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ORIGINAL ARTICLE

Salivary shedding of Epstein–Barr virus and cytomegalovirus in people infected or not by human immunodeficiency virus 1

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Abstract The purpose of this study is to determine the frequency of EBV and CMV DNA detection in saliva of HIV infected and non-HIV individuals and their siblings. The study group comprised 240 individuals. Group 1 comprised of 40 HIV-infected patients, group 2 40 non-HIV individuals, group 3 two siblings for each patient from group 1 (n=80), and group 4 two siblings for each individual from group 2 (n=80). Non-stimulated whole saliva was collected, DNA was extracted, and amplification was performed using a nested PCR protocol. EBV and CMV DNA was detected in 7/40 (17.5%) and 5/40 (12.5%) individuals from group 1, 8/40 (20%) and 3/40 (7.5%) from group 2, 11/80 (13.8%) and 2/80 (2.5%) from group 3, and 8/80 (10%) and 1/80 (1.3%) from group 4, respectively. Five (71.4%) out of seven HIV/EBV coinfected individuals of group 1 had a relative also infected with EBV (OR=11.25, CI [1.75–72.5], p=0.011). Regarding group 2, among the eight non-HIV and EBV-infected individuals, three (37.5%) had a relative also positive to EBV (p=0.320). No individual HIV/CMV coinfected had a relative CMV infected (p=1.00). Also, only one non-HIV and CMV-infected individual had a relative also positive to CMV (p=0.075). EBV and CMV DNA was detected mainly in those who had HIV viral load counts <400/mL (71%, p=0.2 and 100%, p=1, respectively) and those who had CD4 T cells counts between 200 and 400/mm^3 (57%, p=0.544 and 60%, p=0.249, respectively). HIV-infected individuals and healthy controls showed a similar frequency of viral DNA detection. EBV DNA was significantly amplified in saliva of household members of HIV/EBV coinfected individuals.

Keywords EBV · Cytomegalovirus · HIV · Saliva · Transmission

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Introduction

Herpesviruses are ubiquitous in the human population [1]. EBV and CMV usually cause no symptoms in healthy adults and children [2–4], but in immunologically immature and immunocompromised host, they may result in severe opportunistic infections with high morbidity and mortality [2–8]. Those herpes virus can be detected in blood and body secretions including saliva, maternal milk, semen, and vaginal secretions [9–12].

Saliva is a common vehicle of transmission of oral herpesviruses [13, 14]. Some lines of evidence point to an EBV transmission via saliva, by salivary residues left on cups, food, toys, or other objects and when mothers pre-chew food that is then given to their babies [15–18]. CMV may also be transmitted through saliva [9, 10, 12, 19, 20]. The detection rate of EBV DNA by PCR in healthy people

can be very high, in African and American adults [11, 21] and 90% of throat washings in Japanese adults [22]. In one African study, the PCR detection rates of CMV and EBV DNA were, respectively, 78% and 20% in blood donors and 60% and 87.2% among patients infected by human immunodeficiency virus 1 (HIV-1) [23]. A study conducted among Italian individuals showed that the detection rate of EBV DNA was higher in renal transplant patients (65%) than in HIV-infected patients (42%) and healthy controls (17%) [24]. These authors also observed that the CMV DNA detections were considerably lower (4% among HIV-infected patients and none among renal transplant patients). Among Japanese transplant recipients, EBV DNA detection rates were in 24% and 6% samples of blood and plasma, respectively, and CMV in 11% and 5%, respectively [25]. These findings support the hypothesis that EBV and CMV prevalence can vary among patients originating from different geographical areas.

Although many molecular and serological tests studies have been conducted to study the epidemiology of EBV and CMV, the results are still contradictory. Moreover, the possible routes of transmission have also not been well established. Hence, the aim of the present work was to determine the frequency of EBV and CMV detection in saliva of HIV infected and their siblings.

Materials and methods

Subjects

The study population comprised 240 individuals, divided into four groups. Group 1 comprised 40 HIV-infected outpatients of the Infectious and Parasitic Diseases Service of Hospital das Clínicas, Universidade Federal de Pernambuco (UFPE), Recife, Brazil. Group 2 comprised 40 healthy volunteers recruited from dentistry clinics at the UFPE. Group 3 included two siblings of patients from group 1 (n=80). Group 4 comprised of two siblings from group 2 (n=80). Local ethical approval was given to the study, and informed consent was obtained from all the participants.

Demographic informations including gender and age were collected. HIV viral load and CD4 T cells counts were observed in the medical records of the HIV-infected patients. All patients had unstimulated whole saliva collected by the method described by Navazesh et al. (1993) and stored at −20°C for later DNA extraction [26].

