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Viral coinfection in the oral cavity of HIV-infected children: relation among HIV viral load, CD4+T lymphocyte count and detection of EBV, CMV and HSV

Co-infecção viral na cavidade bucal de crianças infectadas pelo HIV: relação entre carga viral, contagem de linfócitos T-CD4+ e detecção de EBV, CMV e HSV

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ABSTRACT: Viral coinfection in the oral cavity associated to HIV infection was evaluated in 180 children from birth to 13 years of age of both sexes. The oral examinations were performed at the Pediatric AIDS Outpatient Clinic, São Lucas Hospital and Clinic Hospital, both in Porto Alegre, Brazil and at the School of Dental Medicine, University Hospital Center, State University of New York at Stony Brook, USA. The aim of this study was to identify the presence of viral infections in the oral cavity. PCR technique was used to determine opportunistic viral infections caused by CMV, EBV, and HSV in mucosal swabs. A high frequency of viral infection was detected in the oral cavity of HIV-infected children determined by the PCR technique. HIV-infected children with viruses had a favorable CD4+T lymphocyte count and unfavorable viral load.

DESCRIPTORS: AIDS-related opportunistic infections; Herpes simplex virus; Cytomegalovirus; Epstein-Barr virus; Viral load; CD4-positive T-lymphocytes.

RESUMO: A relação entre a infecção pelo HIV e a presença de diferentes tipos de vírus na cavidade bucal foi estudada em 180 crianças HIV-positivo, com idades entre zero e 13 anos de idade, de ambos os sexos. Os exames foram realizados nos Ambulatórios de Aids Pediátrica dos Hospitais São Lucas e de Clínicas, ambos em Porto Alegre, RS, Brasil e no Centro Hospitalar Universitário da Universidade Estadual de Nova Iorque, em Stony Brook (EUA). O objetivo desta pesquisa foi usar a técnica da PCR para detectar a presença dos vírus CMV, EBV e HSV na cavidade bucal desses pacientes, independentemente da presença ou não de manifestações estomatológicas relacionadas aos mesmos. Pode-se concluir que foi alta a freqüência de vírus detectados na cavidade bucal das crianças da amostra através da técnica da PCR e que a contagem média de linfócitos T-CD4+ das crianças com a presença dos vírus encontrava-se próxima da normalidade, enquanto a Carga Viral do HIV encontrava-se elevada.

DESCRITORES: Infecções oportunistas relacionadas com o HIV; Vírus do herpes simplex; Citomegalovírus; Vírus Epstein-Barr; Carga viral; Linfócitos T CD4-positivos.

INTRODUCTION

Children and adults infected with the Human Immunodeficiency Virus (HIV) are prone to develop opportunistic viral infections in the oral mucosa mainly by the *Herpesviridae* family members such as the Cytomegalovirus (CMV), the Epstein-Barr Virus (EBV), and the Herpes Simplex Virus (HSV),

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all of which are important etiologic agents of morbidity[14,19,23]. Coinfections of HSV and CMV[19]; and CMV and EBV[23] have been reported in oral ulcers of HIV-infected patients. Despite the unknown pathogenesis of these coinfections[20], the detection of more than one virus in the oral mucosa of HIV-infected patients may have important clinical implications and, therefore, requires further investigation.

Many authors have investigated bacterial, fungal and viral infections in oral lesions of HIV-infected patients utilizing the Polymerase Chain Reaction (PCR) technique[6,13,22]. This technique offers several advantages over other methods. It requires a low quantity of biological material[16] and can detect the viral presence on "early" infections[24]. PCR detection of HSV, EBV, and CMV is highly sensitive and specific, which can aid in the prevention of clinical manifestations of virus-associated oral lesions through the selection of the appropriate therapy.

Many tests are used to evaluate the status of the immune system of HIV-infected patients, specially the CD4+T lymphocyte count and the HIV viral load[5]. The CD4+T lymphocyte count provides an estimation of the immune system status of the HIV-infected individual, and reflects the previous history of the disease[20]. The CD4+ T-lymphocyte count also indicates the necessity for prophylaxis for opportunist infections and helps to evaluate initial antiretroviral therapy or treatment failure[5,8,20]. Children without evidence of immunodeficiency have CD4+T lymphocyte count around 25% and many authors have related the oral lesions in HIV-infected patients to low CD4+T lymphocyte count[10,15,17].

