PCR detection of multiple human herpesvirus DNA in saliva from HIV-infected individuals in Teresina, State of Piauí, Brazil

Detecção por PCR do DNA de vários herpesvírus humanos na saliva de indivíduos infectados pelo HIV em Teresina, Estado do Piauí, Brasil

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ABSTRACT

Introduction: Human herpesviruses are frequently associated with orofacial diseases in humans (HSV-1, EBV, CMV and HHV-8), some can also cause systemic disease (CMV and HHV-8). The transmission of these viruses occurs by contact with infected secretions, especially saliva. Human immunodeficiency virus infection is associated with an increased risk of HHVs and related diseases. Methods: This work aimed to detect HSV-1, EBV, CMV and HHV-8 DNA in saliva of HIV-infected patients from Teresina, northeast Brazil, by PCR and compare these findings with age and sex matched HIV-seronegative individuals. Results: No difference in prevalence was verified between HHV detection in the saliva of HIV-seropositive individuals and controls. The individual frequencies of these viruses in these two populations were different. HIV seropositivity correlated positively with the presence of CMV (OR: 18.2; p = 0.00032) and EBV (OR: 3.44; p = 0.0081). No association between CD4 counts and the prevalence of HHVs in the saliva was observed; however, a strong association was determined between seropositivity and the presence of multiple HHV DNAs in saliva (OR: 4.83; p = 0.0028). Conclusions: These findings suggest the asymptomatic salivary shedding of HHVs is a common event between HIV-seropositive and seronegative individuals from Teresina, Piauí, Brazil, and, especially for HIV-seropositive patients, saliva is a risk factor for the acquisition/transmission of multiple HHVs.

Key-words: Human herpesvirus. Saliva. PCR. HIV seropositive.

INTRODUCTION

Human herpesviruses (HHVs) belong to the Herpesviridae family and are widely distributed viruses that cause benign and malignant disease in animals and humans. Human simplex virus 1 and 2 (HSV-1 and HSV-2), Varicella-zoster virus (VZV), Cytomegalovirus (CMV), Epstein-Barr virus (EBV), Human herpes virus 6 (HHV-6), Human herpes virus 7 (HHV-7) and Human herpes virus 8 (HHV-8) are known to cause infections in humans. Among these, HSV-1, EBV, CMV and HHV-8 are frequently associated with orofacial diseases in humans. Additionally, EBV and HHV-8 are also implicated in systemic diseases. The transmission of these viruses occurs by contact with infected secretions, especially saliva. The majority of HIV infected individuals show no clinical symptoms, but can shed HHVs asymptomatically in saliva. Human immunodeficiency virus (HIV) is one of the most important pathogens of this century. Almost 33 million people are living with HIV. It is clear that HHV-related diseases are a significant problem for HIV-seropositive patients. HIV oral manifestations induced by HHVs are frequently reported and include oral aphthous ulceration, AIDS-associated oral lymphoma, Kaposi sarcoma and oral hairy leukoplakia.

Using PCR and real time PCR to detected HHV viral genomes in saliva, some research groups have been reported the prevalence of these viruses in the saliva of HIV-seropositive patients. The frequencies of these viruses are wide-ranging, varying from 62 to 90% for EBV; 57% for HHV-8; 19 to 72% for CMV and 5 to 16 for HSV-1. In these reports, the prevalence of HHVs was higher in HIV-seropositive patients than seronegative individuals. Thus, the molecular detection of HHVs in body fluids could be predictive of the development of HHV-related diseases and, consequently, would be of great benefit to the HIV-seropositive population. In Brazil, few...
studies exist that determine the frequencies of HHVs in HIV positive patients. The majority of studies report a high frequency of HHV-8 in HIV positive patients, but few studies also report high frequencies of CMV, EBV and HSV-1 and HSV-2. However, there is no available data regarding the serological or molecular frequency of HHVs in the State of Piauí (PI), northeast Brazil. Therefore, this study aimed to determine the prevalence of HSV-1, EBV, CMV and HHV-8 in the saliva of HIV-seropositive patients from Teresina, PI, by PCR and compare these findings with age and sex matched HIV-seronegative individuals.

**METHODS**

**Study population and procedures**

Forty-four HIV-seropositive individuals were recruited from the Natan Portella Institute of Tropical Diseases (Instituto de Doenças Tropicais Natan Portella, IDTNP) in Teresina, PI. Forty age control subjects were recruited from the Federal University of Piauí campus including workers from the IDTNP. All samples were collected in 2008.

