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ORIGINAL ARTICLE

Detection of EBV, CMV and HSV-1 in subgingival samples of HIV positive and negative patients with chronic periodontitis.

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Abstract: Objective: To detect the presence of infection by EBV (Epstein-Barr Virus), CMV (Cytomegalovirus) and HSV-1 (Herpes Simplex Virus type 1) in subgingival samples from HIV- positive patients under HAART (High Activity Antiretroviral Therapy), HIV- positive patients without HAART, HIV-negative patients with chronic periodontitis and healthy controls. Methodology: Crevicular fluid samples of 11 HIV+ patients on therapy were evaluated, 6 without antiretroviral therapy, 7 HIV- negative subjects with chronic periodontitis and 7 periodontally-healthy controls. PI (Plaque index), GI (Gingival Index), PD (probing depth) and CAL (Clinical Attachment Loss) were registered at six sites per each tooth in all teeth and subgingival plaque samples of a tooth were collected per quadrant. Nested PCR was used to detect EBV and endpoint PCR to detect infection by CMV and HSV-1. Results: Clinical parameters showed statistically significant differences between HIV-positive patients and subjects with chronic periodontitis compared with the control group ($p<0.05$). DNA of EBV was detected mainly in HIV-positive patients under HAART, 91% (10/11). DNA of CMV was detected mainly in patients without HAART, 67% (4/6), while HSV-1 was observed in 27% (3/11) of patients under HAART. In the control group no virus was detected. Coinfection was observed in 50% of HIV patients without HAART, 36% of HIV patients with HAART and 14% of HIV-negative with chronic periodontitis. Conclusion: Viral infection was prevalent in HIV patients under HAART and EBV was the primary viral infection detected in HIV-positive patients with chronic periodontitis.

Keywords: *EBV, CMV, HSV-1, Periodontitis, Subgingival.*
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INTRODUCTION.

Periodontal disease is a multifactorial condition caused by a complex community of microorganisms that interact with host cells and tissues, causing the release of a wide range of inflammatory cytokines, chemokines, and mediators that lead to the destruction of periodontal tissues[1,2]. The coexistence of herpesvirus and periodontopathic bacteria, together with the host's local immune response, would be involved in the disease progression[3]. Different Herpesviridae detected in gingival tissue, such as Epstein-Barr virus (EBV) and cytomegalovirus (CMV), have been detected. They seem to play a pathogenic role in periodontitis[4-6].

EBV is transmitted by contact with saliva and remains throughout life in the form of latent infection, usually asymptomatic[7]. EBV or Human Herpesvirus 4 (HH-4) belongs to the genus *Lymphocryptovirus*. Its main feature is the ability to establish itself as a latent infection within the host cell and the ability to induce proliferation of these[8].

The role of herpesviruses in the etiology of periodontal disease has been suggested by their presence in gingival tissue, crevicular fluid and subgingival plaque in teeth with periodontal disease. However, the role of CMV and EBV in the pathogenesis of periodontal disease has not yet been elucidated[3].

A model of periodontal disease in which the activation of herpesvirus results in the suppression of the immune response has been described, leading to the exacerbated growth of periodontal pathogenic bacteria, release of proinflammatory cytokines and chemokines, initiation of cytotoxic and immunopathological events, and the subsequent destruction of periodontal tissue[1]. In HIV-infected patients periodontal disease progresses more rapidly, depending on the degree of immunosuppression. In addition, the presence of virus of the herpesviridae family has been confirmed in subgingival samples of periodontal pockets. This could explain why periodontal disease progresses more rapidly in HIV patients[9].

CMV has been found in 81% of HIV-positive patients affected by periodontitis[10]. Other viruses isolated from these patients are EBV and HSV-8, also known as Kaposi's sarcoma virus. In addition, EBV has been found in 57% of biopsies of periodontal lesions, and HSV in 24% of HIV patients with periodontitis[11].

The aim of this study was to detect the presence of infection by EBV, CMV and HSV-1 in subgingival samples from HIV-positive patients on HAART (High Activity Antiretroviral Therapy), HIV-positive patients without HAART, HIV-negative patients with chronic periodontitis and healthy controls.

MATERIALS AND METHODS.

Patients

Eleven HIV+ patients on therapy, 6 without antiretroviral therapy, 7 HIV patients with chronic periodontitis and 7 periodontally healthy controls were evaluated. All patients were referred from the Graduate Periodotics Program, School of Dentistry, Universidad Central de Venezuela, from January to December 2013. The periodontal diagnosis was established based on a clinical and radiographic study defined in 1999 by the American Academy of Periodontology[12].

