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PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA
MESTRADO EM ODONTOLOGIA
ÁREA DE CONCENTRAÇÃO EM CLÍNICA INTEGRADA

TALITA RIBEIRO TENÓRIO DE FRANÇA

DETECÇÃO DO DNA DOS VÍRUS EBV E CMV NA SALIVA DE INDIVÍDUOS INFECTADOS OU NÃO PELO HIV

Recife – PE

2010

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Dissertação apresentada ao Programa de Pós-Graduação em Odontologia do Centro de Ciências da Saúde da Universidade Federal de Pernambuco, como requisito parcial para obtenção do Grau de Mestre em Odontologia com área de concentração em Clínica Integrada.

Orientador: Profº. Drº. Jair Carneiro Leão

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SECRETARIA

Oziclere Sena de Araújo

Ata da 91ª Defesa de Dissertação do Curso de Mestrado em Odontologia com área de Concentração em Clínica Integrada do Centro de Ciências da Saúde da Universidade Federal de Pernambuco. Recife, 22 de fevereiro de 2010.

Às 09:00(nove horas) do dia 22 (Vinte e dois) do mês de fevereiro do ano de dois mil e dez, reuniram-se no auditório do Curso de Pós Graduação em Odontologia da Universidade Federal de Pernambuco, os membros da Banca Examinadora, composta pelos professores: Profa. Dra. ALESSANDRA DE ALBUQERQUE TAVARES CARVALHO , atuando como presidente, Prof.Dr.EMANUEL SAVIO DE SOUZA ANDRADE da FOP/UPE atuando como primeiro examinador. Prof. Dr. LUIZ ALCINO MONTEIRO GUEIROS, atuando como segundo examinador, para julgar o trabalho intitulado **"DETECÇÃO DO DNA DOS VIRUS EBV E CMV NA SALIVA DE INDIVIDUOS INFECTADOS OU NÃO PELO HIV,** da mestranda TALITA RIBEIRO TENORIO DE FRANÇA, candidata ao Grau de Mestre em Odontologia, na Área de Concentração em CLINICA INTEGRADA, sob orientação do Prof.Dr. JAIR CARNEIRO LEÃO. Dando inicio aos trabalhos o Coordenador do Programa de Pós Graduação em Odontologia Prof. Dr. JAIR CARNEIRO LEÃO, abriu os trabalhos convidando os senhores membros para compor a Banca Examinadora, foram entregues aos presentes cópias do Regimento Interno do Curso de Mestrado em Odontologia, que trata dos critérios de avaliação para julgamento da Dissertação de Mestrado. A presidente da mesa após tomar posse conferiu os membros, seguindo convidou a CD. TALITA RIBEIRO TENORIO DE FRANÇA., para expor sobre o aludido tema, tendo sido concedido trinta minutos. A candidata expôs o trabalho e em seguida colocou-se a disposição dos Examinadores para argüição. Após o término da argüição os Examinadores reuniram-se em secreto para deliberações formais. Ao término da discussão, atribuíram a candidata os seguintes conceitos: Prof. Dr. EMANUEL SAVIO DE SOUZA ANDRADE **(APROVADA),** Prof .Dr. LUIZ ALCINO MONTEIRO GUEIROS,(APROVADA) Profa. Dra. ALESSANDRA DE ALBUQERQUE TAVARES CARVALHO, **(APROVADA),** a candidata recebeu três conceitos **(APROVADA)** é considerada **(APROVADA),** devendo a candidata acatar as sugestões da Banca Examinadora de acordo com o Regimento Interno do Curso, face a aprovação, fica a candidata, apta a receber o Grau de Mestre em Odontologia, cabendo a

Universidade Federal de Pernambuco através de sua Pró-Reitoria para Assuntos de Pesquisa e Pós Graduação, tomar as providências cabíveis. Nada mais havendo a tratar, A Presidente da Banca Examinadora encerrou a sessão e para constar foi lavrada a presente ata que vai por mim assinada , Oziclere Sena de Araújo e pelos demais componentes da Banca Examinadora e pela recém formada mestre pela UFPE, **TALITA RIBEIRO TENORIO DE FRANÇA** Talita Ribeiro Tenório de França

Oziclere Sharp

Recife, 22 de fevereiro de 2010.

Alessandra de Albuquerque Tavares Carvalho

Profa.Dra. ALESSANDRA DE ALBUQUERQUE TAVARES CARVALHO

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Prof. Dr. LUIZ ALCINO MONTEIRO GUEIROS

2° Examinador

Dedicatória

Dedicatória

Dedico este trabalho a um ser humano que oferta seu amor sem exigir nada em troca. Ela é a pessoa que está sempre disposta a me ouvir, que oferece sua mão à palmatória pra livrar a minha da dor. Escuta meus estresses, problemas, desabafos, novidades e alegrias. Pessoa mais paciente não há. Ela é amiga, é guerreira, é minha heroína. É leoa que protege a cria. Ela é Maria da Penha, minha mãe, a pessoa mais especial em minha vida! Obrigada por tudo, sem você nada teria sentido. Eu te amo!

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Agradecimentos especiais

A Deus, por ter abençoado a minha vida e a vida daqueles que amo e iluminado meu caminho tornando possível esse momento.

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EPÍGRAFE

"Se alguma coisa se te opõe e te fere, deixa crescer. É que estás a ganhar raízes e a mudar. Abençoado ferimento que te faz parir de ti próprio."

Saint-Exupéry

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LISTA DE SIGLAS E ABREVIATURAS

EBV	Epstein-Barr vírus
CMV	Citomegalovírus
HIV	Vírus da Imunodeficência Humana
DNA	Ácido desoxirribonucléico
SD	Standard deviation/ Desvio padrão
HAART	Terapia antiretroviral altamente potente
PCR	Reação em cadeia da polimerase
HHV-F1	Human Herpesvirus forward 1
HHV-F2	Human Herpesvirus forward 2
HHV-R1	Human Herpesvirus reverse 1
HHV-R2	Human Herpesvirus reverse 2
$MgCl_2$	Cloreto de Magnésio
dNTPs	Deoxinucleotídeo trifosfatados
min	Minutos
s	Segundos
h	Hora
OR	Odds Ratio
CI	Confidence intervals/ intervalo de confiança
UDI	Usuário de droga injetável

LISTA DE SÍMBOLOS

µL	Microlitros
mM	Milimolar
nM	Namolar
U	Unidade
˚C	Graus Celsius
nm	Nanômetro
bp	Pares de bases

