitis and a group of subjects with periodontitis, according to Aas et al. (2007). Patients with gingivitis had red oedematous gingival tissue with a tendency to bleeding and at least four gingivitis sites with gingival index >0, probing depth <3 mm and attachment loss $=0$. Patients with periodontitis had at least 20 teeth, and at least four sites with bleeding on probing (BOP), probing depth (PD) $\geqslant 4$ mm and clinical attachment loss (CAL) $\geqslant 4$ mm. The CP group was comprised of patients with moderate to severe generalized chronic periodontitis (Armitage 1999) and had at least 20 teeth, PD $\geqslant 4$ mm and CAL $\geqslant 4$ mm at six or more sites, BOP and bone loss on radiographs.

Clinical evaluation

Clinical history and examination of the oral cavity were performed by a trained and calibrated dentist. A questionnaire was administered to gather demographic information, smoking habits and medical history, including type of antiretroviral therapy for the HIV group. Recent laboratory findings (within a month of the clinical examination) were transcribed to a special form created for this study.

HIV-related oral lesions were diagnosed according to the presumptive criteria of EEC-Clearinghouse Classification (1993). Oral biopsies and/or brush cytology were taken when necessary for confirmation of clinical diagnoses.

The following clinical parameters were recorded: probing depth (PD) at six sites/tooth, clinical attachment loss (CAL) at six sites/tooth, observation of clinical mobility and furcation involvement. PD and CAL were measured in millimetres using a manual UNC 15 probe (Hu-Friedy, Chicago, IL, USA). Plaque was scored as being absent or present (0 or 1). Plaque was scored positive if visible before the time of probing. Fourteen periapical radiographs were taken from all the patients.

Sample collection and nucleic acid extraction

The following samples were obtained from the HIV and CP groups:

Saliva

The individuals were not allowed to brush their teeth or eat 30 min. before sampling. Unstimulated saliva was obtained using a collector tube and stored at -70°C. DNA was extracted from the samples by the ammonium acetate and isopropanol method (Rivero et al. 2006). Briefly, 200 μl 4 M ammonium acetate was added to the tube containing the lysate for protein precipitation. The material was homogenized for 20 s, incubated on ice for 5 min. and centrifuged at 13,000 *g* for 3 min. Precipitated protein was observed at the bottom of the tube and the supernatant containing DNA was transferred to another tube. For precipitation of DNA, 600 μl 100% isopropanol was added and the mixture was homogenized and centrifuged at 16,000 *g* for 5 min. The supernatant was discarded and the DNA pellet was washed with 600 μl of 70% ethanol and centrifuged at 16,000 *g* for 2 min. The alcohol was removed and the sample was evaporated dry at room temperature. The DNA pellet was dissolved in 30–50 μl TE buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA, pH 8) and stored at -70°C.

Peripheral blood

Collected by a finger puncture and stored in a filter paper without an anticoagulant. The samples from the filter papers were punched out with a stainless-steel puncher, yielding discs 3 mm in diameter. The samples were stored at -70°C.

Subgingival plaque

After removal of supragingival plaque with sterile curettes, the sites were dried

by air and isolated from saliva contamination using cotton rolls. Two sterile paper points were inserted to the pocket depth in four periodontitis sites in each patient with periodontitis or in one random bleeding site per quadrant in patients with gingivitis. The paper points were kept in place for 20 s. The samples were stored at -70°C.

The nucleid acid extraction of peripheral blood and subgingival plaque samples was based on preferential binding to silica particles in the presence of a high concentration of guanidinium thiocyanate (GuSCN) (Parra & Slots 1996). Briefly, 0.4 ml of sample was mixed with 5 μl of silica particles (Sigma Chemical Co., St. Louis, MO, USA) in 80 μl of lysis buffer (120 g of GuSCN; 100 ml of 0.1 mM Tris HCl, pH, 6,4; 22 ml of 0.2 M EDTA, pH, 8; 2.6 g of Triton $\times$ 100), mixed on a vortex 10 s and kept at room temperature for 10 min. Nucleic acid/silica completes were recovered by centrifugation at 12,000 *g* for 1 min., washed twice in buffer (GuSCN-Tris HCl), twice in 70% ethanol and one time in acetone. Then the sample was dried in a heating block at 56°C for 10 min. The nucleic acid pellet was ressuspended in 100 μl of TE buffer. The sample was incubated at 56°C for 10 min. After centrifugation at 12,000 *g* for 2 min., the supernatant was stored at -70°C.