Subjects in the control group were systemic and periodontally healthy, with no history of periodontal disease. They all signed an informed consent to participate in the study. The research was approved by the Bioethics Committee of the Faculty of Dentistry at Universidad Central de Venezuela. Clinical measurements were performed by a calibrated researcher in the four groups of patients included in the study. Clinical indices evaluated were: gingival index (GI)[13], plaque index (PI)[14], probing depth (PD) and clinical attachment level (CAL). Measurements were performed at six sites per tooth (mesiobuccal, buccal, distobuccal, disto-lingual, lingual and meso-lingual) for all teeth excluding third molars.

Samples of gingival crevicular fluid (GCF) were collected from a tooth with the deepest periodontal pockets, and from the healthy gingival sulcus in the control group. The study sites were isolated with cotton rolls and supragingival plaque was removed with sterile gauze. A sterile paper tip was introduced to the bottom of the sulcus. The paper tips were kept in place for 20 seconds and then transferred to a 1.5ml Eppendorf tube and stored at -80°C until viral analysis. Sampling was performed by a previously trained operator.

Patients diagnosed with HIV were referred to the Graduate Periodotics Program, from the Care Center for Patients with Infectious Diseases, (CAPEI, for its acronym in Spanish), School of Dentistry, Universidad Central de Venezuela. They had a previous diagnosis of HIV infection and laboratory tests with CD4+ count and viral load.

Nucleic acid extraction

The paper tips containing samples of GCF were resuspended in 200µl TE buffer and mixed by vortexing. Extraction of nucleic acids was performed using QIAGEN® DNA mini kit (Qiagen N.V., Germany), following the specifications of the manufacturer. Samples were incuba-

ted at 65°C overnight in ATL buffer, then AL buffer was added, and samples were incubated at 72°C for 10 minutes. Absolute ethanol was added and transferred to a column; washes were performed with buffers W1 and W2. Elution was performed with 200µl of elution solution.

PCR for viral detection

EBV was detected using a nested PCR, following the specifications described by Arreaza *et al.*[15]. External primers contained the sequences 5' CTAGGGGAGAACGTGAA 3'(W1) and 5' CTGAAGGTGAACCGCTTACCA 3' (W2); internal initiators set consisted of 5 'GGTATCGGGCCAGAGGTAAGT 3' (W3); and 5' GCTGGAGGACCCCTTCTAC-3' (W4). Amplification of the EBV genome was performed using 2.5µl of DNA in 25µl of solution, containing 10mmol/L Tris-HCl (pH 8.3), 50mmol/L KCl, 1.2mmol/L $MgCl_2$, 200mmol/L of each dNTP (Invitrogen, USA) and 20pmol of the respective initiator 1.25U Taq DNA polimersa (Invitrogen, USA), and water to a volume of 25µl.

Thirty amplification cycles of 92°C for 45 seconds, 66°C for 30 seconds and 72°C for 45 seconds were performed with external initiators. Then, 2.5µl of amplified material were taken to be amplified with internal primers using 40 cycles under the same conditions; the amplification product is 192pb. For detection of HSV and CMV, "HSV type 1 DNA pol Primer Set Kit" and "Cytomegalovirus Major immediatly Early, primer set kit" were used respectively (Maxim Biotech Inc., USA), following the specifications of the manufacturer.

The reaction mixture for the detection of both viral agents consisted of 40µl of master mix, 0.2µl of Taq DNA polymerase, and nuclease-free water to a final volume of 50µl. A total of 10µl of DNA from the sample were used. The conditions of amplification were as follows: 96°Cx 1min, 35 cycles (94°Cx 1min, 58°Cx1min, 72°Cx1min), 72°Cx1min. Those cases in which an amplification of 105pb was observed were considered positive for HSV-1, and those in which the amplification was 435pb were considered positive for CMV.

Amplicons were detected by 1.5% agarose gel electrophoresis stained with ethidium bromide (1µg/ml), 10µl of the amplified solution were used in each case. The photographic record was made with ChemiDOC™ XRS+(BIORAD, USA).

Statistic Analysis

To determine the existence of statistically significant differences among all the groups studied with statistical variables the Kruskal-Wallis and Mann-Whitney tests were used. Statistical significance was considered for values $p<0.05$.