Immunochromatography assays for HIV-1 and HIV-2 (Inverness Medical) were performed on all controls to insure that they were HIV seronegative. All controls were in good general health. The inclusion criteria for control subjects were: they should be free of symptoms of acute illness (fever, headache, sore throat, body aches and diarrhea) and malignancies at the time of enrollment. The only inclusion criterion for HIV-seropositive patients was seropositivity, regardless of the presence or absence of symptoms. The exclusion criteria for both groups were pregnancy, use of immunosuppressant medications and administration of antiparasitic therapy one week before the study enrollment date. All patients and control subjects were submitted to oropharyngeal examinations in order to identify any oral alterations, such as mouth ulcers, gum bleeding, mouth pain, dry mouth, oral mucosal lesions and cervical lymphadenopathy. Unstimulated whole saliva (3 ml) was collected in a sterile recipient.

The sample were divided into 1.5 ml aliquots and frozen at -20°C until use. Determination of CD4 count was performed by flow cytometry.

**DNA extraction from saliva, primers and polymerase chain reaction**

DNA from 1.5 ml of each saliva specimen was centrifuged and the DNA was isolated from the cell pellet using the phenol-chloroform method described by Anzai-Kanto et al. Two microliters of DNA were used in each PCR reaction. The integrity of the extracted DNA and the exclusion of PCR inhibitors in samples were confirmed by amplifying the human β-globin gene. The primers for CMV genome detection (HSVF 5’- GACCTTGCAGCCCTSTACCC-3’ and CMVR 5’- CTCTCCTCGAATCCCCC-3’) were based on the DNA polymerase gene of CMV (GenBank accession number AB329634.1) and amplified 499 base pairs PCR products. The amplification mixture for CMV contained 10 μl of each primer, 0.1 μM of each dNTP, 0.1 U Taq polymerase, Taq buffer, 1.5 mM MgCl2 concentration, 2 μl template and distilled water in a final volume of 20 μl. PCR amplification was performed at 94°C for 5 min (1 cycle); 94°C for 1 min, 58°C for 1 min and 72°C for 1 min (35 cycles); and 72°C for 10 min (1 cycle). PCR for the detection of HSV-1, EBV and HHV-8 was performed using the oligonucleotide primers and conditions described previously. The specificities of the primer pairs were validated by DNA sequencing of the PCR products. Genomic DNAs extracted from clinical samples were used as positive control standards for HSV-1, CMV and EBV previously confirmed by PCR. The genomic DNA extracted from BCBL-1 cells was used as positive control standard for HHV-8. The amplified PCR products were run on 2% agarose gel stained with ethidium bromide and visualized under a UV light transilluminator. The PCR was performed with strict adherence to the universal quality control guidelines. All reactions performed involved sample preparation, reaction mix preparation, amplification and electrophoresis in separate rooms to avoid cross-contamination.

**Statistical analysis**

Odds ratios (and 95% confidence intervals) were based on 2 x 2 contingency tables and were calculated to assess the association between the presence of a virus and demographics or associated factors (CD4 counts). The significance of measurement was determined by the Chi square (χ2) and Fisher exact tests.

**Ethical**

The present study was approved by the Committee of Ethics in Research at the Federal University of Piauí (UFPI) (protocol number 0240.0.045.000-07) and all patients provided written informed consent as part of the study protocol.

**RESULTS**

The study was conducted in 2008 and according to analysis of the results, the 44 HIV-seropositive individuals were demographically similar to the 40 controls. The demographic analysis indicated that the average age of HIV-seropositive individuals was 35.89 years-old and 33.92 years-old for control subjects. The majority of individuals in both groups were men whose marital status was single (Table 2).

**TABLE 1 - Genome targets and references for HHV genomes and β-globin detected by PCR.**

<table>
<thead>
<tr>
<th>Genome targets</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1 Thymidine Kinase</td>
<td>[18]</td>
</tr>
<tr>
<td>CMV DNA polymerase</td>
<td>This study</td>
</tr>
<tr>
<td>EBV EBNA-1</td>
<td>[19]</td>
</tr>
<tr>
<td>HHV-8 orf64-orf65</td>
<td>[20]</td>
</tr>
<tr>
<td>β-globin b-globin gene</td>
<td>[17]</td>
</tr>
<tr>
<td>HSV: human herpesvirus type 1, CMV: cytomegalovirus, EBV: Epstein-Barr virus, EBNA-1: Epstein-Barr virus nuclear antigen 1</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 2 - Demographic characteristics of the study population.