PCR procedures

The nested PCR method was used to detect viral DNA from HSV-1, HCMV and EBV. The outer and inner oligonucleotides primers that were used, the temperature of melting and the product length are described in Table 1. PCR was performed with a final volume of 25 μl mixture containing 25 pmol of each primer (Invitrogen, Carlsbad CA, USA), 1 U Taq platinum DNA polymerase (Invitrogen), $MgCl_2$, 0.05 mM dNTP mix and 1–10 μl of extracted DNA sample. PCR procedures included a 40-round amplification process. All specimens were tested in duplicate to each of the target microorganisms. In case of conflicting results for PCR reactions (e.g. one replicate positive, one replicate negative), a second PCR set-up was carried out. If replicate results for this second PCR set-up concurred (i.e., both replicates negative or both replicates positive), the conclusion was negative or positive, respectively. If replicate results remained ambiguous, DNA extraction was repeated from the case and a new PCR was performed. Negative and positive controls were included in every reaction: DNA free mix as negative control, DNA from EBV-positive cell B lymphoma diagnosed in our laboratory as positive control for EBV and controls for HSV-1 and HCMV were provided by the Laboratory of Virology of University of São Paulo. The second reaction of the nested-PCR included a negative control containing DNA free mix and also the negative control of the first PCR in which the second run mix was added.

Amplicons were identified by electrophoresis in a 2% agarose gel containing 0.5 μg/ml ethidium bromide.

Table 1. PCR primers for identifying infectious agents, the temperature of melting (TM) and amplicon length

Infectious agent (genbank accession)	Primer	TM (°C)	Product length (pb)	References
HSV 1 (X03101)	1° round 5′-TACATCGGCGTCAT CTACGGGG-3 5′-GGGCCAGGCGCTTGTTGGT GTA-3′	57	331	Tsurumi et al. (1987)
	2° round 5′-GCGTTTATCAACCG CACCTCC-3′ 5′-CAGTTCGGCGGTGAGGAC AAA-3′	56	222	
EBV-1 (S71027)	1° round 5′-AGGGATGCCTGGAC ACAAGA-3′ 5′-TGTGCTGGTGCTGCTGGTGG-3′	56	602	Espy & Smith (1995)
	2°round: 5′-AACTTCAACCCACA CCATCA-3′ 5′-TTCTGGACTATCTGGATCAT-3′	46	116	
HCMV (X17403)	1° round 5′-GAGGACAACGAAATC CTGTTGGGCA-3′ 5′-TCGACGGTGGAGATACTGCTGAGG-3′	56	150	Darlington et al. (1991)
	2° round 5′-ACCACCGCACTGAGG AATGTCAG-3′ 5′-TCAATCATGCGTTTGAAGAGGTA-3′	50	100	

Statistical analysis

Statistical analyses were conducted with SAS software, version 8.2. A descriptive statistical study (means, standard error of the mean) was conducted on age, sex, number of teeth presented and number of teeth with attachment loss. The χ^2 test was applied to compare the two groups regarding sex distribution and smoking habits. The Student *t*-test was used to compare age distribution for both groups. The number of teeth presented in both groups was compared using the Student *t*-test. Significant differences in probing depth and clinical attachment loss between the two groups were determined using Student's *t*-test. The χ^2 test was applied to determine the relationships between any two viruses or two different sampling methods (statistically significant if p value <0.05).

Results

The HIV group comprised 50 HIV-positive patients (35 male and 15 female) with a mean age of 41.2 ± 8.36 years. Twenty-three HIV-positive patients had gingivitis and 27 had periodontitis. The CP group consisted of 50 HIV-negative patients with chronic periodontitis, 28 males and 22 females, mean age 41.7 ± 11.29 years.

The HIV and CP groups were similar regarding age, plaque index, number of teeth and smoking habits. Two HIV-positive patients with gingivitis and three HIV-patients with periodontitis smoked more than 15 cigarettes/day. Six non-HIV patients smoked >15 cigarettes/day. The CP group comprised more females than the HIV group. HIV-positive patients with periodontitis had less teeth affected by periodontal disease than the CP group patients ($p = 0.04$), but the means of clinical attachment level for both groups were similar ($p = 0.31$). A clinical description of both groups is presented in Table 2.