RESULTS.

The evaluation of clinical parameters in the different HIV-positive groups on HAART, HIV without HAART, patients with chronic periodontitis and control group are shown in Table 1.

It was observed that the average age was similar in the four groups (between 28 and 43 years old) with no statistically significant differences ($p>0.05$). The group with chronic periodontitis presented higher values of PD and CAL ($p=0.007$). Similarly, significant differences in

Table 1. Clinical parameters in HIV+ patients without HAART, with HAART, with chronic periodontitis and control group.

	HIV+ onHAART (n=11)	HIV+ with HAART (n=6)	Chronic periodontitis (n=7)	Control group (n=7)
Age	36.36±7	28±6.48	43.14±8.49	43±12
PI	1.3±0.36	1.3±0.27	1.5±0.26	0.8±0.6
GI	1.6±0.54	1.3±0.19	1.6±0.25	0.6±0.4
PD	2,9±0.61	2.6±0.33	3.6±0.52	1.5±0.2
CAL	3.1±0.71	2.8±0.62	3.9±0.71	1.5±0.3
CD4+	386.54±253.57	148±95.75		
Viral Load	115.795±249.688	305.861±313.065		

Plaque index (PI), gingival index (GI), probing depth (PD) and Clinical Attachment Level (CAL).

Table 2. Distribution of EBV, CMV and HSV-1 in subgingival plaque in HIV-positive patients on HAART, HIV-positive without HAART, patients with chronic periodontitis and control group.

	HIV+ with HAART (n=11)	HIV+ without HAART (n=6)	Chronic periodontitis (n=7)	Control group (n=7)
VBE	10/11 (91%)	4/6 (67%)	3/7 (43%)	0/7 (0%)
CMV	2/11 (18%)	4/6 (67%)	2/7 (29%)	0/7 (0%)
VHS-1	3/11 (27%)	0/6 (0%)	0/7 (0%)	0/7 (0%)

Epstein-Barr virus (EBV), Cytomegalovirus (CMV); Herpes Simplex Virus type 1 (HSV-1).

CD4+ cell count among HIV-positive patients with and without HAART were observed (p=0.011).

The presence of EBV, CMV and HSV-1 among all the groups in study are shown in Table 2. The prevalence of viral coinfection among the evaluated groups was observed in 50% of HIV-positive patients on HAART, 36% of HIV-positive patients without HAART, and in 14% of patients with chronic periodontitis.

DISCUSSION.

Microbiological research in human periodontal disease has typically focused on bacteria and to a lesser extent on parasites and yeasts. However, in the last decade the presence of herpesviruses has been shown, including HSV1 and 2, EBV, and CMV in periodontal pockets of patients with periodontitis[16-22].

A systematic review of microbiological studies in patients with periodontal disease in Central and South America by Contreras *et al.*[23] indicates that the genomes of HSV-1, CMV and EBV have been detected in the periodontal pocket, saliva and gingival immune cells. The three viruses have been associated with chronic periodontitis, aggressive periodontitis, acute ulcerative necrotizing gingivitis and periodontal abscess. Zhu *et al.*[19] suggest that EBV and CMV are significantly associated with chronic periodontitis.

In the present study the increased frequency of infection was observed in HIV-positive patients, EBV infection being the most common, followed by CMV and HSV-1; while in the control group no infection was detected. When comparing HIV-positive patients to each other, it was noted that for patients on HAART, EBV and HSV-1 infections were the most frequent, with 91% and 27%, respectively. However, for patients without HAART, a frequency of infection of equal magnitude for EBV and CMV (67% each) was observed, which was not the case for HSV-1.

There are few studies evaluating the frequency of infection by virus in GCF in HIV positive and negative patients with and without HAART. Some studies report a significant relationship between herpesviruses and the risk of chronic periodontitis, however, their findings are inconsistent[19]. Only two similar studies evaluating some of the variables included in this research were found. Large *et al.*[24] evaluated the frequency of infection by EBV, CMV and HSV-1 in two groups of patients, HIV-positive and HIV-negative, both diagnosed with chronic periodontitis, without distinction between the application or non-application of antiretroviral therapy.

Their results indicated a higher frequency of detection of EBV in saliva and subgingival plaque in HIV-positive patients compared to HIV-negative patients. Consequently, the authors suggest an association between the presence of EBV-1 and coinfection by EBV-1 and CVM with the diagnosis of periodontitis in HIV-positive patients[25]. The results of these two studies are similar to those of the present study regarding the type and frequency of the virus detected, although samples are larger. Some studies report that HAART has been shown to reduce the number of opportunistic infections, however these infections may occur and damage the immune system of patients under HAART[9].

