

## LACK OF ASSOCIATION BETWEEN HERPESVIRUS DETECTION IN SALIVA AND GINGIVITIS IN HIV-INFECTED CHILDREN

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### SUMMARY

The aims of this study were to compare the detection of human herpesviruses (HHVs) in the saliva of HIV-infected and healthy control children, and to evaluate associations between viral infection and gingivitis and immunodeficiency. Saliva samples were collected from 48 HIV-infected and 48 healthy control children. Clinical and laboratory data were collected during dental visits and from medical records. A trained dentist determined gingival indices and extension of gingivitis. Saliva samples were tested for herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), and cytomegalovirus (CMV) by nested polymerase chain reaction assays. Thirty-five HIV-infected and 16 control children had gingivitis. Seventeen (35.4%) HIV-infected children and 13 (27%) control children were positive for HHVs. CMV was the most commonly detected HHV in both groups (HIV-infected, 25%; control, 12.5%), followed by HSV-1 (6.2% in both groups) and HSV-2 (HIV-infected, 4.2%; control, 8.3%). The presence of HHVs in saliva was not associated with the presence of gingivitis in HIV-1-infected children ( $p = 0.104$ ) or healthy control children ( $p = 0.251$ ), or with immunosuppression in HIV-infected individuals ( $p = 0.447$ ). Gingivitis was correlated with HIV infection ( $p = 0.0001$ ). These results suggest that asymptomatic salivary detection of HHVs is common in HIV-infected and healthy children, and that it is not associated with gingivitis.

**KEYWORDS:** HIV infection; Herpesvirus; Periodontitis; Gingivitis; Children.

### INTRODUCTION

Herpesviruses are large DNA-enveloped viruses belonging to the *Herpesviridae* family. Herpesviruses are highly disseminated in nature. Of more than 200 known, eight are human pathogens: herpes simplex virus 1 (HSV-1), herpes simplex 2 (HSV-2), varicella zoster virus (VZV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), and human herpesviruses 6, 7 and 8 (HHV-6, -7, -8)<sup>23</sup>. Transmission occurs by contact, and primary infections generally occur early in life, followed by persistence of the virus in the organism. Herpesvirus diseases occur primarily in immunosuppressed individuals; fatal infections in immunocompetent hosts are rare<sup>23</sup>.

Several studies have implicated herpesviruses in the etiology of periodontitis<sup>26-29</sup>. Apparently, periodontal tissue breakdown occurs more frequently and progresses more rapidly in herpesvirus-infected than in herpesvirus-free periodontal sites<sup>26-29</sup>. Herpesviruses may cause periodontal pathosis as a direct result of virus infection and replication, or as a consequence of virally induced impairment of periodontal immune defenses, resulting in heightened virulence of

resident bacterial pathogens<sup>8,26</sup>. The herpesviral-bacterial hypothesis of periodontitis development proposes that active herpesvirus infection initiates periodontal tissue breakdown and that host immune responses against the herpesvirus infection are important components of the etiopathogeny of the disease<sup>28</sup>. The herpesvirus infection triggers the release of proinflammatory cytokines, which have the potential to activate osteoclasts and matrix metalloproteinases and to impair antibacterial immune mechanisms, causing an upgrowth of periodontopathic bacteria<sup>28</sup>.

High frequencies of EBV and CMV genomes have been noted in adults with progressive periodontitis, in localized and generalized aggressive (juvenile) periodontitis, HIV-associated periodontitis, acute necrotizing ulcerative gingivitis, periodontal abscesses, and some rare types of advanced periodontitis associated with medical disorders<sup>26</sup>. Other herpesviruses such as HHV-6, HHV-7, HHV-8, and HSV-1, have also been associated with periodontitis<sup>4,15,19</sup>. In contrast, HSV-2 appears to be uncommon at periodontal sites<sup>7,32</sup>. However, the pathogenesis of herpesviruses in periodontitis has not yet been fully elucidated.

Human herpesviruses (HHVs) have often been detected in the saliva

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of HIV-infected individuals<sup>4,5,7,11,12,15,18</sup>, and several studies have shown that highly active antiretroviral therapy (HAART) does not significantly reduce the prevalence or the load of HHVs in saliva<sup>11,17,22,32</sup>. The elevated frequency of HHVs infections in association with periodontitis in HIV-infected individuals<sup>7,8,11,12</sup> suggests that these viruses play a role in the disease. In children, the prevalence of some oral manifestations associated with HIV infection was reduced after HAART initiation. However, other lesions emerged<sup>24</sup>, as these individuals are prone to develop opportunistic viral infections, especially those caused by *Herpesviridae* family members, in the oral mucosa. Little information is available on HHVs co-infection in the saliva of HIV-infected children.