RESUMO

Os vírus Epstein- Barr (EBV) e Citomegalovirus (CMV) apresentam ampla distribuição na população e são responsáveis pelo desenvolvimento de várias doenças. A saliva pode conter uma grande quantidade desses herpes vírus e é um veículo comum de transmissão horizontal entre indivíduos próximos. O objetivo do presente estudo foi determinar a detecção de DNA do EBV e do CMV na saliva de indivíduos infectados pelo HIV ou não e em seus familiares, avaliando papel exercido pela imunodeficiência na transmissão salivar. O grupo de estudo foi constituído por 240 indivíduos. Grupo 1: 40 indivíduos infectados pelo HIV; grupo 2: 40 indivíduos não-HIV; grupo 3: 2 parentes de cada indivíduo do grupo 1 (n=80), não parceiros-sexuais, residentes no mesmo domicílio; grupo 4: 2 parentes de cada indivíduo do grupo 2 (n=80), não parceiros-sexuais, residentes no mesmo domicílio. Foi coletada saliva não estimulada de cada participante e o DNA foi extraído usando o Kit Geneclean® II (BIO 101, La Jolla, CA, USA). A amplificação do DNA do EBV e do CMV foi realizada usando um protocolo de *nested* PCR. O DNA do EBV e do CMV foi detectado respectivamente em 7/40 (17,5%) e 5/40 (12,5%) indivíduos do grupo 1; 8/40 (20%) e 3/40 (7,5%) indivíduos do grupo 2; 11/80 (13,8%) e 2/80 (2,5%) familiares do grupo 3 e 8/80 (10%) e 1/80 (1,3%) familiares do grupo 4. Entre os 7 indivíduos HIV/EBV co-infectados, 5 (71,4%) tinham um parente também infectado pelo EBV (OR= 11,25, CI [1,75- 72,5], p= 0,011). Com relação ao grupo 2, entre os 8 indivíduos não HIV e infectados pelo EBV, 3 (37,5%) tinham um parente também positivo para EBV (p= 0,320). Entre os 5 indivíduos HIV/CMV co-infectados, nenhum tinha um parente infectado pelo CMV (p= 1,00). Com relação ao grupo 2, entre os 3 indivíduos não HIV e infectados pelo CMV, 1 (33,3%) tinha um parente também infectado pelo CMV (p= 0.075). Baseado nos resultados do presente

estudo, o DNA dos vírus EBV e CMV é freqüentemente amplificado na saliva. Os indivíduos infectados pelo HIV e os controles saudáveis apresentaram uma freqüência de detecção de DNA viral similar. Parentes de indivíduos HIV/EBV co-infectados apresentam um risco maior de adquirir EBV através de contato não sexual.

Palavras-chaves: EBV; Citomegalovirus; HIV; saliva; transmissão.

ABSTRACT

Epstein- Barr virus (EBV) and Cytomegalovirus (CMV) are ubiquitous in the human population causing a wide spectrum of diseases. Saliva can contain high counts of herpesvirus and is a common vehicle of horizontal transmission among close individuals. The objective of the present work was to determine the frequency of EBV and CMV DNA detection in saliva of HIV infected and non-HIV individuals and their relatives, assessing the role of HIV related immunodeficiency in salivary transmission. The study group comprised 240 individuals. Group 1: 40 HIV-infected patients; group 2: 40 non-HIV individuals; group 3: 2 non-sexual partners relatives for each patient from group 1 (n=80); group 4: 2 non-sexual partners relatives for each individual from group 2 (n=80). Each participant had non-stimulated whole saliva collected and DNA was extracted using Geneclean® kit II (BIO 101, La Jolla, CA, USA). EBV and CMV-DNA amplification were performed using a nested PCR protocol. EBV and CMV-DNA were 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. Among the 7 HIV/EBV co-infected individuals, 5 (71.4%) had a relative also infected with EBV (OR= 11.25, CI [1.75- 72.5], p= 0.011). Regarding group 2, among the 8 non-HIV and EBV infected individuals, 3 (37.5%) had a relative also positive to EBV (p= 0.320). Among the 5 HIV/CMV co-infected individuals of group 1, none had a relative CMV infected (p= 1.00). Regarding group 2, among the 3 non-HIV and CMV infected individuals, 1 (33.3%) had a relative also positive to CMV (p= 0.075). Based upon the results of the present study EBV and CMV-DNA is frequently amplified in saliva. HIV infected individuals and healthy controls showed a similar

frequency of viral DNA detection. Relatives of HIV/EBV co-infected individuals are at greater risk of acquiring EBV through non-sexual contact.

Key words: EBV; Cytomegalovirus; HIV; saliva; transmission.

APRESENTAÇÃO

Conhecimentos da biologia, patogenicidade e associações clínicas do EBV e do CMV têm sido expandidos rapidamente nos últimos anos. Testes moleculares e sorológicos têm sido usados para definir a epidemiologia do EBV e do CMV. No entanto, a prevalência do EBV e do CMV na saliva da população brasileira ainda não é bem esclarecida, bem como o modo de transmissão desse vírus em nosso país, principalmente na região Nordeste. É muito importante conhecer a prevalência, para se criar medidas preventivas importantes para saúde pública que possam minimizar a disseminação desses herpesvírus. Esta dissertação foi organizada em forma de artigo, submetido a um periódico científico na área de ciências da saúde. A revisão de literatura usada no artigo foi realizada através de buscas nas bases de dados PubMed e Scopus. O artigo **"EBV and CMV DNA detection in saliva of HIV and non-HIV infected individuals"** apresenta a saliva como um importante veículo de transmissão dos herpesvírus humanos, principalmente em pacientes imunocomprometidos, como é o caso dos indivíduos infectados pelo HIV. Este trabalho determinou a freqüência de detecção do DNA dos vírus EBV e CMV na saliva de indivíduos infectados ou não pelo HIV e em seus familiares e avaliou o papel da imunodeficiência na transmissão salivar desses herpesvírus humanos.

Palavras-chave: EBV; Citomegalovirus; HIV; saliva; transmissão

PRESENTATION

Knowledge of biology, pathogenicity and clinical associations of EBV and CMV have been expanded rapidly in recent years. Molecular and serological tests have been used to define the epidemiology of EBV and CMV. However, the prevalence of EBV and CMV in saliva of Brazilian population is not well understood, as well as the mode of transmission in our country, especially in the Northeast. It is very important to know the prevalence in order to create important public health preventive measures that can minimize the spread of herpesvirus. This work was organized in article, submitted to a scientific periodic specialize in the area of Health Sciences. The literature review of the article was performed through PubMed and Scopus databases. The article "EBV and CMV DNA detection in saliva of HIV and non HIV-infected individuals" presents saliva as an important vehicle for transmission of human herpevirus, especially in immunocompromised patients, such as HIV-infected individuals. This study determined the frequency of detection of EBV and CMV DNA in saliva of HIV infected individuals or not and their relatives and assessed the role of immunodeficiency in salivary transmission of human herpesvirus.

Keywords: EBV, cytomegalovirus, HIV, saliva, transmission

EBV AND CMV DNA DETECTION IN SALIVA OF HIV AND NON-HIV INFECTED INDIVIDUALS

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SUMMARY

Epstein- Barr virus (EBV) and Cytomegalovirus (CMV) are ubiquitous in the human population causing a wide spectrum of diseases. Saliva can contain high counts of herpesvirus and is a common vehicle of horizontal transmission among close individuals. The objective of the present work was to determine the frequency of EBV and CMV DNA detection in saliva of HIV infected and non-HIV individuals and their relatives, assessing the role of HIV related immunodeficiency in salivary transmission. The study group comprised 240 individuals. Group 1: 40 HIV-infected patients; group 2: 40 non-HIV individuals; group 3: 2 non-sexual partners relatives for each patient from group 1 (n=80); group 4: 2 non-sexual partners relatives for each individual from group 2 (n=80). Each participant had non-stimulated whole saliva collected and DNA was extracted using Geneclean® kit II (BIO 101, La Jolla, CA, USA). EBV and CMV-DNA 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. Among the 7 HIV/EBV co-infected individuals, 5 (71.4%) had a relative also infected with EBV (OR= 11.25, CI [1.75- 72.5], p= 0.011). Regarding group 2, among the 8 non-HIV and EBV infected individuals, 3 (37.5%) had a relative also positive to EBV (p= 0.320). Among the 5 HIV/CMV co-infected individuals of group 1, none had a relative CMV infected (p= 1.00). Regarding group 2, among the 3 non-HIV and CMV infected individuals, 1 (33.3%) had a relative also positive to CMV (p= 0.075). Based upon the results of the present study EBV and CMV-DNA is frequently amplified in saliva. HIV infected individuals and healthy controls showed a similar frequency of viral DNA detection. Relatives of HIV/EBV co-infected individuals are at greater risk of acquiring EBV through non-sexual contact.

