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Major Article

Current status of herpesvirus identification in the oral cavity of HIV-infected children

Raquel dos Santos Pinheiro[1], *Dennis de Carvalho Ferreira*[2], *Flávia Nóbrega*[1], *Norma Suely de Oliveira Santos*[3], *Ivete Pomarico Ribeiro de Souza*[1] *and Gloria Fernanda Barbosa de Araujo Castro*[1]

[1]. Departamento de Odontopediatria, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ. [2]. Departamento de Microbiologia Medica, Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ. [3]. Departamento de Virologia, Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ.

ABSTRACT

Introduction: Some viruses of the *Herpesviridae* family are frequently the etiologic agents of oral lesions associated with HIV. The aim of this study was to identify the presence of herpes simplex virus types 1 and 2 (HSV-1, HSV-2), Varicella Zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), human herpesvirus type 6, type 7 and type 8 (HHV-6, HHV-7 and HHV-8) in the oral cavity of HIV-infected children/adolescents and verify the association between viral subtypes and clinical factors. **Methods:** The cells of oral mucosa were collected from 50 HIV infected children/adolescents, 3-13 years old (mean age 8.66). The majority (66%) of selected were girls, and they were all outpatients at the pediatric AIDS clinic of a public hospital in Rio de Janeiro. Nested-PCR was used to identify the viral types. **Results:** Absence of immunosuppression was observed in 66% of the children. Highly active antiretroviral therapy (HAART) was used by 72.1% of selected and moderate viral load was observed in 56% of the children/adolescents. Viral types were found in 86% of the children and the subtypes were: HSV-1 (4%), HSV-2 (2%), VZV (4%), EBV (0%), HCMV (24%), HHV6 (18%), HHV-7 (68%), HHV8 (0%). **Conclusions:** The use of HAART has helped to reduce oral lesions, especially with herpes virus infections. The health professionals who work with these patients should be aware of such lesions because of their predictive value and the herpes virus can be found circulating in the oral cavity without causing lesions.

Keywords: Herpesvirus. Polymerase chain reaction. Human immunodeficiency virus.

INTRODUCTION

Oral manifestations in human immunodeficiency virus (HIV)-infected children are common. The prevalence of these lesions ranges from 20 to 80% depending on patient immune suppression, and they are considered as markers of infection and predictors of the progression of HIV disease to acquired immunodeficiency syndrome (AIDS)[1].

After the introduction of highly active antiretroviral therapy (HAART) some changes could be observed, such as the prevalence of some oral manifestations and the emergence of other lesions in this group of individuals[1]. Human immunodeficiency virus -infected children and adults are prone to opportunistic viral infections in the oral mucosa, mainly from the *Herpesviridae* family members such as herpes simplex virus (HSV), the cytomegalovirus (CMV) and Epstein-Barr virus (EBV), all of which are important etiologic agents of morbidity[2-4]. This family of infectious agents is composed of three groups of viruses and has a distinct characteristic of being able to persist in latency and they depend on some stimulus for their reactivation, as occurs in cases of immunosuppression promoted by HIV infection. Herpesviruses have a single double-stranded deoxyribonucleic acid (DNA) molecule in their composition and notably, this group of viruses has been able to infect cells involved in human host[5-6].

The human herpes simplex virus I and II (HSV-I and HSV-II), are usually associated with labial and genital lesions, respectively. However, genital herpes can be a consequence of HSV-I and it is similar to HSV-II in the oral cavity. The human herpesvirus 3 (Varicella Zoster Virus - VZV) causes primary varicella, mainly in children and reinfection may be the cause of the herpes zoster (HZ). The human herpesvirus 4 (EBV) is associated with infectious mononucleosis, Burkitt's lymphoma and nasopharyngeal carcinoma[1-6]. The human herpes virus 5 (CMV) can cause pneumonia, rhinitis and severe hepatitis, particularly in individuals who have blood transfusions. Herpesvirus 6 (HSV-6) causes sudden exanthema, as it regulates the expression of cluster of differentation (CD4), and it is associated to multiple sclerosis. Human herpesvirus 7 (HSV-7) is considered the etiologic agent of exanthema subitum, occurring primarily in children, and is accompanied by high fevers and rashes and the human herpesvirus type 8 (HSV-8) is associated with Kaposi's sarcoma and can cause death especially in immune suppressed patients[5-7].