<table>
<thead>
<tr>
<th>Serostatus</th>
<th>Sex</th>
<th>Marital status</th>
<th>Age range (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV seropositive</td>
<td>32M; 12W</td>
<td>31S; 13 Ma</td>
<td>25-59 (35.89 ± 7.98)</td>
</tr>
<tr>
<td>HIV seronegative</td>
<td>29M; 11W</td>
<td>23S; 17 Ma</td>
<td>21-51 (33.92 ± 9.25)</td>
</tr>
</tbody>
</table>


None of the HIV-seropositive patients presented signs or symptoms of oral lesions caused by any HHVs or symptomatic Kaposi sarcoma. Twenty HIV-seropositive patients presented secondary diseases: visceral leishmaniosis (7/20), tuberculosis (5/20), fungal meningitis (3/20), oral candidiasis (3/20), Pneumocystis carinii pneumonia (2/20) and genital herpes (1/20). Among the HIV-seronegative individuals, only five (5/40) presented oral herpes.

After PCR assays, 75% of HIV-seropositive patients (33/44) had detectable salivary HHV DNAs. Among the 40 HIV-negative subjects, salivary HHVs were detected in 31 (77.5%) of the 40 samples tested. In the HIV-seropositive group, the most prevalent
In our cohort, 70.4% of HIV-seropositive patients were taking highly active antiretroviral therapy (HAART) and this therapy could alter the frequency of oral lesions caused by these viral agents. Besides the high frequencies of HHVs in saliva from HIV-infected patients, none of these patients presented oral lesions due to HHV activity. This could be explained by the fact that HHVs are frequently shed asymptomatically in the saliva of HIV-infected patients under HAART[14, 32, 33].

The frequency of HHVs in these patients indicates that asymptomatic HHV shedding in saliva is a common event even in the presence of HAART. One of the Brazilian public health priorities is to make HIV medications available free of charge to all HIV-seropositive patients[34]. The consequences of universal access to HAART include a reduction in the number of deaths by AIDS and a reduction of the incidence of opportunistic diseases[34]. Therefore, the present data, in conjunction with other reports, corroborate the hypothesis that HAART has little effect on HHV shedding in saliva, because all HHVs tested in this study were frequently detected in HIV-infected patients. However, the statistical analysis only showed a risk for CMV and EBV shedding in the saliva of HIV-seropositive patients. These data are consistent with other studies that showed high CMV and EBV salivary shedding among more immunosuppressed patients in comparison with HIV-seronegative individuals[47, 10]. Even in patients submitted to HAART, EBV and CMV shedding appears to persist at a higher frequency compared with HIV-uninfected controls[48, 12]. This finding is in agreement with other studies in Brazil that also showed a high prevalence of CMV and EBV in HIV-seropositive patients[15, 35].

Besides the differential prevalence, the most relevant data from this study is the high prevalence of HHV coinfec tion in HIV-seropositive patients. The majority of the HIV-seropositive group presented at least two detectable HHVs in the saliva. A 17-fold increase in the odds of triple infection detection in saliva was demonstrated. This data is consistent with other studies that reported a high prevalence of multiple HHVs in the saliva of HIV-seropositive patients and suggests that salivary shedding of all HHVs are a common event among patients receiving HAART treatment[4, 10, 35]. The presence of HHV coinfec tion in HIV-positive individuals could be due to a lack of immunosurveillance by virus-specific CD8+ cytotoxic T lymphocytes and virus specific CD4+ T cells[36, 37].

In summary, the observations reported herein suggest that the presence of HHVs in saliva is a frequent event in HIV-infected individuals from northeastern Brazil and seropositivity increases the risk of multiple infections in these patients, even in the presence of HAART therapy. This study represents the first report of the prevalence of multiple salivary HHVs in HIV-seropositive patients and HIV-seronegative individuals from Teresina, PI, northeast Brazil.

Saliva, blood, cerebrospinal fluid, tissue and skin lesion samples have all been used for HHV diagnosis by PCR[31, 32]. Since HHVs have different susceptibilities to antiviral drugs, rapid and precise diagnosis of the HHVs involved in a specific pathology is very important.

As expected, after PCR assays for HSV-1, EBV, CMV and HHV-8, a high prevalence of HHVs in saliva of HIV seropositive and seronegative individuals was observed: 75% for HIV seropositive and 77.5% for seronegative. The present data is consistent with other studies that showed evidence of infection with HHVs in the majority of the world's populations[27-30].

Interestingly, a strongly associated was verified between seropositivity and the presence of multiple HHV DNAs in saliva (OR 4.83, p = 0.0028). Saliva of HIV-positive patients showed a high frequency of coinfection by three different HHVs (OR 17.1429, p = 0.0008) (Table 4). Viruses most likely to be simultaneously present in the saliva of HIV-seropositive patients were HSV-1 and EBV, CMV, HSV-1 and EBV, HV-8, HSV-1 and EBV, and HSV-1, CMV and HV-8 (data not shown).

**DISCUSSION**

**ACKNOWLEDGMENTS**

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**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.
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