The aims of this study were to detect HHVs in the saliva of HIV-1-infected children in comparison with healthy control children, and to evaluate possible associations between viral infection and gingivitis and immunodeficiency stage.

## MATERIAL AND METHODS

**Samples:** The ethics committees of the Hospital Universitário Clementino Fraga Filho and Institute of Pediatrics and Childcare Martagão Gesteira, Universidade Federal do Rio de Janeiro (UFRJ), Brazil, approved the study protocol. The parents of all children involved in the study provided written informed consent in accordance with Resolution 196/96 of the Brazilian Ministry of Health.

The study population was composed of patients attending the UFRJ School of Dentistry between August 2009 and July 2010. Participants were selected by convenience sampling during initial appointments for dental treatment. The HIV-1-infected group was made up of 48 children of both sexes, ranging from six to 12 years old, who were patients at the Institute of Pediatrics and Childcare Martagão Gesteira, UFRJ, with definitive diagnoses of HIV infection. The following medical history data were extracted from their medical records: diagnosis of HIV infection, results of most recent (closest to the day of saliva sample collection; maximum interval, three months) laboratory tests (viral load, CD4 and CD8 counts, and CD4/CD8 ratio) and use of anti-retroviral agents (at the time of saliva sample collection). The immunodeficiency stages of HIV-infected individuals were defined using CD4 counts, according to the classification of the Centers for Disease Control and Prevention<sup>6</sup>.

The control group consisted of 48 healthy children, ranging from seven to 12 years old, who attended the UFRJ Pediatric Dentistry Clinic and showed no clinical evidence of systemic or chronic disease. They were considered clinically healthy because they were receiving no medical treatment for any disease and showed no clinical sign of immunosuppression, systemic disease, and/or had no history of a risk factor for HIV infection. These data were collected through medical anamnesis with the patients' parents and the attending physician. Children in the control group did not undergo testing to confirm serological HIV negativity because there was no reason to justify this procedure, which the local ethics committees therefore disallowed.

Prior to saliva sample collection, all children in the HIV-1-infected and control groups underwent oral and oropharyngeal examinations by a trained and calibrated dentist to identify oral manifestations such as gum bleeding, mouth ulcers, oral mucosal lesions, and cervical lymphadenopathy. The gingival index was assessed using a sterile

periodontal probe. Gingivitis was considered to be present when gingival bleeding occurred on probing<sup>1</sup>. The extension of gingivitis was classified according to the System for the Classification of Periodontal Diseases and Conditions<sup>2</sup>; patients with gingivitis at <30% of sites surveyed were classified as having localized gingivitis, and those with >30% of surveyed sites affected were classified as having generalized gingivitis.

Individuals were not allowed to brush their teeth or eat for one h before providing saliva samples. Five milliliters of paraffin-stimulated saliva were collected in a sterile container. The samples were kept in an ice-filled cooler and submitted for laboratory analysis within two h.

**Sample processing:** The saliva samples were centrifuged and pelleted, and DNA was extracted using the Wizard<sup>®</sup> Genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

**Virus detection:** All samples were subjected to human  $\beta$ -globin gene amplification to determine the integrity and quality of extracted DNA and to avoid false-negative results<sup>3</sup>. Specimens were analyzed using conventional polymerase chain reaction (PCR) assays, as described previously, to detect the presence of HSV-1/2, VZV, EBV, and CMV<sup>30</sup>. PCR products were detected using 1.2% agarose gel electrophoresis and staining with ethidium bromide.