Despite the unknown pathogenesis of these co-infections HIV/HSV[4], the detection of more than one virus in the oral mucosa of HIV-infected patients may have important clinical implications[1]. Thus our study aimed to detect the viruses of

Address to: Profa. Gloria Fernanda Castro. Depto Ortodontia e Odontopediatria/ FO/UFRJ. Rua Araguaia, 994, Bl 2, Apt 304, Freguesia, Jacarepaguá, 22745-270 Rio de Janeiro, RJ, Brasil.
Phone: 55 21 2562-2101; Fax: 55 21 2562-2098
e-mail: gfbacastro@yahoo.com.br
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Herpesviridae family in the oral cavity of HIV infected children/adolescents and associate the detection of the viral genomes with immunosuppression (CD4%), viral load (VL), gender and use of HAART.

METHODS

Subjects

HIV-infected children/adolescents between 3 and 13 years old were investigated. This study included 50 children/adolescents who were outpatients at the *Instituto de Puericultura e Pediatria Martagão Gesteira* (IPPMG) of the Federal University of Rio de Janeiro, Brazil and the SIDA/AIDS Project in Pediatric Dentistry. The patients had weekly medical appointments, as part of the clinical routine. The samples and data were collected from June 2008 to September 2009. The goal of this study was explained to the legal caretakers of the children and those who agreed to participate had to fill in an identification form and sign the term of consent.

Children/adolescents had their definitive diagnosis for HIV infection confirmed by enzyme-linked immunosorbent assay (ELISA) and Western Blot tests[8]. Information on age, gender, HIV transmission, time of HIV diagnosis, delivery, laboratorial exams (viral load and CD4+ lymphocytes count), and use of antiretroviral therapy were obtained from the medical records. The laboratorial exams used were the ones closest to the time of data collection. The immunological classification was based on patients CD4 count, according to CDC criteria. We considered AIDS when patient have CD4 count <15% and patients were considered to be using HAART when the antiretroviral therapy involved the use of three or more drugs.

Oral samples

After obtaining a signed informed consent from the patients's parents or guardians, oral squamous cells were obtained by swab® (CB Products Ind. e Com LTDa) with a cotton-tipped applicator from tongue, cheeks, palate, floor of mouth, for buccal and cheek mucosas. The swabs were placed in Eppendorf tubes filled with 500µl of virus transport medium (composed of salt solution, plus gentamicin 500µg/ml and fungizone 20µg/ml) and kept at 4°C until required for DNA extraction. This technique was performed previously by Otero et al.[9].

Laboratorial analysis

Viral nucleic acid was extracted from 200µl of the sample using Wizard® Genomic DNA Purification KIT (Promega, Madison, WI, USA). Briefly, in a 1.5ml Eppendorf tube, 200µl of nuclei lysis solution and 2µl of ribonuclease (RNase) solution were added to 200µl of the clinical sample. The sample was mixed by inverting the tube 3-5 times, incubated at 37ºC for 30min, and then cooled to room temperature for 5min. Protein precipitation solution (130µl) was added, and the tube was vortexed vigorously for 10-20s and placed on ice for 5min. The tube was centrifuged at 13,000xg for 4min at room temperature and the supernatant was transferred to a new tube containing 600µl of isopropanol. The sample was mixed by inversion and incubated at room temperature for 30min; centrifuged at 13,000xg for 1min and the supernatant was discarded; 600µl of 70% ethanol was added and the tube was gently inverted to wash the pellet and centrifugation was performed at 13,000xg for 1min. The ethanol was aspirated; the tube was inverted onto a clean absorbent paper and the pellet was air-dried for 10-15min. Finally, 60µl of DNA rehydration solution was added and the DNA was rehydrated by incubating the solution at 65ºC for 1h. The DNA was stored at 40ºC until required.