Table 3 shows the CD4+ cell counts, viral load, medication used, co morbidities and HIV-related oral lesions

Table 2. Sample description and periodontal variables of HIV and CP groups

Variables	HIV group		CP group	
	gingivitis	periodontitis	periodontitis	*p* value
Number of patients	23	27	50	
Age (mean ± SD)	41.65 ± 8.36	41.22 ± 7.13	41.74 ± 11.29	$p = 0.95$*
% of faces with plaque (mean ± SD)	43.72 ± 5.41	45.33 ± 6.32	42.23 ± 5.65	$p = 0.23$*
Number of teeth (mean ± SD)	24.13 ± 3.63	22.17 ± 4.22	23.72 ± 4.57	$p = 0.13$*
Number of teeth with CAL >4 mm (mean ± SD)	0	11.0 ± 5.7	15.46 ± 7.12	$p = 0.04$*
PD (mean ± SD)	1.88 ± 0.30	2.63 ± 1.01	2.98 ± 0.68[a]	
CAL (mean ± SD)	2.25 ± 0.65	3.21 ± 0.85	3.45 ± 1.21[b]	
% of females	30%		60%	$p = 0.002$†
Smokers	7	8	17	$p = 0.67$†

*Student *t* test.
†Chi-square test.
[a]Student *t* test, $p < 0.0001$ *versus* HIV group (gingivitis).
[b]Student *t* test, $p < 0.0001$ *versus* HIV group (gingivitis).
SD, standard deviation; CP, chronic periodontitis; CAL, clinical attachment level; PD, probing depth.

Table 3. Clinical and immunological condition of HIV positive patients

Variable	Category	Frequency (%)
Medication used[a]	None	10 (20)
	HAART	40 (80)
	Antifungal therapy	2 (4)
	Other antibacterial therapy	3 (6)
CD4+ (cells/mm^3)	<200 cell/mm^3	5 (10)
	200–499 cell/mm^3	23 (46)
	>500 cell/mm^3	21 (42)
	Not determined	1 (2)
Viral load (copies/ml)	Indetectable	29 (58)
	<10,000 copies	14 (28)
	>10,000 copies	7(14)
Comorbidities*	Tuberculosis	4 (8)
	Pneumocistes carinii pneumonia	4 (8)
	Vaginal candidiasis	10 (20)
	Syphilis	5 (10)
	Gonohrea	3 (6)
	Anemia	6 (12)
	Meningitis	1 (2)
	Anogenital herpes	5 (10)
	Herpes zoster	6 (12)
	Hepatitis A, B or C	14 (28)
	HCMV retinitis	1 (2)
	Neuropatia	4 (8)
	Toxoplasmosis	1 (2)
Oral manifestations	Labial herpes	6 (12)
	Oral hairy leukoplakia	5 (10)
	Lichen planus	1 (2)
	Kaposi's sarcoma	1 (2)
	Candidiasis	5 (10)

*Some cases presented more than one systemic disease.
[a]Some patients use more than one drug.

presented in 50 HIV patients. Eighty per cent of the patients of the HIV group had CD4 cell counts above 200 cell/mm^3, and 58% (29/50) of the patients had an undetectable viral load. Only 10 patients of the HIV group did not have any antiviral drug prescription. Three patients reported that they had received sulphamethoxazole for respiratory infections that had occurred at least 6 months before enrolment in the study. All the 40 patients who were on medication were under HAART therapy. Regarding co-morbidities, 40 HIV-positive patients presented at least one associated disease or condition: hepatitis was the most frequently disease, followed by vaginal candidiasis, anaemia, herpes zoster, anogenital herpes and syphilis.

The frequencies of detection of HSV-1, EBV-1 and HCMV in patients of both groups are presented in Table 4. HIV-positive patients diagnosed with gingivitis were grouped together with HIV-positive patients diagnosed with periodontitis because the frequencies of detection of the viruses were similar between the groups for the samples studied (χ^2 test, $p > 0.05$). EBV-1 was detected statistically more frequently in the subgingival sites and saliva of patients with periodontitis from the HIV group than the CP group (χ^2 test, $p = 0.01$ and $p = 0.009$, respectively).

In the HIV group, HSV-1 was detected in similar frequencies in subgingival plaque, saliva and blood samples (6%, 18% and 6%, respectively), the same occurring with HCMV (82%, 72% and 68%). But in this group, EBV-1 was detected in subgingival plaque (72%) and saliva samples (62%) more frequently than in peripheral blood (18%) ($p < 0.005$, χ^2 test).