In addition to the immune system abnormalities, HIV-infected Brazilian children can be affected by the lack of appropriate caregiver supervision[4]. The low education level of caregivers of the HIV-infected Brazilian children can be related to the poor compliance with the antiretroviral treatment, thus affecting the child's health[4,5].

The aim of this research was to detect the presence of some viruses (EBV, CMV and HSV) in the oral cavity of HIV+ children by the PCR technique and study the relation among these virus types with the HIV viral load and CD4+T lymphocyte count.

METHODS

This research was approved by the hospitals' Research Ethics Committees.

Subjects

HIV-infected children between zero and 13 years of age were investigated. This study included 180 subjects: 143 Brazilians (Pediatric AIDS Outpatient Clinic, São Lucas Hospital, Pontifical Catholic University of Rio Grande do Sul, and Clinic Hospital, Federal University of Rio Grande do Sul – both in Porto Alegre, Brazil); and 37 Americans (Pediatric Infectious Disease Clinic, University Hospital Center, School of Dental Medicine, State University of New York at Stony Brook, NY, USA). All patients had CD4+T lymphocyte count (expressed in % value/cytometric technique) and HIV viral load (expressed in $\log_{10}$ value/NASBA method) determined.

Oral samples

Oral swabs were collected after obtaining a signed informed consent from the children's parents or guardians. Oral swabs (saliva and epithelium cells) were collected with sterile cotton tipped applicators[14] (Citmed, Citronelle, AL, USA) from the buccal mucosa bilaterally, regardless of the presence of oral lesions. Specimens were placed in test tubes (Eppendorf AG, Hamburg, Germany) containing 500 µl of sterile phosphate buffered saline (PBS – Merck KGaA, Darmstadt, Germany). DNA was extracted by the organic method (phenol: chloroform:IAA – Invitrogen Corporation, Carlsbad, CA, USA)[22].

PCR assay

DNA extracted from the buccal swabs was used to amplify HSV (type 1 and type 2)[18], CMV[12], and EBV[2] by two-step (semi-nested and nested) PCR amplifications. All specimens were tested for the presence of the β-globin gene to ensure the presence of DNA. All PCR reactions had positive (stored DNA obtained from a patient with HSV, CMV or EBV disease) and negative controls (stored DNA obtained from a patient without HSV, CMV or EBV disease) and a reaction mix. Some samples were lost during the laboratory process. The primer sequences used in the PCR reactions are shown in Table 1.

CMV was amplified with CMV-1/CMV-2 in the first and CMV-1/CMV-4 in the second round. EBV was amplified with EBV-1/EBV-2 in the first round and EBV-3/EBV-4 in the second. HSV was amplified with primers HSV-1/HSV-2 in the first round and HSV-3/HSV-4 in the second round. The final products included a 220 bp fragment of the Major

Immediate Early Antigen (MIE) gene from CMV; a 209 bp product of the EBNA-1 gene from EBV and a 142 bp fragment of the D gene of HSV.

The PCR reaction mix contained 0.4 µM of the appropriate primer (Invitrogen Corporation, Carlsbad, CA, USA), 1 X PCR Buffer (Invitrogen Corporation, Carlsbad, CA, USA), 200 µM of each dNTP (Invitrogen Corporation, Carlsbad, CA, USA), 1.25 units of Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA, USA) and 1.5 mM (HSV), 2.0 mM (CMV) or 2.5 mM (EBV) of $MgCl_2$ (Invitrogen Corporation, Carlsbad, CA, USA) in a final volume of 50 µl. Viral DNA, human DNA and reaction controls were included in each run. DNA amplification was performed in an automated thermal cycler (MJ Research, Waltham, MA, USA). Reactions were brought to 95°C for 10 min, followed by thirty cycles consisting of a denaturing step for 30 s at 94°C, annealing step for 30 s at 50°C (CMV), 60°C (EBV), 50°C (HSV first round) or 60°C (HSV second round), and an extension step for 30 s at 72°C. A final extension step at 72°C was carried out for 5 min. A total of 5 µl of the first round product was used in the second round of amplifications. Aliquots of 15 µl of the PCR product were analyzed on 2% agarose gel (Merck KGaA, Darmstadt, Germany) containing 0.5 g/ml of ethidium bromide (Merck KGaA, Darmstadt, Germany) and visualized under ultraviolet light (Bio-Rad Laboratories Inc., Hercules, CA, USA). The PCR sensitivities were the same in children from Brazil and from the USA: 0.5 pg for CMV; 50 ng for EBV; 5×10^{-5} pg for HSV.