The human viruses targeted in this study were the following: HSV-1/2, VZV, EBV, HCMV, HHV-6, HHV-7 and HHV-8. A multiplex nested-polymerase chain reaction (PCR) approach was used to simultaneously detect HSV-1/2, VZV, EBV and HCMV according to Tafreshi et al.[10]. Nested PCR assays were used for detection of HHV-6[11], HHV-7[12], and HHV-8[13]. Aliquots of 5µl of extracted DNA were used as templates in each individual PCR reaction for virus detection. All PCR reactions and cycling parameters for virus detection are summarized in a previous study[9].

To confirm the specificity of the PCR products and to differentiate between viral types presenting the same PCR product size, the amplified DNAs of positive samples were purified using the Wizard SV gel and PCR Clean-Up system kit (Promega), and the sequences determined using the BigDye® Terminator Cycle Sequencing Kit and the ABI PRISM® 3100 automated DNA sequence (Applied Biosystems, Foster City, CA, USA). DNA sequences were edited using the software Chromas (Technelysium Pty Ltd, Australia) and compared to the DNA sequences available in the GenBank (www.ncbi.nlm.nih.gov) using the BLAST tool.

Statistical analysis

A descriptive analysis was performed. Also, an univariate analysis were performed estimating the crude odds ratios (OR) with their respective 95% confidence intervals (CI) to verify the associations between the presence of herpesviruses and, immunosuppression, AIDS, viral load and use of HAART ($p<0.05$). For means comparisons the Mann-Whitney test was used.

Ethical considerations

The protocols were approved by the local ethical committee (21/07 - CEP/IPPMG).

RESULTS

The mean age of the children was 8.66 (SD 3.12) and 66% were female (33/50). The mean of CD4+ lymphocytes count was 40.43% (SD 92.04) and according to CDC criteria the absence of immune suppression was observed in 64% (32/50) and AIDS was observed in only 10% (5/50); also, 72% (36/50) were under HAART. Other personal and medical data is show in **Table 1**.

The HSV DNA was detected in 86% (43/50) of the children/adolescents. Thirty children (69.7%) were infected with only one type of herpes, twelve (27.9%) with 2 types and one (2.3%) with 3. The distribution according to the types of HSV can be observed in **Table 1**.

No difference was found in patients with or without HSV co-infection according to gender or age. Also, in the HSV co-infected patients (n=43), the absence of immunosuppression was confirmed in 67.4%, AIDS in 9.3% and 72.1% of them were under HARRT. Any association was observed between HSV co-infection and presence of immunosuppression, AIDS, viral load and use of HAART **(Table 2)**.

TABLE 1 - Personal and medical data from HIV-Infected children (n=50).

Personal data	HIV infected children n	%
Gender	17	34.0
male	33	66.0
female		
Age (years)	8.66 (±3.20)	
Age group		
3 to 10 years	34	68.0
> 10 years	16	32.0
Medical data	**HIV infected children**	
	n	**%**
HIV transmission		
vertical	50	100.0
Immunological classification*		
1	32	64.0
2	13	26.0
3	5	10.0
AIDS		
yes	5	10.0
no	45	90.0
Viral load		
detectable	30	60.0
not detectable	20	40.0
HAART		
yes	36	72.0
no	14	28.0
HSV co-infection	**HIV infected children**	
	n	**%**
Presence of HSV co-infection		
yes	43	86.0
no	7	14.0
Type of HSV		
HSV-1	2	4.0
HSV-2	1	2.0
EBV	no detected	
HCMV	12	24.0
VZV	2	4.0
HHV-6	9	18.0
HHV-7	34	68.0
HHV-8	no detected	