First-round PCR reactions consisted of the addition of 5  $\mu$ L of extracted DNA to 20  $\mu$ L of PCR mix containing 0.5  $\mu$ M of each of the primers HHV-F1 and HHV-R1, 0.125  $\mu$ M of each of the primers VZV-F1 and VZV-R1, and 1x PCR buffer; 1.5 mM MgCl<sub>2</sub> and 0.2 mM of deoxyribonucleotide triphosphates; and 2.5 U of GoTaq DNA polymerase (Promega). First-round PCR was carried out as follows: one cycle at 94 °C for three min, followed by 35 cycles at 94 °C for 45 s, 65.5 °C for one min, 72 °C for one min, and final extension at 72 °C for seven min. For nested PCR, 0.5  $\mu$ L of first-round product was transferred to 25  $\mu$ L PCR mix similar to that described above, but containing second-round primers (HHV-F2, HHV-R2, VZV-F2, and VZV-R2), at the same concentrations as in the first round. PCR conditions were the same as in the first round, except that the annealing temperature was changed to 63 °C. Positive and negative controls were included in each run. Infected cell cultures were used as positive controls for HSV-1 and HSV-2 (Vero cells), EBV (Daudi cells), and CMV (MRC-5 cells). For VZV, clinical samples obtained from patients with varicella diagnoses confirmed by PCR amplification and sequencing analysis were used as positive controls. Negative controls consisted of saliva samples previously demonstrated to be HHVs. The expected sizes of the PCR products for first-round and nested PCRs, respectively, were: HSV-1/2, 742 and 493 pb; VZV, 650 and 356 pb; EBV, 748 and 499 pb; and CMV, 817 and 565 pb.

Because some PCR products had very similar sizes, sequencing analysis was used to confirm their specificity and to differentiate HSV-1 and HSV-2. Amplified DNA from all HSV-positive samples and three CMV-positive samples was purified using the Wizard SV gel and PCR clean-up system kit (Promega), and sequences were determined using the BigDye terminator cycle sequencing kit and the ABI PRISM 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) using the same PCR primers. DNA sequences were edited using the Chromas software (Technelysium Pty. Ltd., Brisbane, QLD, Australia) and compared with the DNA sequences available in GenBank (<http://www.ncbi.nlm.nih.gov>)

using the BLAST tool (<http://www.ncbi.nlm.gov/BLAST>).

**Statistical analysis:** Using the Epi-Info statistical program (version 3.5.1., CDC, Atlanta, GA, USA), data from the two groups were compared using the Mann-Whitney test for means of continuous variables (age) and the  $\chi^2$  test and Fischer's exact tests for categorical variables (sex, presence of HV types in saliva, and gingivitis). Within the HIV-infected group, the  $\chi^2$  and Fischer's exact tests were used to verify correlations between the presence of HVs in saliva and immunosuppression, HAART, and the presence of oral manifestations.

## RESULTS

The mean age of the 48 HIV-1-infected children was 9.58 years; 45.8% of these subjects were male, 70.8% were receiving HAART, and 52.1% had no immunosuppression. The mean age of healthy control children (47.9% male) was 9.04 years. These groups did not differ in terms of age or sex ( $p > 0.05$ ). Other clinical and medical data from the study subjects are shown in Table 1.

**Table 1**

Demographic, clinical, and immunological characteristics of children in the HIV-1-infected and control groups

Variable	HIV-1-infected subjects	Control subjects
Age (years), mean (range)	9.58 (6-12)	9.04 (7-12)
Sex	Male 22 (45.8%) Female 26 (54.2%)	Male 23 (47.9%) Female 25 (52.1%)
HAART	Yes, 34 (70.8%) No, 14 (29.2%)	<200; 9 (18.7%)
CD4 <sup>+</sup> count (cells/ $\mu$ L)	200-499; 14 (29.2%) >500; 25 (52.1%)	
Gingivitis	Yes, 35 (72.9%) No, 13(27.1%)	Yes, 16 (33.3%) No, 32 (66.7%)
Oral findings		
Candidosis	n = 4	- <sup>1</sup>
Linear gingival erythema	n = 5	-
Angular cheilitis	n = 1	-
Oral ulcer	n = 1	-

HAART = highly active antiretroviral therapy; <sup>1</sup> -: absence of symptoms.

Seventeen (35.4%) of the 48 HIV-1-infected children were positive for HHVs: 6.2% (3/48) were positive for HSV-1, 4.2% (2/48) for HSV-2, and 25.0% (12/48) were positive for CMV. In the control group, 13/48 (27.0%) children were positive for HHVs: 6.2% were positive for HSV-1, 8.3% (4/48) for HSV-2, and 12.50% (6/48) were positive for CMV. No VZV, EBV or co-infection with those viruses was detected in either group (Table 2). No significant difference was observed in HHVs detection in

the saliva of HIV-1-infected and healthy children ( $p = 0.167$ ); however, HSV-2 was more common in the control group and CMV infection was more common in immunocompromised HIV-1-infected children. Sequence analysis confirmed the PCR results and allowed differentiation between HSV-1 and HSV-2 strains.