A larger number of studies have been conducted on patients with periodontitis. Grenier *et al.*[17] evaluated the

presence of herpesvirus in patients with periodontitis and periodontally healthy patients, finding that the prevalence of CMV and HSV in GCF was higher in patients with periodontitis, and that the prevalence of CMV increased in proportion to the depth of the pocket.

Sharma *et al.*[18] compared the presence of CMV and EBV in patients with chronic and aggressive periodontitis with healthy individuals, reporting higher prevalence of EBV in patients with chronic periodontitis, while CMV infection was higher in patients with aggressive periodontitis. The highest frequency for both viral agents was observed in deep pockets. Rones *et al.*[26] demonstrated *in vitro* positivity for HSV-1 in epithelial cells and fibroblasts in the area of the gingival sulcus cells, suggesting that these cells could be reservoir of latent virus. Furthermore, Petrovic *et al.*[27] reported the presence of HSV-1 in GCF by PCR, indicating that the presence of this virus coincides with a high degree of tissue destruction in patients with chronic periodontitis. These observations were confirmed by Das *et al.*[28]. In the present study detection frequency for EBV and CMV observed in patients with periodontitis is consistent with previous reports, confirming their presence in patients with periodontal disease and deep periodontal pockets.

The present study evaluated coinfection between these herpesviruses, finding that the highest rate occurred in HIV positive patients without HAART (36%) and in patients with chronic periodontitis (14%). Coinfection with these viruses may increase the complexity of the clinical picture. Botero *et al.*[29] found a correlation between periodontal pathogen detection, CMV and deep pockets. These authors suggest that viral infections are acquired at an early age and that the prevalence in the population increases between 30-35 years old.

The results obtained in this research reinforce the hypothesis that herpesviruses may be involved in increased periodontal destruction, supporting the evidence that herpesviruses have a pathogenic role in the etiology of periodontal disease.

In conclusion, there is a higher frequency of herpesvirus infection in HIV-positive patients, the most prevalent being EBV in the HIV-positive group with HAART, and CMV in the HIV-positive group without HAART.

Detección de VEB, CMV y VHS-1 en muestras subgingivales de pacientes VIH positivos y negativos con periodontitis crónica.

Resumen: Detectar la presencia de infección por VEB (Virus Epstein-Barr), CMV (Citomegalovirus) y VHS-1 (Virus Herpes simple tipo 1) en muestras subgingivales de pacientes VIH-positivos bajo HAART (Terapia Anti Retroviral de Alta Actividad), VIH-positivos sin HAART, pacientes VIH-negativos con periodontitis crónica y controles sanos. Metodología: Se evaluaron muestras de fluido crevicular de 11 pacientes VIH+ bajo terapia, 6 sin terapia antiretroviral, 7 sujetos VIH–negativo con periodontitis crónica y 7 controles periodontalmente sanos. Se registró el IP (Índice de placa), IG (Índice Gingival), PS (Profundidad del Sondaje) y NIC (Nivel de Inserción Clínica) en seis sitios por diente en todos los dientes y se recolectaron muestras de placa subgingival de un diente por cuadrante. Se empleó PCR anidada para detectar VEB y PCR punto final para identificar la infección con CMV y VHS-1. Resultados: Los parámetros clínicos mostraron diferencias estadísticamente significativas entre pacientes VIH-positivos y sujetos con periodontitis crónica comparados con el grupo control ($p<0.05$). El ADN de EBV fue detectado principalmente en pacientes VIH-positivos bajo HAART con 91% (10/11). El ADN de CMV se detectó principalmente en pacientes sin HAART, 67% (4/6), mientras que VHS-1 se observó en 27% (3/11) de los pacientes bajo HAART. En el grupo control no se detectó ningún virus. La coinfección fue observada en 50% de los pacientes VIH sin HAART, 36% de los VIH con HAART y 14% de los VIH negativos con periodontitis crónica. Conclusión: La infección viral fue predominante en los pacientes VIH bajo HAART y VEB fue la principal infección viral detectada en los pacientes VIH positivos y con periodontitis crónica.

Palabras clave: *EBV, CMV, VHS-1, Periodontitis, Subgingival*

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