In the CP group, the frequencies of detection of HSV-1 in subgingival plaque, saliva and peripheral blood were similar (16%, 24% and 8%) as were the frequencies of detection of HCMV (80% in subgingival plaque and saliva and 84% in peripheral blood). In this group, EBV-1 was detected more frequently in subgingival plaque (48%) than in peripheral blood (24%) ($p = 0.01$, χ^2 test).

There was a lack of agreement (low sensitivity, low specificity, χ^2 test $p > 0.05$) between the sampling methods to detect EBV-1 and HSV-1 in both HIV-positive and HIV-negative groups of patients, meaning that the identification of EBV-1 and HSV-1 in subgingival plaque was not accompanied by salivary detection or peripheral blood detection. There was a significant agreement between subgingival plaque and saliva (sensitivity = 0.87, specificity = 0.7 and χ^2 test $p < 0.001$) and subgingival sampling and peripheral blood in the detection of HCMV, with high sensitivity (0.87) and specificity (0.7) and χ^2 test $p < 0.001$ for the CP group, but in the HIV-positive group although the sensitivity was for the detection of HCMV in saliva (0.75) and peripheral blood (0.76) compared with subgingival plaque samples, the specificity was low (0.0 for saliva compared with subgingival plaque

Table 4. Distribution of Human Herpes simplex virus -1 (HSV-1), Epstein–Barr-1 virus (EBV-1) and human cytomegalovirus (HCMV) in subgingival samples, saliva samples and peripheral blood samples from both groups of patients

	HIV group			CP group		
	subgingival samples	saliva samples	peripheral blood samples	subgingival samples	saliva samples	peripheral blood samples
HSV-1 positive	3 (6%)	9 (18%)	3 (6%)	8 (16%)	12 (24%)	4 (8%)
EBV-1 positive	36 (72%)†	33 (62%)¥	9 (18%)	24 (48%)†	20 (40%)¥	12 (24%)
HCMV positite	41 (82%)	36 (72%)	34 (68%)	40 (80%)	40 (80%)	42 (84%)

†$p = 0.01$ (χ^2 test).
¥$p = 0.009$ (χ^2 test).

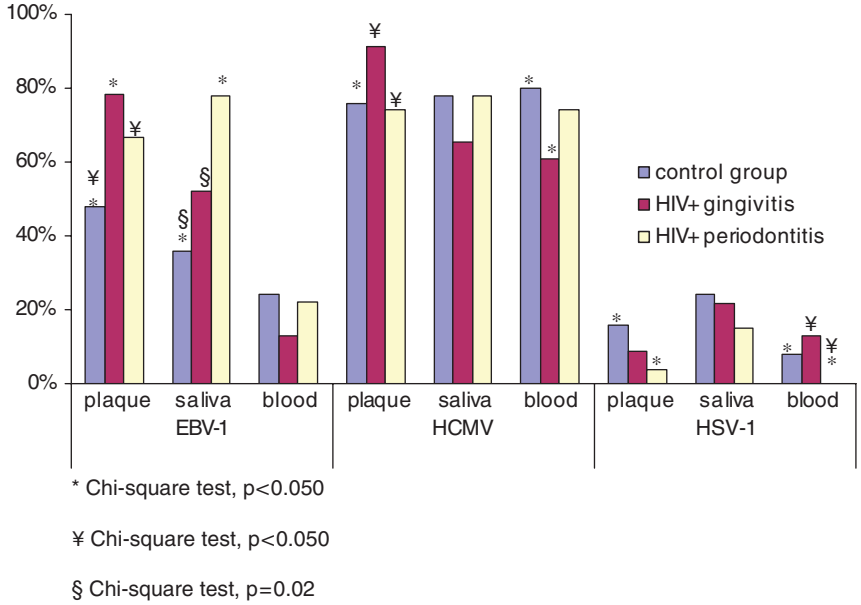

Fig. 1. Detection of EBV-1, HCMV and HSV-1 in subgingival samples, saliva and peripheral blood in HIV group (23 HIV positive patients with gingivitis and 27 HIV positive patients with periodontitis) and CP group (50 HIV negative patients with chronic periodontitis).