HIV: human immunodeficiency virus; **AIDS:** acquired immunodeficiency syndrome; **HAART**: highly active antiretroviral therapy; **HSV:** herpes simplex virus; **EBV:** Epstein-Barr virus; **HCMV**: Human cytomegalovirus; **VZV**: Varicella Zoster virus; **HHV**: Human herpesvirus. *1994 revised classification system for human Immunodeficiency Virus Infection in children less than 13 years of age (CDC): 1 - absence of Immunessupression (CD4%>25); 2 - moderate Immunessupression (CD4%=15-24); 3 - severe Immunessupression (CD4%<15).

TABLE 2 - Medical data from HSV/HIV co-infection.

	HSV/HIV co-infection (n=50)				
	yes (n=43)		no (n=7)		Odds ratio
Presence of:	n	%	n	%	(95%CI)
Immunessupression*					
yes	14	32.6	4	57.1	0.36 (0.07-1.84)
no	29	67.4	3	42.9	1.00
AIDS					
yes	4	9.3	1	14.3	0.61 (0.05-6.47)
no	39	90.7	6	85.7	1.00
Viral load					
detectable	26	60.5	4	57.1	0.87 (0.17-4.39)
not detectable	17	39.5	3	42.9	1.00
Use of HAART					
yes	31	72.1	5	71.4	1.03 (0.17-6.06)
no	12	27.9	2	28.6	1.00

HSV: herpes simplex virus; **HIV:** human immunodeficiency virus; **95%CI:** confidence intervals; **AIDS:** acquired immunodeficiency syndrome; **HAART**: highly active antiretroviral therapy. *Presence of immunosupression was considered when CD4 count was less than 24% (CDC).

DISCUSSION

The use of HAART reduces AIDS related oral manifestations. Treatment with HAART improves the immune function[14] and decreases mortality, morbidity, and opportunistic infections in HIV-infected persons[15]. In Brazil, an evaluation accuracy of HIV related oral lesions in predicting immune and virological failure in HIV-infected children treated with HAART demonstrated that oral lesions had moderate sensitivity, high specificity, and positive predictive value for forecasting immune failure[16]. Nevertheless, this evidence has been described in patients with HIV infection under HAART use, where these viruses were shedding in saliva, but not showing the clinical oral manifestations compatible with the infectious agents detected[17]. Grando et al. in a study conducted with HIV-infected children in Brazil and in the USA, also showed a high prevalence of co-infection HIV/HSV. Among 180 children, 116 (89.4%) were co-infected. Infection by CMV was found in 105 (58.3%) children, EBV infection in 177 (98.3%) children and HSV in 56 (31.1%). HIV-infected children with associated viruses had favorable CD4+ T lymphocyte counts and unfavorable viral loads[2]. Compared with our study we found less prevalence of coinfection by HSV, EBV and CMV and a higher prevalence of HHV-7 and HHV-6. The prevalence of HSV-1 infection increases progressively from childhood, the seroprevalence being inversely related to the patient's socioeconomic background[1]. Brazilian literature reports an HSV prevalence ranging from 1.3 to 24% in HIV-infected children, confirming our finds[3,18].

Leggot in 1992 reported a prevalence of 21% of VZV among Indian HIV infected children[19]. A retrospective cohort study determined the incidence of HZ in children infected with HIV perinatally, and assessed the impact of HAART and VZV immunization on primary varicella and HZ incidence and identified the risk of factors for HZ in a pediatric HIV clinic from 1989 to 2006. The incidence rates during three intervals, between 1989 and 1996, 1997 and 1999, and 2000 and 2006, were compared following the introduction of HAART (in 1996) and VZV vaccination (in 1999). In 356 patients followed for 1721 person-years, the incidence of HZ was 30.0 per 1000 person-years during the 1989-1996 period, 31.9 per 1,000 person-years during the 1997-1999 period, and 6.5 per 1,000 person-years during the 2000-2006 period. No difference was observed in the incidence-rate ratio calculated between the periods 1989-1996 and 1997-1999. However, a significant difference in HZ