Key words: EBV; Cytomegalovirus; HIV; saliva; transmission.

INTRODUCTION

Herpesvirus are ubiquitous in the human population (Jenkins *et al*., 2003). In healthy adults and children EBV and CMV usually causes no symptoms (Flaitz *et al*, 1996; Boppana *et al*., 2001; Ross *et al*., 2006), but in the immunologically immature and immunocompromised host they may result in severe opportunistic infections with high morbidity and mortality (Flaitz *et al*, 1996; Doniger *et al*. 1999; Boppana *et al*., 2001; Sitki-Green *et al*., 2002; Nishiwaki *et al*., 2006; Ross *et al*., 2006; Rafailidis *et al*., 2008).

Saliva can contain high counts of herpesvirus and is a common vehicle of herpesvirus horizontal transmission among close individuals (Miller *et al*., 2005; Sahin *et al*., 2009). EBV is probably transmitted via saliva, through intimate oral contact, by salivary residues left on cups, food, toys, or other objects and when mothers pre-chew food that is then given to babies (Cohen, 2000; Balfour *et al*., 2005; Fafi-Kremer *et al*., 2005; Slots, 2006). CMV could be transmitted through saliva, breastfeeding, sexual contact and spread from children who frequently acquire CMV from the mother (Bello & White, 1991; Sharland *et al.*, 2002; Hecker *et al*., 2004; Meier *et al*., 2005; Wilms *et al*., 2008). In addition, CMV can be transmitted by blood transfusion and solid organ transplantation (Stratta, 1993).

In Japan, EBV DNA was detected in 90% of throat washings from healthy adults (Ikuta *et al*., 2000). In another study conducted with patients who were in good systemic health, EBV DNA was detected in saliva of 81.5% (Dawson *et al*., 2009). The prevalence of CMV and EBV in healthy blood donors was 77.6% and 20.0% and among the HIV-AIDS patients 59.2% and 87.2%, respectively (Adjei *et al*., 2008). Ammatuna *et al*. (2001) determined that the prevalence of EBV-DNA in HIV-infected

patients was 42.1%, in renal transplant patients was 65% and in healthy uninfected controls was 16.6%. They also observed that CMV-DNA was detected in 3.5% of HIV1 and in none of transplant patients. Other studied reported that the detection rates for viral DNA in whole blood and plasma from transplant recipients were 23.8% and 5.9% for EBV, 11.2% and 5.3% for CMV, respectively (Wada *et al*., 2007). Mbulaiteye *et al*. (2006) reported that the rate of EBV DNA detection in saliva from asymptomatic Ugandan children was 90% and in their mothers was 79%.

Although the molecular and serological tests have been used to define the EBV and CMV epidemiology, its exact prevalence in Brazil, mainly in Northeast, is still uncertain and possible routes of transmission have not been well established. Clarify this data represents an essential and auxiliary tool to the development of methods which help to limit the dissemination of herpesvirus infection. Therefore, the aim of the present work was to determine the frequency of EBV and CMV detection in saliva of HIV infected and non-HIV individuals and assessing the role of HIV related immunodeficiency in salivary transmission among HIV individuals and their relatives.

RESULTS

The 40 HIV-infected individuals were demographically similar to the 40 non-HIV controls. Demographic findings (gender, age, skin color and marital status) were expressed in table 1. The mean of age in each group was: group 1, 38.2 (SD= 10.7); group 2, 28.8 (SD= 12.1); group 3, 31.8 (SD= 16.7); group 4, 37.6 (SD= 17.8).

Oral manifestations was observed only in the group of HIV infected individuals in which 4/40 (10%) participants had oral ulcerations, 3/40 (7.5%) had pseudomembrane candidosis, 2/40 (5%) had leukoplakia and 1/40 (2.5%) had

erithroplakia. These diagnoses are based only on clinical findings, without histological confirmation. The type of relative relationship was shown in table 2. The majority of relatives in group 3 was sons (38/80 [47.5%]) of individuals of group 1 and in group 4, the majority was brothers (25/80 [31.3%]) of individuals of group 2 (Table 2).

The majority of HIV-infected patients were adequately controlled as determined by HIV loads and CD4 T cells counts. In the group of HIV infected individuals, 35/40 (87.5%) had viral load counts lower the minimum limit of 400 copies (<min.lim.400) and 5/40 (12.5%) individuals had HIV viral load higher than this limit (>min.lim.400). It was observed that 18/40 (45%) had CD4 T cells counts equal to or greater than 500/mm^3, 19/40 (47.5%) had counts between 200- 400/mm^3 and 3/40 (7.5%) had counts lower than 200/mm^3.

Oral manifestations were present mainly in those patients who had viral load counts lower the minimum limit of 400 copies (8/35 [22.8%]). Regarding CD4 T cells counts, oral ulcerations and leukoplakia were more prevalent in patients who had counts equal to or greater than 500/mm^3 (2/18 [11.1%] and 3/18 [16.7%], respectively). Pseudomembrane candidosis and erithroplakia were most frequently found in patients who had counts between 200- 400/mm^3 (2/19 [10.5%] and 1/19 [5.3%], respectively).

EBV- DNA was detected in 15/80 (18.8%) individuals from groups 1 and 2; 7/40 (17.5%) were from the HIV-infected (group 1) and 8/40 (20%) were from non-HIV individuals (group 2) (p= 1.0). CMV-DNA was detected in 8/80 (10%) individuals from groups 1 and 2; 5/40 (12.5%) were from the HIV-infected (group 1) and 3/40 (7.5%) were from non-HIV individuals (group 2) (p= 0.71). Figures 1 and 2 show some PCR results. In the group of HIV infected patients, EBV- DNA was detected

mainly in those who had viral load counts <min.lim.400 (5/7 [71.4%], p=0.2) and those who had CD4 T cells counts ranging 200 to 400/mm^3 (4/7 [57.1%], p=0.544). CMV-DNA was detected only in those who had viral load counts <min.lim.400 (5/5 [100%], p=1) and those who had CD4 T cells counts ranging 200 to 400/mm^3 (3/5 [60%], p=0.249)

It was also possible to amplify EBV- DNA from 19/160 (12.9%) relatives; 11/80 (13.8%) relatives of HIV infected participants and 8/80 (10%) from relatives of non-HIV (p=0.6). Among the 7 HIV/EBV co-infected individuals, 5 (71.4%) had a relative EBV infected (OR= 11.25, CI [1.75- 72.5], p= 0.011). Regarding group 2, among the 8 non-HIV and EBV infected individuals, 3 (37.5%) had a relative also positive to EBV (p= 0.320) (Table 3).