**Table 2**

Herpesviruses detected in saliva from HIV-1-infected and healthy control children

Virus <sup>1</sup>	HIV-1-infected subjects (%) N = 48	Control subjects (%) N = 48	p
HSV-1	3 (6.2)	3 (6.2)	1.00
HSV-2	2 (4.2)	4 (8.3)	0.458
CMV	12 (25.0)	6 (12.5)	0.117
Total	17 (35.4)	13 (27.0)	0.167

<sup>1</sup>EBV and VZV were not detected.

HIV-1-infected individuals were classified into three immunologic categories: no evidence of suppression (CD4<sup>+</sup> > 500 cells/ $\mu$ L; CD4<sup>+</sup> % > 25), moderate suppression (CD4<sup>+</sup> = 200-499 cells/ $\mu$ L; CD4<sup>+</sup> % [15-24]), and severe suppression (CD4<sup>+</sup> < 200 cells/ $\mu$ L; CD4<sup>+</sup> % < 15)<sup>19</sup> (Table 1). Twelve of 25 (48.0%) children with no evidence of immunosuppression, 2/14 (14.3%) children with moderate immunosuppression, and 3/9 (33.3%) children with severe immunosuppression were HHVs positive. No correlation was found between HHV infection and the degree of immunosuppression ( $p = 0.447$ ).

Eleven of 34 (32.4%) individuals undergoing HAART and 6/14 (42.8%) children not receiving HAART were HHVs positive. However, no significant correlation between HHVs detection in saliva and receipt of HAART was observed ( $p = 0.489$ ).

Thirty-five of 48 (72.9%) HIV-1-infected children had gingivitis at the time of sample collection; 10 (28.6%) were positive for HHVs infection. In the control group, six of 16 (37.5%) children with gingivitis were HHVs positive. No significant correlation between the presence of HHVs in saliva and the presence and extension of gingivitis was observed within each group, HIV-1-infected children ( $p = 0.104$ ) and healthy control children ( $p = 0.251$ ), or when the HIV-1-infected group was compared with the control group ( $p = 0.491$ ). However, HIV infection was strongly correlated with gingivitis ( $p = 0.0001$ ).

Four (8.3%) HIV-1-infected children had candidosis, five (10.4%) had linear gingival erythema (LGE), one (2.1%) had an oral ulcer, and one (2.1%) had angular cheilitis (Table 1). One subject with candidosis and LGE and one subject with angular cheilitis were HSV-1 positive; one subject with LGE was CMV positive. HHV detection in saliva was not correlated with any oral symptom.

## DISCUSSION

Herpesviruses, most commonly CMV, EBV, and HSV-1, have been detected in oral samples from immunosuppressed and immunocompetent individuals with gingivitis<sup>7,9,11-13,15,16,18,25,33</sup>.

Median CMV detection rates in healthy periodontium and in individuals with gingivitis are about 8% and 33%, respectively<sup>27</sup>. CMV has been detected in 25-49% of immunocompetent individuals and 40-62% of HIV-infected individuals with gingivitis<sup>11-13,16,33</sup>. Previous studies have not been able to demonstrate a clear association between the presence of CMV and gingivitis because the virus was detected at high frequencies in control groups. The present study found CMV in the saliva of 25% of HIV-1-infected children and 12.5% of healthy control children. Although CMV was detected more often in immunocompromised children, CMV could not be clearly associated with gingivitis.

HSV-1 is detected less frequently than CMV and EBV in the saliva of individuals with periodontitis<sup>12,13,25</sup>, but its detection has been described in patients with gingivitis<sup>11,16</sup>. The present study found HSV-1 in the saliva of 6.2% (3/48) of subjects from both groups.

HSV-2 is rarely detected in saliva<sup>7,20,27,31,32</sup>, but it was detected in both groups in the present study. Two HIV-1-infected boys aged 10 and 11 years were HSV-2 positive; one of these subjects, a severely immunosuppressed (CD4<sup>+</sup> count = 149 cells/ $\mu$ L [11.27%]) boy who was not undergoing HAART, had gingivitis. The other HIV-1-infected, HSV-2-positive child had no evidence of immunosuppression (CD4<sup>+</sup> count = 724 cells/ $\mu$ L [29%]) and was receiving HAART. Four children (aged 7-9 years) in the control group were HSV-2 positive; three of them had gingivitis.