Laboratory methods

DNA was extracted from saliva by Geneclean® II (BIO 101, La Jolla, CA, USA). Extracts underwent PCR to amplify a segment of β-globin DNA using primers GH20 (5'-CAACTTCATCCACGTTCACC-3') and PC04 (5′-GAAGAGCCAAGGACAGGTAC-3′) [27]. EBV and CMV DNA amplification was performed by applying a nested PCR protocol using primers HHV-F1, HHV-R1, HHV-F2, and HHV-R2 within the highly conserved regions shared between CMV and EBV as previously described [28]. Primer sequences used are shown in Table 1.

First-round PCR reactions consisting of 3 μL of extracted DNA was added to 27 μL PCR mix containing: 0.5 μM of each primers HHV-F1 or HHV-R1, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 2.5 U Taq DNA polymerase (Invitrogen® Brazil). PCR was carried out as follows: 94°C for 3 min (predenaturation), 35 cycles each cycle consisting of 94°C for 45 s, 65.5°C for 1 min, 72°Cfor 1 min, with a final extension at 72°C for 7 min. For nested PCR, 0.5 μL of first-round product was transferred to 29.5 μL of an identical PCR mix but containing second-round primers with the same concentration as the first round. PCR conditions were the same as for first-round PCR. Positive and negative controls were included in each run. PCR amplicons were then electrophoresed through an agarose gel and visualized through a transilluminator. Precautions against PCR contamination were taken, such as: DNA extraction and preparation of master mix performed in different places, use of separate pipettes and filter tips, frequent change of gloves, and use of sodium hypochlorate solution to decontaminate surfaces.

The consensus primers used were designed to amplify a highly conserved region common to HSV-1, HSV-2, CMV, and EBV. CMV amplicons could be identified by their distinct molecular weight (corresponding to their length, 565 bp). It was not possible to identify amplicons originating from HSV-1, HSV-2, and EBV because their similar molecular weights, corresponding to lengths 493 and 499 bp, respectively. Nested PCR products were therefore digested with TaqI restriction endonuclease (Invitrogen® Brazil). Fragments sizes obtained were: 88, 93, and 312 bp for HSV-1; 88, 99, 144, and 167 bp for HSV-2; 21, 229, and 249 bp for EBV [28] and the products visualized after agarose gel electrophoresis.

Data analysis

Absolute and percentage distributions and statistics measures were obtained. Pearson chi-square or Fisher exact tests were used when it was not possible to use chi-square. Standard deviation (SD), odds ratio (OR), and 95% confidence intervals (CI) were verified by means of inferential statistics. The significance level used in statistical tests was 5% (0.05). Statistical calculations were performed using SPSS version 13.0 software.

Table 1 Primers sequences used for amplifying CMV and EBV DNA (Tafreshi et al. 2005) [28]

Primers	Primer function	Primers sequences (5′ to 3′)	Size of amplications (bp)
HHV-F1	First round—PCR (outer sense)	GTCGTGTTTGACTTTGCCAGC	748 (EBV) 817 (CMV)
HHV-R1	First round—PCR (outer antisense)	GTCTTGCGCACCAGATCCAC	
HHV-F2	Second round—PCR (inner sense)	GCATCATCCTGGCTCACAACC	499 (EBV) 565 (CMV)
HHV-R2	Second round—PCR (inner antisense)	GTCCGTGTCCCCGTAGATG	

Results

Information regarding gender and age are shown in Table 2. The mean ages (in years) in each group were: group 1, 38.2 (SD=10.7); group 2, 28.8 (SD=12.1); group 3, 31.8 (SD=16.7); and group 4, 37.6 (SD=17.8). The majority of siblings in group 3 were sons (23/80 [28.8%]), and in group 4, the majority were brothers (25/80 [31.3%], Table 3).

Most of HIV-infected patients were under adequate clinical control as assessed by HIV viral load and CD4 T cells counts. Thirty-five (88%) of the HIV-infected individuals had viral loads lower than 400 copies/mL. Eighteen (45%) of the patients had CD4 T cell counts equal to or greater than 500/mm^3, 19 (48%) between 200 and 400/mm^3, and 3 (7.5%) lower than 200/mm^3. Oral lesions were observed only in group 1, four (10%) with oral ulcerations and three (8%) with pseudomembrane candidiasis.