CMV- DNA was amplified from 3/160 (2%) relatives; 2/80 (2.5%) relatives of HIV infected participants and 1/80 (1.3%) from relatives of non-HIV (p=1). Among the 5 HIV/CMV co-infected individuals of group 1, none had a relative CMV infected (p= 1.00). Regarding group 2, among the 3 non-HIV and CMV infected individuals, 1 (33.3%) had a relative also positive to CMV (p= 0.075) (Table 4).

It was not observed association between EBV and CMV positivity and sex, marital status, demonstrable oral manifestations, viral load, CD4 T cell count and type of relative relationship (p>0.05). However, only in group 4 (relative of non-HIV patient), EBV and CMV positivity was associated to age; 2/8 individuals EBV positive had age ranging from 18 to 38 years, 3/8 had age ranging from 38 to 59 years and 3/8 had age over 59 years (p=0.035). The only individual CMV infected in group 4 had age over 59 years (p=0.018).

DISCUSSION

Epidemiological evidence and laboratory data show that EBV and CMV transmission occurs both horizontally and vertically (Cohen, 2000; Sharland *et al.,* 2002; Hecker *et al*., 2004; Balfour *et al*., 2005; Fafi-Kremer *et al*., 2005; Meier *et al*., 2005; Miller *et al*., 2005; Slots, 2006; Wilms *et al.,* 2008; Sahin *et al*., 2009). Several studies demonstrated that these herpesvirus could be transmitted through close contacts and saliva (Cohen, 2000; Druce *et al*, 2002; Balfour *et al*., 2005; Fafi-Kremer *et al*., 2005; Miller *et al*., 2005; Mbulaiteye *et al*., 2006; Slots, 2006; Dawson *et al*., 2009; Sahin *et al*., 2009). Factors that govern acquisition and transmission of EBV and CMV should be further studied. Establish the genotype of these herpesvirus in the individual and relative is important to affirm the exact association and confirm that saliva is a potential source of EBV and CMV transmission.

The results of this study show that EBV and CMV-DNA frequency in saliva was similar in the groups of HIV infected individuals and non-HIV individuals and their relatives. Our results show that relatives of HIV/EBV co-infected individuals are at greater risk of acquiring EBV through non-sexual contact. It is possible that EBV infection can be primarily transmitted non-sexually in the studied population. Therefore, non-sexual transmission mainly through close interpersonal (especially between mother and child and among siblings) contact of non-intact skin or mucous membranes with saliva, may be the primary mode of transmission, similar to that suggested in previous reports (Cohen, 2000; Fafi-Kremer *et al*., 2005; Miller *et al*., 2005; Slots, 2006; Adjei *et al*., 2008; Sahin *et al*., 2009).

The prevalence of these herpesvirus reported in the present study corroborates previously published reports that shown rates ranging from 22 to 90% for EBV and from 1.2 to 31% for CMV (Kunimoto *et al*, 1992; Falk *et al*., 1997; Lucht

et al., 1998; Ikuta *et al*., 2000; Ammatuna *et al*., 2001; Idesawa *et al*., 2004; Miller *et al*., 2005; Rasti *et al*., 2005; Miller *et al*., 2006; Mabulaiteye *et al*., 2006; Yamamoto *et al*., 2006; Wada *et al*., 2007; Watanabe *et al*. 2007; Adjei *et al*., 2008; Dawson *et al*, 2009; Rosenthal *et al*., 2009). In United States, EBV and CMV were detected in 90% and 31% HIV infected patients, respectively, compared with 48% and 2%, respectively, of samples from controls (Miller *et al*., 2006). Other study conducted in United States observed that CMV DNA was detected in saliva of only one patient (1.5%) who was in good systemic health (Dawson *et al*., 2009). In Japan, EBV DNA was identified in 90% of healthy adults and in 38% of saliva from healthy children (Ikuta, 2000). Another study in Japan showed that EBV-DNA was detected in 23% of EBV seropositive healthy adults (Kunimoto *et al.,* 1992). In Brazil, EBV-DNA was identified in 23 (77%) patients while CMV was observed only in 2 (6%) (Watanabe *et al*., 2007).

We did not observe significant difference in the frequency of EBV and CMV DNA detection in patients HIV infected and the healthy controls. Generally, the prevalence of these herpervirus appears higher in HIV-seropositive patients (Lucht *et al*., 1998; Bregel-Pesce *et al*., 2002; Fidouh-Houhou *et al*., 2001; Lampinem *et al*., 2000; Triantos *et al*., 2004; Mbulaiteye *et al*., 2006). Studies that quantified EBV DNA levels in saliva and peripheral blood observed that EBV is variably detected in healthy subjects (Ikuta *et al*., 2000; Stevens *et al*., 2001; Leung *et al*., 2004).

The wide range of detection of EBV and CMV may be attributed to differences in the studied populations and methodological approaches (Imbronito *et al*., 2008). The rates detected are influenced by the biological properties of the virus, method of detection, frequency of sampling, oral health, social behaviors and immunological status of the patient (Miller *et al*., 2004; Mbulaiteye *et al*., 2005; Mbulaiteye *et al*.,

2006). This fact could justified why we did not observe a high prevalence of these herpesvirus in the population studied and why we did not observe a significant difference when compared de frequency of EBV and CMV in group 1 and 2. In one study that examined multiple herpesvirus, rates of detection in saliva of HIV-seropositive patients were similar to that of the general population, except for CMV (Fons, 1994). Other study observed that there was no statistically significant difference in the overall seroprevalence of CMV between HIV-AIDS patients and HIV-seronegative healthy blood donors (Adjei *et al*., 2008).

The group of HIV infected individuals showed a high CD4 T cells count and a low HIV viral load. All patients, except for the healthy volunteers, were under antiretroviral therapy when the samples were collected. This may explain why no oral lesions associated to EBV and CMV were observed and indicates that herpesvirus are frequently shed asymptomatically in the saliva of HIV infected individuals who take HAART. It is known that this therapy increases CD4 cells, which are important in the host defenses against oportunistic organisms (Komatsu *et al*., 2005). The infection would not necessarily evolve to a clinical manifestation, liable to happen only if the immune response of the host in controlling the viral replication is suppressed (Ammatuna *et al*., 1998). Studies about EBV in healthy individuals have been made (Eisenberg *et al*., 1992; Mao & Smith, 1993; Felix *et al*., 1992; Scully *et al*., 1998; Sitki-Green *et al.,* 2003) and now it is accepted that infection by the these herpesvirus is often subclinical in the presence of a healthy immune response (Sitki-Green *et al.,* 2003).

Our results show that relatives of HIV/EBV co-infected individuals are at greater risk of acquiring EBV through non-sexual contact. These results were similar to those found in the literature (Cohen, 2000; Fafi-Kremer *et al*., 2005; Slots,

2006). Saliva can contain high genome-copy counts of herpesvirus and is a common vehicle of herpesvirus horizontal transmission among close individuals (Miller *et al*., 2005; Sahin *et al*., 2009). The sample consists of patients and their relatives, non-sexual partner that cohabitating the same household. These patients and their relatives probably have intimate oral contact, they could share eating utensils and food or other objects. In addiction, other factors associated with low socio-economic conditions are directly associated with low hygiene conditions and a possible contribution to the spread of the virus through salivary fluids (Mbulaiteye *et al*., 2005). According other studies, our findings suggest that person-to-person contact could be a mechanism of EBV transmission in this population (Cohen, 2000; Balfour *et al*., 2005; Fafi-Kremer *et al*., 2005; Slots, 2006; Miller *et al*., 2005; Mbulaiteye *et al*., 2006; Sahin *et al*., 2009).