A recent review of HHVs in periodontitis showed that EBV is detected in association with gingivitis in 20% of cases and with healthy periodontium in 8% of cases<sup>27</sup>. Several studies have described EBV detection rates of 48-90% in the saliva of HIV-infected individuals<sup>9,14,16,17</sup> and 17-40% in the saliva of healthy individuals<sup>5,11,14-16,22</sup>. Surprisingly, the present study did not detect EBV in HIV-1-infected or healthy children with or without gingivitis.

Few studies have reported VZV excretion in saliva. Such excretion is usually observed in stressed individuals<sup>20</sup> or those with herpes zoster manifestations<sup>10,21</sup>. WANG *et al.*<sup>32</sup> detected VZV DNA in the saliva of 5.1% (3/59) of HIV-positive subjects and 1.9% (1/53) of healthy control subjects. They detected VZV in individuals undergoing HAART and concluded that such an event is infrequent in the saliva of asymptomatic HIV-positive persons and that HAART does not reduce the risk of asymptomatic VZV excretion<sup>32</sup>. Accordingly, VZV has not been associated with periodontal disease<sup>27</sup>. Consistent with these findings, the present study did not detect VZV in its study population.

In this study, HHVs detection in the saliva of HIV-1-infected and healthy children with and without gingivitis was compared. Although sample size potentially limits the statistical power of the results, the study's findings are comparable to those reported in the literature. CMV was the most prevalent virus detected in both groups, followed by HSV-1 and HSV-2. EBV and VZV were not detected in either group. No association was demonstrated between HHV detection in saliva and the presence of gingivitis. No association between the detection of HV DNA in saliva and the level of immunosuppression in HIV-1-infected children was observed. Moreover, HAART did not seem to reduce virus shedding. However, a strong correlation between HIV infection and gingivitis was confirmed.

## RESUMO

### Ausência de associação entre a detecção de herpesvírus na saliva e gengivite em crianças infectadas pelo HIV

Os objetivos deste estudo foram detectar a presença de herpesvírus humanos (HHVs) na saliva de crianças infectadas pelo HIV, em comparação com controles saudáveis e avaliar a associação entre infecção viral, gengivite e imunodeficiência. Para este fim, foram colhidas amostras de saliva de 48 crianças HIV-positivas e 48 controles saudáveis. O índice gengival e extensão de gengivite foram determinados por um dentista treinado. Informações clínicas e laboratoriais foram obtidas durante a consulta odontológica e dos registros médicos. As amostras de saliva foram testadas para detecção de vírus herpes simplex tipos 1 e 2 (HSV-1 e HSV-2), vírus da varicela-zoster (VZV), vírus Epstein-Barr (EBV) e citomegalovírus (CMV) através de *nested*-PCR. Trinta e cinco crianças HIV-positivas e 16 crianças do grupo controle apresentavam gengivite. Dezesete (35,4%) crianças HIV-positivas e 13 (27%) crianças controle testaram positivo para a presença de HHVs. CMV foi o vírus mais comum detectado em ambos os grupos (25% HIV-positivas e 12,5% de controle), seguido por HSV-1 (6,2% de ambos os grupos) e HSV-2 (4,2% HIV-positivas e 8,3% de controle). Não houve associação entre a detecção de HHVs na saliva e a presença de gengivite em crianças HIV-positivas ( $p = 0,104$ ) ou crianças saudáveis ( $p = 0,251$ ), ou com imunossupressão em indivíduos HIV-positivos ( $p = 0,447$ ). Foi observada uma correlação entre a infecção por HIV e a presença de gengivite ( $p = 0,0001$ ). Os resultados sugerem que a detecção salivar assintomática de HHVs é comum entre crianças HIV-positivas e crianças saudáveis, e não está associada à gengivite.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## AUTHORS' CONTRIBUTIONS

GFC, IPRS, and NS conceived and designed the study and analyzed the data. RAO, FN NN, RCS, and RSL were responsible for data and sample collection and PCR analysis. All authors have read and approved the final manuscript.

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