TABLE 1 - Primer sequences* used in the semi-nested and nested PCR technique.

Virus type	Primer sequences (5' - 3')	
CMV Gene: MIE	CMV-1	5' CAT AAT CTC ATC AGG GGA GC 3'
	CMV-2	5' TTG GGC TAA CTA TGC AGA GC 3'
	CMV-4	5' AGC TGC ATG ATG TGA GCA AG 3'
EBV Gene: EBNA-1	EBV-1	5' ATC GTG GTC AAG GAG GTT CC 3'
	EBV-2	5' ACT CAA TGG TGT AAG ACG AC 3'
	EBV-3	5' AAG GAG GGT GGT TTG GAA AG 3'
	EBV-4	5' AGA CAA TGC ACT CCC TTA GC 3'
HSV Gene: D	HSV-1	5' TGC TCC TAC AAC AAG TC 3'
	HSV-2	5' CGG TGC TCC AGG ATA AA 3'
	HSV-3	5' ATC CGA ACG CAG CCC CGC TG 3'
	HSV-4	5' TCT CCG TCC AGT CGT TTA TCT TC 3'

*The primer sequences were obtained from Krajden *et al.*[12] (1996) (CMV); Cinque *et al.*[2] (1993) (EBV) and Powell *et al.*[18] (1990) (HSV). CMV: Cytomegalovirus; EBV: Epstein-Barr Virus; HSV: Herpes Simplex Virus.

RESULTS

It was possible to identify HSV in 116 children (79 Brazilians/37 Americans), CMV in 105 children (68 Brazilians/37 Americans) and EBV infection in 177 children (140 Brazilians/37 Americans) by PCR.

Viral detection by PCR

HSV, CMV and EBV results obtained analyzing the Brazilian and American children are presented in Table 2. According to the Chi-squared test, there were no differences in CMV infection between Brazilian and American children ($p = 0.110$); there were more Brazilian children infected with EBV and HSV than American children ($p = 0.001$).

Figures 1, 2, and 3 show the reaction products of the nested PCR for CMV, EBV and HSV after electrophoresis (Bio-Rad Laboratories Inc.,

TABLE 2 - Frequency (f) and percentage (%) of Brazilian and American children as detected by PCR.

Virus type	Children with different virus types		
	Brazil f/n (%)	USA f/n (%)	Total f/n (%)
CMV	13/68 (19.12%)	3/37 (8.11%)	16/105 (15.24%)
EBV	79/140 (56.43%)	10/37 (27.07%)	89/177 (50.28%)
HSV	50/79 (63.29%)	6/37 (16.22%)	56/116 (48.28%)
Total	142/143 (99.30%)	19/37 (51.35%)	161/180 (89.44%)

CMV: Cytomegalovirus; EBV: Epstein-Barr Virus; HSV: Herpes Simplex Virus.