CMV DNA was found in saliva of non-HIV individuals and also in saliva of their relative, however this association was not statistically significant. This result is different from most studies found in the literature. It is known CMV could be transmitted through saliva (Bello & White, 1991; Druce *et al*, 2002; Miller *et al*, 2005; Wilms *et al.*, 2008). However, one study with African-Americans childrens, observed that there was no association between CMV infections of the children and their mothers (Wilms *et al*., 2008). The rates detected could be influenced by the method of detection used, frequency of herpesvirus in the sample and social behaviors (Miller *et al*., 2004; Mbulaiteye *et al*., 2006). All that, as well as different patient populations studied, could explain the different observations obtained.

Determining the EBV and CMV real prevalence may be difficult once the technique used, PCR, is prone to false positive and false negative results. Serology, however, may sometimes be preferred rather than PCR (Leao, 1999). However, the

use of PCR in the present work is justifiable due to sensitivity of the test and ability to amplify small amounts of the target sequence as showed in previously studies (Mabruk *et al*., 1994; LaDuca *et al*, 1998; Lucht *et al*, 1998; Clementi, 2000; Johnson *et al*., 2000; Tanaka *et al*., 2000; Druce *et al*., 2002; Hara *et al*., 2002; Kubar *et al*., 2005; Tanaka *et al*., 2009). 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 a frequent change of gloves and sodium hypochlorate solution to decontaminate surfaces were used to prevent contamination.

The decision to use non-sexual relatives was made in other to avoid confusion with other routes of transmission. Our study was limited because the participants number (40 cases and 40 controls). However, we found a significant *p* value in the case group. Our results showed a significant association between the presence of EBV DNA in saliva of HIV infected patients and their relatives.

Based upon the results of the present study EBV and CMV DNA is frequently amplified in saliva. HIV infected individuals and healthy controls showed a similar frequency of detection of these herpesvirus. Relatives of HIV/EBV co-infected individuals are at greater risk of acquiring EBV through non-sexual contact.

METHODS

Subjects

The study population comprised 240 individuals, divided in 4 groups. Group 1 comprised forty HIV-infected outpatients of the Infectious and Parasitic Diseases Service of Hospital das Clínicas, Universidade Federal de Pernambuco (UFPE),

Recife, Brazil. Group 2 was the control group and has 40 healthy volunteers recruited from Dentistry Clinics at the UFPE. Group 3 included 2 non-sexual partners relatives cohabitating with each patient from group 1 (n=80). Group 4 comprised 2 non-sexual partners relatives cohabitating with each individual from group 2 (n=80). Local ethical approval was given to the study and informed consent was obtained from all the participants.

We collected demographic information, including gender, age, sex, marital status and family relationships. In group 1, HIV viral load and CD4 T cells counts were also assessed by medical record. Oral examinations were performed by a single trained examiner. Unstimulated whole saliva was collected at a single visit by the method described by Navazesh *et al*. (1993). All samples were collected in the hospital where the study was performed and, then, identified and stored at -20 ^{0}C for later DNA extraction.

Laboratory methods

DNA was extracted from saliva by Geneclean® kit II (BIO 101, La Jolla, CA, USA). Extracts underwent PCR to amplify a segment of β-globin DNA using GH20 (5' CAACTTCATCCACGTTCACC 3') and PC04 (5 'GAAGAGCCAAGGACAGGTAC 3') primers to determine the presence of cellular DNA (Leao *et al*., 1999). EBV- and CMV-DNA amplification was performed using a nested consensus PCR protocol (Tafreshi *et al*., 2005). Primers consensus assigned as HHV-F1, HHV-R1, HHV-F2 and HHV-R2 within highly conserved regions of CMV and EBV were used for nested PCR (Tafreshi *et al*., 2005). Primer sequences used are illustrated in Table 5.

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, HHV-R1 (Invitrogen®

Brazil), 1X PCR buffer (Invitrogen® Brazil), 1.5 mM $MgCl_2$ (Invitrogen® Brazil), 0.2 mM dNTPs (dNTp mixture, 100 nM solution, Amresco ®), 2.5 U Taq DNA polymerase (Invitrogen® Brazil). First round PCR was carried out using the following programs: 94 ˚C for 3 min (predenaturation), 35 cycles at 94 ˚C for 45 s, 65.5 ˚C for 1min, 72 ˚C for 1min, with a final extension at 72 ˚C for 7min. For the 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.

Seven microliters of PCR product were stained with 3 µL of blue/orange loading buffer (Promega®, Madison, WI, USA) and electrophoresed through a 1.5 % agarose gel (Pronadisa Conda ® Laboratories - Madrid, Spain) containing 6.25 µL of ethidium bromide (Promega® Madison, WI, USA) in tris-borate-EDTA 1X (Amresco® Inc, Ohio, USA). All DNA fragments were visualized through a transilluminator (Vilber Lourmat®, France) with a wavelength of 302 nm; photographs were taken with the photo-documentation system (Vilber Lourmat ®, France).

The consensus primers used were designed within a highly conserved region of HSV-1, HSV-2, CMV and EBV. CMV amplified products were accurately identified by their molecular weight (size of amplicon= 565 bp). It was not possible to identify those products of HSV-1, HSV-2 and EBV by their molecular weight because they have very similar weight, 493, 493 and 499 bp, respectively. These viruses were identified by double digestion of amplicons. To identify EBV the nested PCR products for each viral sample was digested with TaqI restriction enzyme (Invitrogen® Brazil). The digestion mixture consisted of 8 µL of second round PCR product, 2 µL of appropriate enzyme buffer, five units of enzyme and nuclease free water to give a

final volume of 20 and 20 µL mineral oil also was added to prevent evaporation. The reaction mixture was incubated for 4 h at 65 °C. Digested products were separated by 2 % agarose gel electrophoresis. Fragments size after Taq I digestion 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 (Tafreshi *et al*., 2005).

Data analysis

Absolute and percentage distributions and statistics measures were obtained. It was used Pearson chi-square or Fisher Exact tests when it was not possible to use Chi-square. Standard deviation (SD), Odds Ratio (OR) and confidence intervals (CI with 95%) 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.

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TABLES

Table 1 – Demographic findings of groups 1, 2, 3 and 4.

Variables	HIV		Non-HIV		Relative of HIV		Relative 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
• **Gender**								
Male	29	72.5	6	15	32	40	31	38.8
Female	11	27.5	34	85	48	60	49	61.3
• **Marital status**								
Single	22	55	31	77.5	58	72.5	47	58.8
Married	17	42.5	9	22.5	17	21.3	29	36.3
Widow	1	2.5	-	-	5	6.3	4	5
• **Skin color**								
Leucoderma	2	5	18	45	27	33.8	34	42.5
Faioderma	19	47.5	21	52.5	37	46.7	40	50
Melanoderma	19	47.5	1	2.5	16	20	6	7.5
TOTAL	**40**	**100**	**40**	**100**	**80**	**100**	**80**	**100**

Quantitative analysis

Table 2 – Type of relative relationship

Relative relatioship	Relatives of HIV infected		Relative of non-HIV	
	N	%	n	%
Father	3	3.8	9	11.3
Mother	12	15	21	26.3
Son	38	47.5	12	15
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 3 – EBV-DNA occurrence in HIV infected and non- HIV infected individuals and their relatives.