EBV DNA was detected in seven (17.5%) of group 1 and eight (20%) in group 2 ($p>0.05$). In group 1, EBV DNA was detected mainly in those who had viral load counts <400/mL (5/7 [71%]), and those who had CD4 T cells counts between 200 and 400/mm^3 (4/7 [57%]). EBV DNA was also amplified from 11 people in group 3 (13.8%) and 8 (10%) in group 4. Among the seven group 1 patients with EBV DNA detected in saliva, five (71%) had a sibling in whom EBV DNA was also amplified (OR=11.25, CI [1.75–72.5], $p=0.011$). Among the eight group 2 patients with EBV DNA detected in saliva, three (38%) had a sibling member in whom EBV DNA was also amplified ($p=0.320$, Table 4).

CMV DNA was detected in five (12.5%) of group 1 and three (7.5%) in group 2. In group 1, CMV DNA was detected only in those who had viral load counts <400/mL [5/5 [100%], $p=1$) and those who had CD4 T cells counts between 200 and 400/mm^3 (3/5 [60%]). CMV DNA was also amplified from two people in group 3 (2.5%) and one (1.3%) in group 4. Among the five group 1 patients with CMV DNA detected in saliva, none had a sibling in whom CMV DNA was also amplified. Among the three group 2 patients with CMV DNA detected in saliva, one (33.3%) had a sibling in whom CMV DNA was also amplified (Table 5).

Table 2 Information about gender and age of groups 1, 2, 3, and 4

Variables	HIV	Non-HIV	Siblings of HIV	Siblings of non-HIV
	n (%)	*n* (%)	*n* (%)	*n* (%)
Age				
18–38	21 (52.5)	33 (82.5)	56 (70)	42 (52.5)
39–59	18 (45)	6 (15)	15 (18.8)	29 (36.3)
>59	1 (2.5)	1 (2.5)	9 (11.3)	9 (11.3)
Total	40	40	80	80
Gender				
Male	29 (72)	6 (15)	32 (40)	31 (38.8)
Female	11 (27.5)	34 (85)	48 (60)	49 (61.3)
Total	40	40	80	80

Quantitative analysis

Discussion

Epidemiological evidence and laboratory data show that EBV and CMV transmission occurs both horizontally and vertically [9, 10, 12–18, 20]. Several lines of evidence show that this herpes virus could be transmitted through close contacts via saliva [11, 13–18, 21]. Nonsexual transmission mainly through close interpersonal contact of

Table 3 Type of relative relationship

Relative relatioship	Relatives of HIV infected		Relatives of non-HIV	
	N	%	*N*	%
Father	3	3.8	9	11.3
Mother	12	15	21	26.3
Son	23	28.8	4	5
Daughter	15	18.8	8	10
Brother	22	27.5	25	31.3
Cousin	1	1.3	2	2.5
Brother-in-law	–	–	2	2.5
Friend	–	–	4	5
Grandmother	–	–	1	1.3
Uncle	–	–	3	3.8
Nephew	4	5	1	1.3
Total	80	100.0	80	100.0

Qualitative analysis

Table 4 EBV DNA occurrence in HIV-infected and non-HIV-infected individuals and their relatives

EBV							
Group	EBV in relatives	Positive		Negative		Total		*p* value
		n	%	*n*	%	*n*	%	
HIV infected	Yes	5	71.4	6	18.2	11	27.5	$p=0.011^{a,b}$
	No	2	28.6	27	81.8	29	72.5	
	Total	7	100.0	33	100.0	40	100.0	
Non-HIV	Yes	3	37.5	5	15.6	8	20.0	$p=0.320^{a}$
	No	5	62.5	27	84.4	32	80.0	
	Total	8	100.0	32	100.0	40	100.0	

[a] Significant difference at 5.0% level

[b] Using Fisher's exact test

non-intact skin or mucous membranes with saliva may be the primary mode of transmission [13–15, 17, 18, 23].

The results of this study show that the rates of detection of EBV and CMV DNA were similar in HIV-infected individuals and non-HIV-infected individuals and their siblings. CMV DNA was found in saliva of non-HIV individuals and their siblings; however, this association was not statistically significant and differs from most studies found in the literature, which suggest that this virus can be transmitted through saliva [12, 13, 19, 29]. On the other hand, an evaluation of African–American children revealed that CMV maternal infection is not associated with their children's viral status [12].

The prevalence of these herpesvirus in the present study is in accordance with previous studies that shown infection rates ranging from 22% to 90% for EBV and from 1.2% to 31% for CMV [11, 13, 21–25, 30–38]. In United States, EBV and CMV were detected in 90% and 31% HIV-infected patients compared with 48% and 2% non-HIV individuals, respectively [35]. Other study conducted in the same country detected CMV DNA in saliva of only 1.5% of the sample [21]. Yet, 90% of healthy Japanese adults and 38% healthy children presented EBV DNA in saliva [22]. Another study in Japan showed that EBV DNA was detected in 23% of EBV seropositive healthy adults [30]. Only one study evaluated the prevalence of EBV and CMV in Brazil and identified EBV in 77% of patients and CMV in 6% [37] of the population studied.