incidence was observed between the periods 1989-1999 and 2000-2006. The incidence of primary VZV infection and HZ in the 57 patients who received the VZV vaccine was 22.3 per 1,000 and 4.5 per 1,000 person-years, respectively. During the time of primary VZV infection, HAART was protective against HZ and increased the HZ-free survival rates[20]. These data agree with those described by Tangsinmankong et al. in a study with 61 children infected with HIV perinatally, who were initiated with and maintained on HAART for longer than 6 months. The results of that study showed that seven episodes of IRD occurred, and that all episodes were cutaneous HZ. Children who developed HZ had significantly lower baseline CD4+ and CD8 T cell counts compared with children who were HZ-negative. HZ occurred in only seven of the 34 children who participated in this study, due to the virological and immunologic success of HAART. In children with a previous history of VZV infection, the risk of developing HZ after HAART was higher in those without a protective level of varicella-specific IgG (50%, or five of 10 subjects) compared to those with seroprotection (10%, or two of 20)[21].

EBV is the etiological factor of hairy leukoplakia (OHL), according to many authors. OHL has rarely been described in the HIV-positive pediatric population, with a prevalence varying from 0 to 22.5%. Our results were in accordance with the literature[20,22-27]. However, Grando et al. described the EBV prevalence as being 56.43% in Brazilian children and 27.1% in the US children[2]. In a study of Brazilian children, clinical and subclinical OHL was identified in 1.7% and 16.7% of the cohort, respectively. The total prevalence (clinical and subclinical) was 18.3% (22 cases)[28]. Note that in these studies in which a high prevalence of OHL was observed, laboratory steps were necessary to complement the different clinical study outcomes. A Brazilian report described the case study of a 12-year-old girl with undiagnosed HIV who presented a clinical OHL that was confirmed by a histopathologic examination. The laboratory data reported a viral load of 170,000 copies/mL and a CD4 count of 4%. This study demonstrated how OHL can be an early indicator of an undiagnosed HIV infection[28,29].

In this study HHV-7 and HHV-6 were found in 68% and 18% of the oral swab samples, respectively. This is the first study to report such a high occurrence of HHV-7 in the oral cavity of HIV infected children/adolescents. Nevertheless the association of HHV-6 and HHV-7 shows an increase of frequency in HIV-positive individuals detected in samples from chronic marginal periodontitis and in acute apical abscesses by endodontic origin from patients without imunossupression[17,30,31]. And recently, in saliva samples of children and adolescents with chronic renal failure[9]. Previously, these herpes viruses (HHV-6 and HHV-7) were associated with *roseola infantum* in early childhood, with fever and exanthematic diseases[32]. However more recently they have been related to the fact that during the course of HIV infection there has been an extensive reactivation of the latent state of these two herpesviruses[33].

AIDS-associated Kaposi Sarcoma (KS) has become prominent in several regions of the world, particularly in Africa. It is predominantly found in heterosexual adults and children in parts of Africa, but is most prevalent among HIV-1 positive homosexual men in the Western world. The majority of researchers who have studied co-infections of HIV and HHV-8 in children did not report findings of oral KS lesions[1].

The involvement of herpesviruses in the oral cavity of different groups of patients and the variation in these detection values could occur due to the viral diagnostic methods used, the clinical status of these patients and the geographical occurrence of these pathogens. And similar to what occurs in oral infections, other factors may also contribute to explain the frequencies described such as climatic conditions, sexual profile, psychological stress, immunosuppression and infectious diseases[34-39].

So this work could conclude that children infected with HIV have a high prevalence of infection caused of the *Herpesvirideae* family. But no association with gender, immune suppression, viral load and use of HARRT could be observed. The health professionals who take care of these patients should be aware of oral lesions because of their predictive value and herpes viruses can be found circulating in the oral cavity without causing lesions.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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