Group	EBV in relatives	EBV						p value
		Positive		Negative		Total		
		n	%	n	%	n	%	
HIV infected	Yes	5	71.4	6	18.2	11	27.5	$p^{(1)} = 0.011$*
	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	$p^{(1)} = 0.320$
	No	5	62.5	27	84.4	32	80.0	
	TOTAL	**8**	**100.0**	**32**	**100.0**	**40**	**100.0**	

(*) – Significant difference at 5.0% level
(1) – Using Fisher's exact test

Table 4 – CMV-DNA occurrence in HIV infected and non- HIV infected individuals and their relatives.

Group	CMV in relatives	CMV						p value
		Positive		Negative		Total		
		n	%	n	%	n	%	
HIV infected								
	Yes	-	-	2	5.7	2	5.0	$p^{(1)} = 1.000$
	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	$p^{(1)} = 0.075$
	No	2	66.7	37	100.0	39	97.5	
	TOTAL	**3**	**100.0**	**37**	**100.0**	**40**	**100.0**	

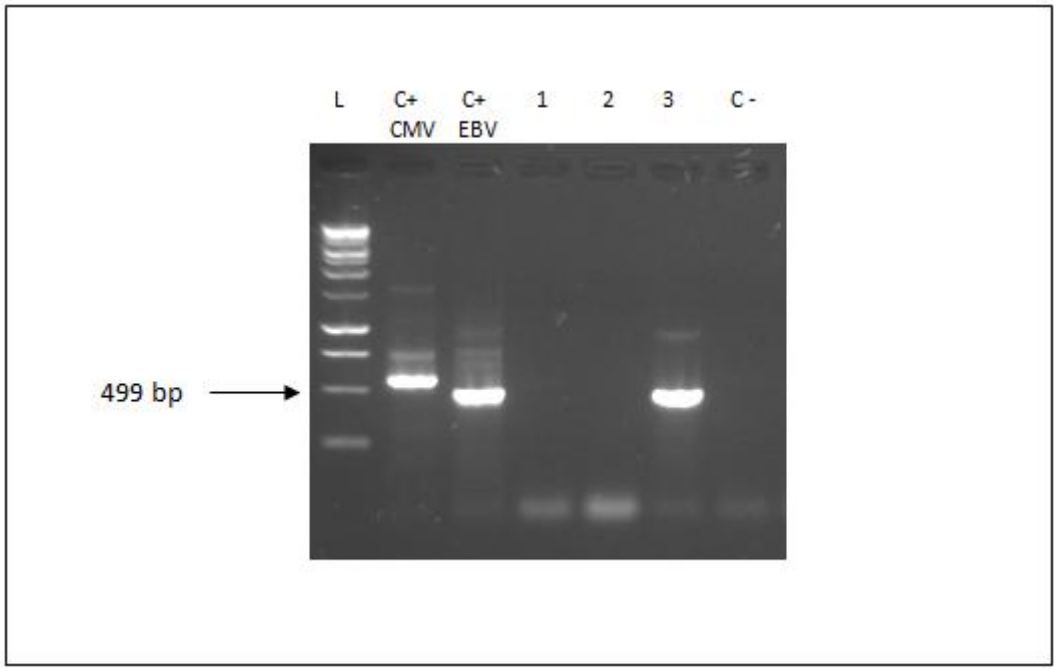
(*) – Significant difference at 5.0% level
(1) – Using Fisher's exact test.

Table 5: Primers sequences used (Tafreshi *et al*., 2005)

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	

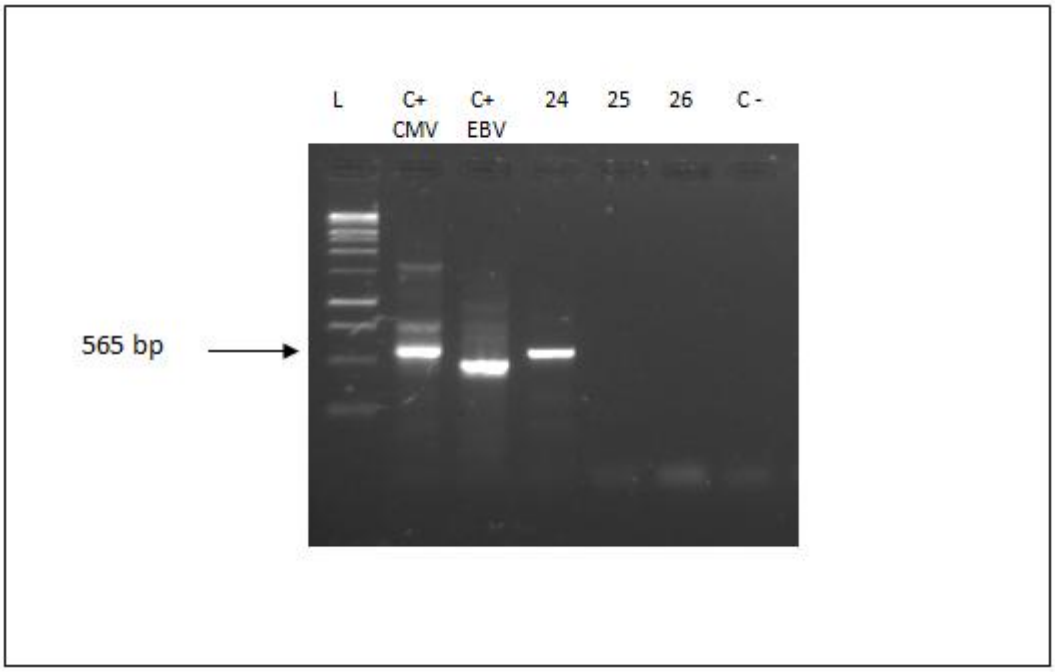
FIGURES

Figure 1: PCR results showing the detection of EBV-DNA



L: ladder; C+ CMV: CMV positive control; C+ EBV: EBV positive control; 1, 2: EBV and CMV DNA negative; 3: EBV-DNA positive; C-: negative control. The arrow indicates the amplicon size (499 bp).

Figure 2: PCR results showing the detection of CMV-DNA



L: ladder; C+ CMV: CMV positive control; C+ EBV: EBV positive control; 24: CMV-DNA positive; 25, 26: EBV and CMV DNA negative; C-: negative control. The arrow indicates the amplicon size (565 bp).