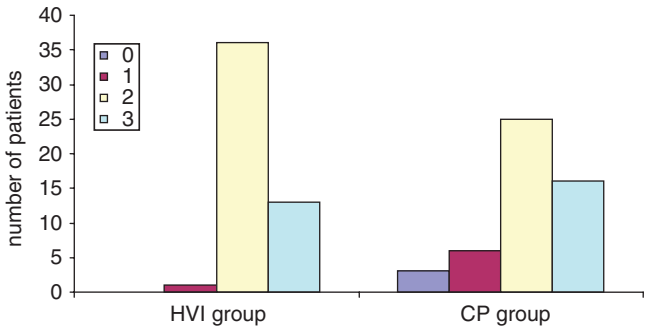

Fig. 2. Detection of viruses' co-infection (zero to three viruses present) in HIV group and CP group.

and 0.06 to peripheral blood compared with subgingival plaque).

The frequencies of the viruses were also evaluated considering the periodontal diagnosis of the patients in the HIV group (gingivitis or periodontitis) (Fig. 1) and compared with the frequencies in the CP group. EBV-1 was detected more frequently in subgingival plaque and saliva of HIV-positive patients diagnosed with gingivitis or periodontitis than in the CP group.

The patient was considered positive to the virus if at least one sample was positive. The co-infection of the viruses in HIV and CP groups is presented in Fig. 2. All HIV-positive patients were positive to at least one of the herpesviruses in at least one sample and there was no viral detection in only three patients of the CP group.

Two HIV-positive patients (5%) were positive to EBV-1 in the three studied samples (subgingival plaque, peripheral blood and saliva) and 16 (32%) were positive to HCMV in the three studied samples.

In the CP group, no patient was positive to HSV-1 in the three sampling methods, only one subject (2.5%) was positive to EBV-1 in the three studies samples, while 31 patients (62%) were positive to HCMV in the three samples. Thirty (60%) patients were negative to HSV-1 in the three samples, 9 (18%) subjects were negative to EBV-1 in the samples and 2 (5%) were negative to HCMV in the samples.

The identification of HSV-1, EBV-1 and HCMV in plaque, saliva and blood and according to the immunological condition of HIV-patients is presented in Fig. 3. No statistical analysis was performed due to the small number of patients with a CD4 count lower than 200 cells/mm^3 (five patients).

Five HIV-positive patients presented hairy leukoplakia and EBV-1 was detected in saliva in all cases. Six HIV-positive patients had labial herpetic lesions and HSV-1 was detected in the saliva in all cases. The patient diagnosed with lichen planus also had oral hairy leukoplakia and candidiasis, and this patient was positive for EBV-1 in subgingival plaque and saliva samples and positive for HCMV in saliva. The patient diagnosed with Kaposi's sarcoma was positive to EBV-1 in the subgingival plaque sample and positive to HCMV in all three samples. One patient had HCMV retinitis and in this patient HCMV was detected in subgingival plaque and peripheral blood.

Discussion

The present study evaluated the presence of viruses HSV-1, EBV-1 and HCMV in samples of subgingival plaque, saliva and peripheral blood of periodontal compromised HIV-positive and HIV-negative patients. Of interest, HIV-positive subjects presented EBV-1 in subgingival and salivary samples in higher frequencies than HIV-negative patients with chronic periodontitis but

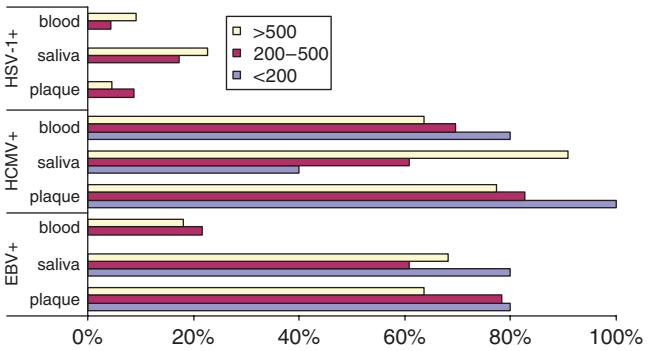

Fig. 3. Identification of HSV-1, EBV-1 and HCMV in plaque, saliva and blood from HIV positive patients, according to the CD4 counts (<200, between 200 and 500 and >500 cells/mm^3).

the detection of HSV-1 and HCMV was similar in both groups.

The severity of periodontal destruction was similar in the 27 HIV-positive patients with periodontitis and in the 50 HIV-negative patients diagnosed with chronic periodontitis. However, because the HIV group had fewer teeth with CAL >4 mm (11.0 ± 5.7 teeth *versus* 15.46 ± 7.12 teeth in the CP group), it seems that the periodontal destruction may be more severe in these HIV-positive patients.