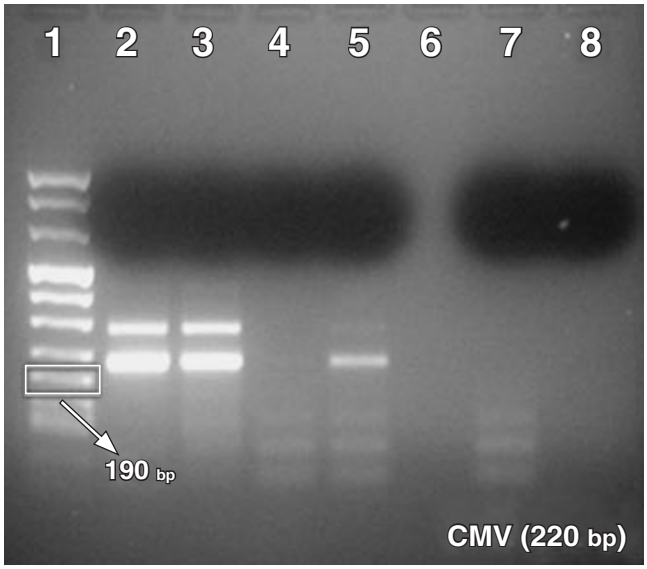

FIGURE 1 - Amplicons of nested PCR for CMV (Cytomegalovirus). Column 1: molecular weight marker VIII. Column 2: positive control. Columns 3 and 5: positive samples. Column 4: negative sample. Column 6: empty column. Column 7: negative control. Column 8: PCR reaction mix without DNA.

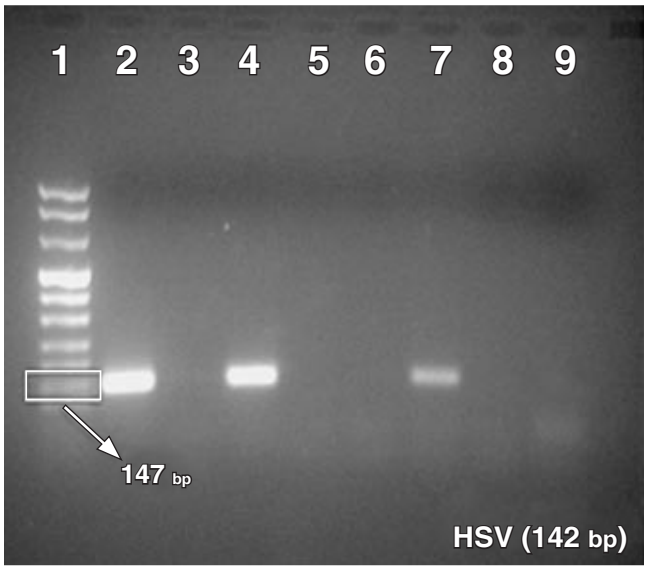

FIGURE 3 - Amplicons of nested PCR for HSV (Herpes Simplex Virus). Column 1: molecular weight marker VIII. Column 2: positive control. Columns 4 and 7: positive samples. Columns 3, 5, 6 and 8: negative samples. Column 9: PCR reaction mix without DNA.

Hercules, CA, USA) in 2% TAE-agarose gel (Merck KGaA, Darmstadt, Germany) containing 0.5 g/ml of ethidium bromide and visualized under ultraviolet light. Table 3 shows the results of the Sensitivity Test for CMV, EBV and HSV.

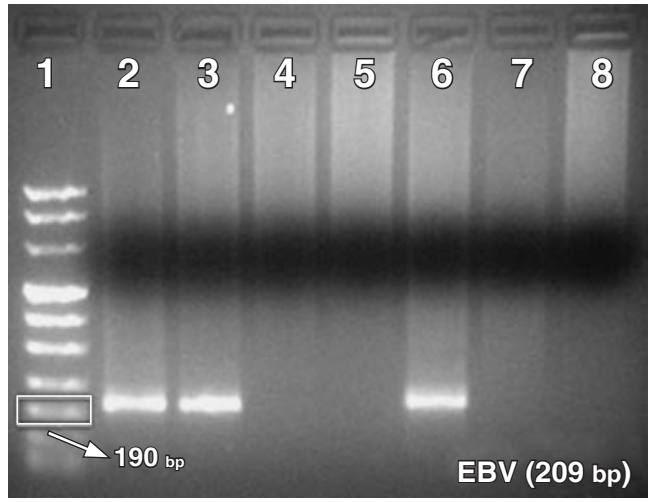

FIGURE 2 - Amplicons of nested PCR for EBV (Epstein-Barr Virus). Column 1: molecular weight marker VIII. Column 2: positive control. Columns 3 and 6: positive samples. Columns 4, 5 and 7: negative sample. Column 8: negative control.