Determining EBV and CMV prevalence may be difficult once the technique used, PCR, is prone to false positive results. Serology may sometimes be preferred rather than PCR [27]. However, the use of PCR in the present work is justifiable due to sensitivity of the test and the ability to amplify small amounts of the target sequence as showed in previous studies [29, 32, 39–46]. In our study, precautions against PCR contamination were taken. Nuclease-free water was used as a negative control and the extraction of DNA and preparation of master mix were performed in different places, using separate pipettes and filter tips. Furthermore, precautions such as frequent change of gloves and sodium hypochlorate solution to decontaminate surfaces were used to prevent contamination.

The wide range of detection of EBV and CMV may be attributed to differences in the studied populations [22, 47]. A feature of herpesvirus is variation in incidence in different geographic regions. Variation in human host genetic factors, environmental factors, or viral factors can explain this geographic variation. Chang et al. [48] affirm that EBV is associated with different malignancies in different geographic regions but this fact remains unclear and may be related to the genotypic variability. Moreover, the detection rates are also influenced by the method of

Table 5 CMV DNA occurrence in HIV-infected and non-HIV-infected individuals and their relatives

CMV							
Group	CMV in relatives	Positive		Negative		Total		*p* value
		n	%	*n*	%	*n*	%	
HIV infected	Yes	–	–	2	5.7	2	5.0	$p=1.000^{a}$
	No	5	100.0	33	94.3	38	95.0	
	Total	5	100.0	35	100.0	40	100.0	
Non-HIV	Yes	1	33.3	–	–	1	2.5	$p=0.075^{a}$
	No	2	66.7	37	100.0	39	97.5	
	Total	3	100.0	37	100.0	40	100.0	

[a] Using Fisher's exact test

detection, frequency of sampling, oral health, social behaviors, and immunological status of the patients [11, 49, 50]. EBV and CMV diagnoses are based mostly in serological testing but PCR methods have been used to enable the diagnosis of specific viral infections [43]. This could partially explain a low prevalence of these herpesviruses in the population studied and a slight difference in the frequency of EBV and CMV between groups 1 and 2.

Almost all HIV-infected individuals were under adequate clinical control of HIV infection and using HAART, presenting high CD4 T cells count and low HIV viral load. This may explain why no oral lesions associated to EBV and CMV were observed and indicates that herpesvirus is frequently shed asymptomatically in the saliva of HIV-infected individuals who take HAART, similar to an immunologically healthy patient [6]. The infection would not necessarily evolve to a clinical manifestation, liable to happen only if the host immune response is suppressed [51].

Our results show that EBV DNA was significantly amplified in saliva of siblings of HIV/EBV coinfected individuals. The sample consists of patients and siblings, cohabitating the same household. These patients and their relatives probably have intimate oral contact, they could share eating utensils and food or other objects. It is possible that, in this population, EBV DNA have been spread from HIV-infected individuals to their relatives or from relatives to HIV-infected individuals. Saliva can contain high genome-copy counts of herpesvirus and is a common vehicle of herpesvirus horizontal transmission among close individuals [13, 14, 50]. According to some studies, our findings suggest that person-to-person contact could be a mechanism of EBV transmission [11, 13–18].

Nevertheless, it cannot be affirmed that the risk of EBV infection in household members of HIV-infected individuals was higher than the non-infected group because EBV and CMV serology was not evaluated. In addition, serology is the best test for evaluating acute versus remote infection in individuals [52]. Seropositivity means infection following exposure, and only part of infected individuals will shed the viruses in saliva since viral DNA in saliva is a measure of oral shedding after exposure and infection. Furthermore, it is also possible that EBV or CMV infection have occurred many years earlier in childhood, so HIV infection could not have contributed to further transmission.

The decision to use nonsexual relatives was made in other to avoid confusion with other routes of transmission and can help to better understand the role of saliva in herpesvirus shedding. In addition, our study was limited because serology and sequencing of the viral DNA could not be performed. However, based upon the results of the present study, it is possible to conclude that EBV and CMV DNA are amplified in saliva. HIV-infected individuals and healthy controls have similar frequency of detection of this herpesvirus and that EBV DNA is frequently amplified in saliva of siblings of HIV/EBV coinfected individuals.

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Conflict of interest The authors declare no conflict of interest.

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