The accuracy to detect pathogens relies on the sampling methods. Because herpesviruses have been implicated in the pathogenesis of periodontal diseases, an important issue is how to detect these viruses. In periodontal research, the detection of herpes viruses has been performed by sampling subgingival plaque single sites (Kubar et al. 2004) or pooled sites (Contreras & Slots 1996), by sampling saliva (Saygun et al. 2005) or gingival biopsies (Contreras et al. 2001). Capillary blood collection and dried blood storage in a paper card avoids venipucture. It is recognized as a useful and rapid method to detect HCMV in neonates (Barbi et al. 2006; Soetens et al. 2008). In this study, HSV-1 and HCMV were detected in similar frequencies for the three sampling methods and EBV-1 was detected more frequently in subgingival plaque and saliva when compared with peripheral blood detection. In spite of similar frequencies of detection, there was a lack of agreement between the methods for the detection of EBV-1 and HSV-1, meaning that the occurrence of the virus in the subgingival plaque is not usually accompanied by the occurrence of the virus in saliva or peripheral blood. Possibly, the combination of subgingival plaque samples and saliva samples may indicate more accurately whether a patient is virus positive or negative. There was an agreement among the sampling methods only to HCMV, probably due to the high rate of occurrence of this virus in subgingival plaque, saliva and peripheral blood.

EBV-1 was the only virus detected more frequently in HIV-positive patients than in HIV-negative patients. EBV-1 was not detected in only two HIV-positive patients and in 10 healthy patients with chronic periodontitis. The prevalence of EBV-1 in subgingival plaque and saliva of HIV-positive either diagnosed with gingivitis or periodontitis occurred with a statistically higher frequency when compared with chronic periodontitis patients. The higher frequencies of EBV-1 in oral sites may be explained by the immunosuppressant condition of HIV-positive patients, and also the presence of EBV-1 in subgingival samples may be due to the recruitment of EBV-infected lymphocytes to sites of chronic periodontal inflammation (Miller et al. 2006) when EBV-1 may be spread from B lymphocytes to monocytes, which then enter the epithelium and initiate productive viral infection of keratinocytes (Tugizov et al. 2007).

In this study, in accordance to the findings of Boldogh et al. (1996), Fons et al. (1994) and Miller et al. (2006), HSV-1 was the less frequently recovered virus in HIV-positive and -negative patients, and presented a similar prevalence in all sites in both groups of patients. Our results showed the presence of HSV-1 in 24% of saliva samples of healthy patients with chronic periodontitis, which is much higher than the 1.2% observed by Miller et al. (2006) in the saliva of healthy patients and the 10% observed by Druce et al. (2002) in a diverse range of clinical samples. Possibly, periodontitis lesions may be a source of salivary HSV-1 or these differences may also be attributed to the different prevalence of the virus in different populations. The frequencies of subgingival samples positive to EBV-1 and HCMV in chronic periodontitis patients resemble the results obtained by Kubar et al. (2005), who found 46% of the subgingival samples to be positive to EBV-1 and 78% to be positive to HCMV. Although HCMV infects salivary glands in newborns and infants and may be reactivated in immunocompromised patients (Merigan & Resta 1990), there was no statistical difference in the frequency of HCMV in subgingival plaque or saliva of HIV-infected patients or systemically healthy patients. Periodontal sites may be the source for viruses in saliva (Saygun et al. 2005). This hypothesis may be plausible to EBV-1, because it was present at lower frequencies in peripheral blood than in saliva and subgingival plaque, but not to HCMV and HSV-1, which were detected in similar frequencies in subgingival plaque, saliva and peripheral blood. It would be important to further investigate whether periodontal sites harbour more copies of the virus than the peripheral blood or whether they present viral activity in periodontal pockets; otherwise, the presence of the viruses in the oral environment could only be a result of the systemic condition of the patient.