Figure 4 shows an example of PCR sensitivity (CMV) test. Although there is a difference in the sample size between Brazilian and American patients, when comparing the data obtained from the two countries, no statistical differences were observed between the number of Brazilian and American children with viral infections in the oral cavity (Chi-squared test, p = 0.659).

Relationship between viral infection in the oral cavity; CD4+T lymphocyte count and HIV viral load

The average ± standard deviation of CD4+T lymphocyte count (%) and HIV viral load (log_{10}) were determined to evaluate a possible relationship between viral infection in the oral cavity and the general health status of the patient. Table 4 shows that the mean CD4+T lymphocyte count for all patients infected with viruses (HSV, CMV and/or EBV) were higher than 25%, thus suggesting no evidence of immunossuppression[20]. There was no statistical difference between mean CD4+T lymphocyte count of patients with different virus types in their oral cavity as shown by the Variance Analysis (ANOVA) test.

The mean HIV viral load of all patients infected with viruses (HSV, CMV and/or EBV) was higher than 1,000 copies of HIV/ml (Table 5). There were no statistical differences among the mean viral load of patients with different viruses in the oral cavity according to the Variance Analysis (ANOVA).

TABLE 3 - Results of the Sensitivity test for CMV, EBV and HSV.

Virus type	Sensitivity Test	
	Brazil	USA
CMV	0,5 pg	0,5 pg
EBV	50 ng	50 ng
HSV	5×10^{-5} pg	5×10^{-5} pg

CMV: Cytomegalovirus; EBV: Epstein-Barr Virus; HSV: Herpes Simplex Virus.

Table 6 shows the frequency of HSV, CMV and EBV in the oral cavity of our study cohort. It was observed that the number of Brazilian children (29.37%) without viruses was similar to the number of American children (45.95%), according to the Chi-squared test (p = 0.372). Sixty-three Brazilian children (44.06%) and 17 American children (45.95%) had at least one virus type present. Seven American (18.92%) and 35 Brazilian children (24.48%) had two different virus types at the same time. Only 3 Brazilian children (2.10%) had HSV, CMV and EBV oral coinfection.

DISCUSSION

Since CMV viruses are endemic in children populations from developing countries[1,24], a higher number of CMV infections was expected in Brazil. The development of the antiviral therapy could be responsible for the control of CMV infection in HIV-infected individuals[9]. Since 1984, CMV-related ulcers in HIV-infected children had been reported[1,6,9]. The pathology of CMV in oral ulcerations remains unknown[19].

EBV is the etiologic agent of an illness that can affect children and young adults, called Infectious Mononucleosis[3,24]. Approximately 90% of the global population has acquired EBV in a non-symptomatic way[24] and the virus remains latent in the lymph nodes and pharynx cells. In immunodeficient patients, Infectious Mononucleosis could be severe and the EBV recurrence can cause lymphoma[21], Kaposi's Sarcoma[24] and hairy leukoplakia[7,8].

HSV infection is considered the most frequent viral infection in HIV-infected patients. The early HSV infection and its high frequency in HIV-infected children correlates with the rapid evolution of the disease, suggesting a worsening prognosis for the patient. In immunodeficient patients, as well as in HIV-infected patients, HSV infection can be severe, clinically atypical, more painful and with a long-term duration[11].