Apêndices

APÊNDICE A: Termo de Consentimento Livre e Esclarecido

UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS DA SAÚDE
PROGAMA DE PÓS- GRADUAÇÃO EM ODONTOLOGIA
MESTRADO EM ODONTOLOGIA
ÁREA DE CONCENTRAÇÃO EM CLÍNICA INTEGRADA

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO
Carta convite

Você está sendo convidado(a) a participar, como voluntário(a), da pesquisa **"Estudo da transmissão salivar do EBV e do CMV em pacientes co-infectados ou não pelo HIV"**, que tem por objetivos: avaliar possível rota de transmissão não-sexual do EBV e CMV em pacientes co-infectados ou não pelo HIV, determinar a freqüência de detecção do EBV e CMV nesses pacientes, avaliar a existência de associação significativa entre a presença desses vírus na saliva de pacientes co-infectados ou não pelo HIV e a prevalência do EBV e CMV em familiares residentes no mesmo domicílio. Você será esclarecido(a) sobre a pesquisa em qualquer aspecto que desejar. Sua participação não é obrigatória e, a qualquer momento, você poderá desistir de participar e retirar seu consentimento, sem que isso acarrete qualquer penalidade. Após ser esclarecido(a) sobre as informações a seguir, no caso de aceitar fazer parte do estudo, assine ao final deste documento, que está em duas vias. Uma delas é sua e a outra é do pesquisador responsável.

Os procedimentos da pesquisa envolvem coletar a saliva e submetê-la a um exame laboratorial, que vai dizer se há presença ou não do vírus EBV e do CMV nesse material. Os riscos inerentes à coleta de saliva são mínimos. É possível que você sinta-se constrangido durante a coleta e ao responder algumas perguntas. Afora isso, o procedimento não apresenta riscos como por exemplo de contaminação uma vez que o material utilizado é todo descartável e o pesquisador estará sempre tomando todos os cuidados de biossegurança.

É importante que você saiba que o pesquisador irá tratar a sua identidade com padrões profissionais de sigilo. O preenchimento da ficha, os resultados do exame laboratorial e da pesquisa serão enviados para você, se você assim o desejar, e permanecerão confidenciais. Você não será identificado(a) em nenhuma publicação que possa resultar deste estudo.

Pela sua participação no estudo, você não receberá qualquer valor em dinheiro, mas terá a garantia de que todas as despesas necessárias para a realização da pesquisa não serão de sua responsabilidade. Apesar de não receber nenhum benefício direto, você será beneficiado ao tomar conhecimento se possui ou não o vírus EBV e/ou CMV, sendo alertado sobre as formas de transmissão do vírus, como tratá-lo e receberá encaminhamento para o tratamento. Ademais, ganha ainda a sociedade como um todo que terá respondida uma questão de saúde pública importante, o que indiretamente pode se constituir num ganho adicional a você.

Você também tem a garantia de que, em qualquer etapa do estudo, terá acesso aos pesquisadores responsáveis para esclarecimento de eventuais dúvidas. Se desejar, pode entrar em contato com o orientador da pesquisa Profº Dr. Jair

Carneiro Leão ou com a Drª Talita Ribeiro Tenório de França, que podem ser encontrados no endereço: Rua Profº Moraes Rego, s/ nº, Cidade Universitária, Recife-PE, CEP: 50670-901, fone: (81) 21268818 ou 9218-7306.

CONSENTIMENTO DO PARTICIPANTE

Eu,_____,RG_____**,**abaixo qualificado, DECLARO para fins de participação em pesquisa, na condição de sujeito objeto da pesquisa/representante legal do sujeito objeto da pesquisa, que fui devidamente esclarecido do Projeto de Pesquisa intitulado: **Estudo da transmissão salivar do EBV e CMV em pacientes co-infectados ou não pelo HIV**, desenvolvido pelo orientador Dr. Jair Carneiro Leão e pela pesquisadora Drª. Talita Ribeiro Tenório de França, do Programa de Pós- Graduação em Odontologia da Universidade Federal de Pernambuco.

DECLARO que após convenientemente esclarecido pelo pesquisador e ter entendido o que me foi explicado, consinto voluntariamente em participar/que meu dependente legal participe desta pesquisa.

Recife, de de 200_

Qualificação do declarante

Objeto da Pesquisa
Nome:.....
RG:..... .....Data de nascimento:..... / ..... / ..... Sexo: M ( )F ( )
Endereço:.....nº.....complemento:.....
Bairro:.....Cidade:.....Cep:.....Tel.:.....

_____
Assinatura do Declarante

DECLARAÇÃO DO PESQUISADOR

DECLARO, para fins de realização de pesquisa, ter elaborado este documento e que obtive, de forma apropriada e voluntária, o consentimento livre e esclarecido do declarante acima qualificado para a realização desta pesquisa.

Recife, de de 200_

_____
Pesquisador responsável

_____
testemunha 1 (membro não participante da pesquisa)

_____
testemunha 2 (membro não participante da pesquisa)

APÊNDICE B: Ficha do paciente HIV

FICHA DO PACIENTE HIV

Identificação:_____
Data da coleta:___/___/___
Endereço:_____
_____
Data de nascimento: ___/___/____ Idade:___ Cor:_____Sexo:_____
Estado civil: _____ Profissão:_____
Nacionalidade:_____ Naturalidade:_____
Telefones:_____

Tempo de infecção:_____

Categoria de exposição:

☐ Homossexual ☐ Heterossexual ☐ UDI Outra:_____

Carga Viral: _____ cópias/ml

Data do exame: ___/___/____

Contagem de células CD4+: _____ células/mm³

Data do exame: ___/___/___

Uso de medicação:

☐ Sim ☐Não

Qual?_____

Manifestações orais:
_____tempo de aparecimento:_____
_____tempo de aparecimento:_____

Declaro estar de acordo com todas as informações citadas acima:

_____ Data: ___/____/_____
Assinatura do paciente

_____
Assinatura do pesquisador (a)

APÊNDICE C: Ficha do paciente não HIV

FICHA DO PACIENTE NÃO HIV

Identificação:_____
Data da coleta:___/___/___
Endereço:_____
_____
Data de nascimento: ___/___/____ Idade:___ Cor:_____Sexo:_____
Estado civil: _____ Profissão:_____
Nacionalidade:_____ Naturalidade:_____
Telefones:_____

História Médica:
_____
_____
_____

História Odontológica:
_____
_____
_____

Outras manifestações orais:
_____tempo de aparecimento:_____
_____tempo de aparecimento:_____
_____tempo de aparecimento:_____
_____tempo de aparecimento:_____

Declaro estar de acordo com todas as informações citadas acima:

_____ Data: ___/____/_____
Assinatura do paciente

_____
Assinatura do pesquisador (a)

ANEXO A: Parecer do Comitê de Ética e Pesquisa da UFPE (CEP UFPE)

SERVIÇO PÚBLICO FEDERAL
UNIVERSIDADE FEDERAL DE PERNAMBUCO
Comitê de Ética em Pesquisa

Of. N. º 318/2008 - CEP/CCS — Recife, 08 de outubro de 2008

Registro do SISNEP FR – 207356
CAAE – 0225.0.172.000-08
Registro CEP/CCS/UFPE Nº 231/08
Título: "Estudo da transmissão salivar do EBV e CMV em pacientes co-infectados ou não pelo HIV".

Pesquisador Responsável: Talita Ribeiro Tenório de França

Senhora Pesquisadora:

Informamos que o Comitê de Ética em Pesquisa envolvendo seres humanos do Centro de Ciências da Saúde da Universidade Federal de Pernambuco (CEP/CCS/UFPE) registrou e analisou, de acordo com a Resolução N.º 196/96 do Conselho Nacional de Saúde, o protocolo de pesquisa em epígrafe, aprovando-o e liberando-o para início da coleta de dados em 07 de outubro de 2008.