All the HIV-infected patients were positive to HCMV, with at least one of the sampling sites positive to HCMV, and only two healthy patients with chronic periodontitis were negative to HCMV in saliva, subgingival plaque and peripheral blood simultaneously. The frequency of detection of HCMV in the saliva of HIV-infected patients (68%) in this study is much higher than reported in the literature (31%) (Miller et al. 2006), and the detection in subgingival samples of HIV-negative patients with periodontitis (76%) was similar to the 68.8% frequency reported in aggressive periodontitis (Kubar et al. 2004). In HIV-infected patients, HCMV was observed in similar frequencies in the patients who had gingivitis or periodontitis. In the present study, HCMV was detected as frequently in peripheral blood samples as oral samples in both groups of patients. Because de Ory et al. (2004) observed that approximately 80% of healthy Spanish women 30–40

years old were seropositive to HCMV and Cunha et al. (2002) observed that 34% of HIV patients were positive to HCMV in blood leucocytes, it is plausible that the detection of HCMV in oral samples could evidence the serological infection of HCMV. The frequencies of EBV-1, HCMV and HSV-1 were similar on comparing smoking and non-smoking patients in both groups of patients (data not shown).

Eighty per cent of the HIV-positive patients in this study were under HAART therapy. One of the primary missions of Brazil's National STD/AIDS Program (NSAP) is to make HIV medications available free of charge to all citizens who need them through the public health care system. The programme of universal access to the antiretroviral therapy, together with other actions, such as the use of chemoprophylaxis for the most important opportunistic infections, led to an expressive reduction in the number of deaths by AIDS and also reduced by 60–80% the most common opportunistic processes related to severe immune deficiency in HIV-positive patients, such as cryptococcosis, Kaposi's sarcoma and tuberculosis. It seems that antiretroviral therapy has little effect on human herperviruses in subgingival sites and saliva, because HSV-1, HCMV and EBV-1 were frequently recovered from HIV-infected patients with gingivitis and periodontitis. These data, combined with previous findings (Miller et al. 2006), suggest that HAART, with its targeted specificity, does not significantly influence the rate or the load of asymptomatic shedding of herpes viruses in saliva. In these patients, periodontal diseased sites might be a reservoir of these viruses, favouring the occurrence of other viral diseases, such as oral hairy leukoplakia and Kaposi's sarcoma. The present finding of frequent detection of EBV-1 and HCMV in the subgingival environment of these patients provides the rationale for further studies evaluating the role of periodontal treatment in reducing herpes viral load and as a preventive measure for the installation of viral-related pathologies.

In this study, CD4 counts had no impact on the clinical severity of periodontal disease, with 62% of HIV-infected patients with gingivitis and 63% of HIV-infected patients with periodontitis presenting a CD4 count lower than 500 cell/mm^3, but it should be pointed out that the progression and severity of periodontal destruction may also depend on the viral load, the antiretroviral therapy and antibiotic therapy. No statistical analysis was performed evaluating the detection of the viruses according to the CD4 counts, because only five patients had <200 CD4 cell/mm^3. Because of the small number of patients with viral load $>10{,}000$ copies/ml, the association between the viral load and the clinical parameters or the detection of the viruses could not be determined. One study suggested that the progression of periodontal disease did not differ between HIV-infected and non-infected women and CD4 counts and viral load had no effect in PD and CAL in HIV-infected patients (Alves et al. 2006) but two others demonstrated that elevated HIV viral loads in the serum and gingival crevicular fluid were indeed associated with increased periodontal attachment loss, and the prevalence of both periodontitis and gingivitis was significantly greater in the CD4 <200 group than in the CD4 >200 group (Maticic et al. 2000, Ranganathan et al. 2007).

In conclusion, our study showed that HCMV is broadly distributed in patients with periodontal disease and EBV-1 is more frequently detected in periodontal disease in HIV-positive patients than in HIV-negative patients.

Acknowledgements

The authors would like to thank Dr. Wellington Peruzzetto for his assistance during the clinical examinations. This study was supported by grants from FAPESP (04/07147-8).

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Address:
Ana Vitória Imbronito
Disciplina de Periodontia – FOUSP
Av Prof Lineu Prestes, 2227
CEP 05508-000
Cidade Universitária São Paulo
Brazil
E-mail: anaimbronito@uol.com.br

Clinical Relevance

Scientific rationale for the study: HCMV and EBV-1 have been implicated in the aetiology of periodontal disease and these viruses have also been associated with opportunistic diseases in HIV patients.

Principal findings: EBV-1 was more frequently recovered from oral sites of HIV-positive patients than from healthy patients with chronic periodontitis.

Practical implications: Subgingival sites of periodontally compromised HIV-positive patients may be a reservoir of viruses, and the results of this study indicated that all HIV patients presented at least one of these viruses in oral samples.