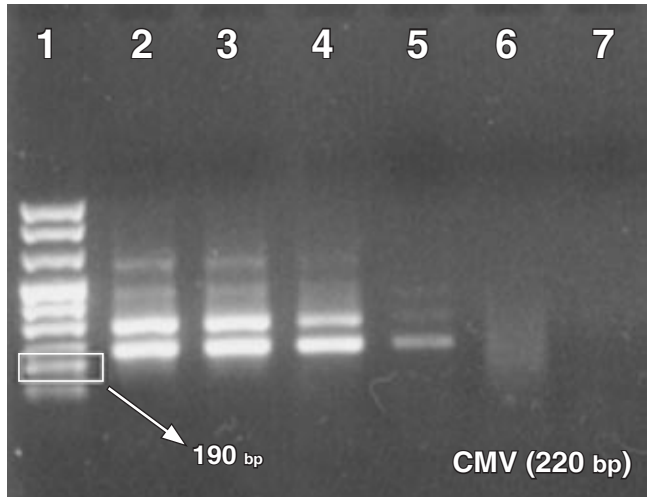

FIGURE 4 - PCR sensitivity for CMV (Cytomegalovirus). Column 1: molecular weight marker VIII. Column 2: 10 ng. Column 3: 5 ng. Column 4: 50 pg. Column 5: 0.5 pg. Column 6: 0.05 pg. Column 7: PCR reaction mix without DNA.

Twenty-five percent of CD4+T lymphocyte count means no evidence of immunosuppression. One thousand copies of HIV/ml ($\log_{10}$ = 3.0) is considered a significant viral load[20]. The absence of clinical manifestations in the HIV-infected children of this study was possibly due to the presence of their high CD4+T lymphocyte count. The children who had viruses detected in their oral cavities had more than 1,585 copies of HIV/ml ($\log_{10}$ = 3.2), which is considered a high viral load[20].

Before the AIDS era, *Herpesviridae* coinfection in human tissue was rare. Presently, this is not the case. Coinfection with HSV and CMV has been described in many anatomic sites, such as the nervous system, skin, esophageal area, and lips. CMV infection often presents severe, painful and long-term duration oral ulcerations[19]. Viral and fungal coinfection has been also observed, in addition to viral coinfection with CMV and EBV in oral ulcers of HIV-infected patients[23].

CONCLUSION

A high frequency of viral infection was detected in the oral cavity of HIV-infected children determined by the PCR technique. EBV was the virus most commonly found, followed by HSV and CMV. HIV-infected children with viruses had a favorable CD4+T lymphocyte count and unfavorable viral

TABLE 4 - Relationship between virus type and CD4+T lymphocyte counts (in % unit) of Brazilian and American children (average ± standard deviation).

Virus type	Frequency (f)/ sample (n) of positive results	CD4+T lymphocyte counts (%) Average ± standard deviation
CMV	16/105 (15.24%)	30.65 ± 12.74
EBV	89/177 (50.28%)	24.76 ± 11.61
HSV	56/116 (48.28%)	26.11 ± 12.87

CMV: Cytomegalovirus; EBV: Epstein-Barr Virus; HSV: Herpes Simplex Virus.

TABLE 5 - Relationship between virus type and viral load (in $\log_{10}$ unit) of Brazilian and American children (average ± standard deviation).

Virus type	Frequency (f)/ sample (n) of positive results	Viral Load ($\log_{10}$) average ± standard deviation
CMV	16/105 (15.24%)	4.21 ± 2.30
EBV	89/177 (50.28%)	4.24 ± 0.90
HSV	56/116 (48.28%)	3.96 ± 0.81

CMV: Cytomegalovirus; EBV: Epstein-Barr Virus; HSV: Herpes Simplex Virus.

TABLE 6 - Frequency (f) and percentage (%) of Brazilian and American children with virus coinfection.

Viral coinfection present in the oral cavity of the same HIV-infected children*		Country		
		Brazil (n = 143) f/n (%)	USA (n = 37) f/n (%)	Total (n = 180) f/n (%)
0 virus		42/143 (29.37%)	17/37 (45.95%)	59/180 (32.78%)
1 or more virus types	1 virus type	63/143 (44.06%)	17/37 (45.95%)	80/180 (44.44%)
	2 virus types	35/143 (24.48%)	7/37 (18.92%)	42/180 (23.33%)
	3 virus types	3/143 (2.10%)	-	3/180 (1.67%)

*Chi-squared test (p = 0.372).

load. Multiple viral coinfections were also observed in the oral cavity of our cohort. Early viral infection was detected in the oral cavity of the patients, despite the absence of clinical manifestations.

Considering the complexity of the viral infection therapy in HIV-infected patients, it is very important to identify the opportunistic agents present in the oral cavity as soon as possible. The clinician must consider the caregiver concerns, medication history, clinical and laboratory findings in order to provide appropriate care for these children.

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