Ressaltamos que o pesquisador responsável deverá apresentar relatório ao final da pesquisa (31/01/10).

Atenciosamente

Prof. Geraldo Bosco Lindoso Couto
Coordenador do CEP/ CCS / UFPE

A
Mestranda Talita Ribeiro Tenório de França
Mestrado em Odontologia – CCS/UFPE

ANEXO B: Normas da Revista Journal of General Virology

Journal of
General Virology

Journal of General Virology

Instructions for Authors – HTML version last modified 5 January 2010

Submission of papers for publication

Bench>Press online submission system:

Authors must submit their papers via our online submission and peer-review system, Bench>Press. Papers should be prepared as described in these Instructions. Click here for more details.

Enquiries concerning online submission and peer review should be directed to vir-bp-feedback@highwire.stanford.edu.

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 Continuous line numbering is used in the text to facilitate the peer-review process. This can be added in Microsoft Word on PCs by selecting **File: Page Setup: Layout: Line numbers: Add line numbering**. On Macs, select **File: Page Setup: Settings->Microsoft Word: Margins: Layout: Line numbers: Add line numbering**.

 If a single PDF or Word file containing any figures/tables is uploaded, the figures/tables must be included **at the end** of the file, **not** within the text.

 The paper complies with the restrictions on paper length. The total number of words in the main text of the paper, and in the Summary, together with the number of figures and tables, **must be stated** on the title page.

 Permission has been obtained for any citations of personal communications or unpublished results; this should be confirmed in a covering message.

 Citation of references in the text and References list conforms with JGV style.

 Use and definition of abbreviations conforms with JGV style.

 Papers that are cited, but not yet published, are supplied as additional files.

 Any supplementary material associated with the paper is supplied as a supplementary file, for peer review with the paper.

Papers that exceed the length limits will not be considered.

Any general queries should be directed to the Editorial Office staff: *Tel* +44 118 988 1833; *Fax* +44 118 988 1834; *E-mail* jgv@sgm.ac.uk.

Scope of the Journal

The *Journal of General Virology* (JGV) aims to publish papers that describe original research in virology and contribute significantly to their field. It is concerned particularly with fundamental studies. Papers must be in English. Standard papers, short communications and review articles are published.

Papers that describe new materials or methods without applying them to research are generally not acceptable. Preliminary or inconclusive data will not be published. Data that differ only in a minor way from previously published results are not acceptable; this applies, for instance, to comparisons of nucleotide sequences with closely similar published sequences. Papers dealing with clinical or epidemiological aspects of virology are welcome, provided that they contain new information on basic biological processes or virus–host interactions.

Prior publication

As part of the online submission process on Bench>Press, the individual submitting the paper will be asked to check a box confirming that material contained in the paper has not been previously published and is not under consideration for publication elsewhere. This is a required field; the submission cannot be completed until this box has been checked.

Authorship

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Authors may mount a PDF file of their accepted manuscript on their own or their institution's website or on a centrally organized repository (such as PubMed Central), provided that the PDF is not publicly available until 12 months after online publication in the journal. The PDF file must correspond exactly to the accepted version of the manuscript. Authors may not mount a PDF of the final published version (with the exception indicated below), although they should include a link to the published version. Author manuscripts must not be mounted less than 12 months after publication in the online version of JGV (again with the exception indicated below); nor must they be mounted on a server for the purpose of commercial sale or systematic external distribution by a third party (e.g. via an e-print server).

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Authors who pay for immediate open access through our Open Option scheme may deposit the published PDF file of their paper in PubMed Central (or other repositories) at the time of publication with no delay to public access, in addition to their paper being freely available to all without a subscription immediately on online publication.

Form of papers submitted

Restrictions on paper length

JGV publishes standard papers, short communications and reviews. These are restricted in length as described below, in order to improve the quality and clarity of the Journal. Any manuscripts submitted that exceed these limits will be returned immediately to the authors. The total number of words in the main text of the paper, and in the Summary, together with the number of figures and tables, **must be stated** on the title page. Papers lacking this information will not be reviewed until it is provided. New submissions, revised versions and resubmissions must **all** comply with these limits.

The Editors wish to emphasize that the review process and scientific criteria for acceptance of standard papers and short communications are identical.

Standard ('full-length') papers. The maximum permitted length of these is as follows: text (Introduction, Results, Discussion, Methods and figure legends, but excluding references and tables), 5500 words; number of tables and figures

combined, eight; summary, 250 words. There are no restrictions on the number of references.

From January 2010, the Methods section of standard ('full-length') papers should be included **after the Discussion**, and before any Acknowledgements and the References. This is now **compulsory** for all manuscripts submitted to JGV.

Standard papers are divided into the following sections: Summary, Introduction, Results, Discussion, **Methods**, Acknowledgements and References. Repetition of content between sections must be avoided. A combined Results and Discussion section is permitted.

Short communications. The maximum permitted length of these is as follows: main text (including figure legends, but excluding tables and references), 2500 words; number of tables and figures combined, three; summary, 150 words. There are no restrictions on the number of references.

Short communications must report completed work, not preliminary findings: they are an alternative format for describing smaller pieces of work. They contain Summary and Reference sections, but the main text is not divided into sections. Methods should be described briefly within the text, not within figure legends.

Review articles. These aim to give an overview of a particular subject suitable for a wide audience. The maximum permitted length of these is as follows: main text (including figure legends, but excluding tables and references), 11 000 words; summary, 250 words; number of references, 150. Most reviews are invited. Unsolicited reviews will be considered but intending authors should consult the Editor-in-Chief in advance.

General style and layout

In addition to reading the following, intending authors are advised to examine a current copy of the Journal for the layout and conventions used.

The past tense should be used throughout in describing new results, and the present tense in referring to previously established and generally accepted results. The *Concise Oxford Dictionary* (Oxford University Press) should be used as the standard for spelling and the CBE manual *Scientific Style and Format* (6th edn, Cambridge University Press) as an editorial guide. Authors are encouraged to use their own style of writing, provided that it is concise and conforms to normal English usage. Authors whose first language is not English are encouraged to have their paper checked by a native English speaker prior to submission.

Title page. The title page should provide the following information: title of paper, name(s) of author(s), name and address of the institution(s) where the work was performed, including the full postal address (plus telephone and fax numbers and e-mail address, if applicable) of the author to whom correspondence should be sent; a shortened running title of not more than 55 letters and spaces; and footnotes to indicate present addresses of any authors who are no longer at the institution where the work was performed. The name of the corresponding author will appear on the

title page of the published paper; the corresponding author's e-mail address will also be given, unless a specific request is made for these to be withheld.

Summary. The Summary is likely to be read by more people than the full paper, and many abstracting services use authors' summaries without modification. It is therefore important that this section is clear and comprehensible in its own right. The Summary should be in the form of a single paragraph. References should **not** be cited and any abbreviations used **must** be defined.

Introduction. The Introduction should give a concise background to the present study. It should not present an extended review (up-to-date review articles should be cited wherever possible), nor should it anticipate the Results or Discussion in any detail.

Results. This section should describe concisely the rationale of the investigation and its outcomes. Overall interpretation of the data belongs in the Discussion. The Results section may be divided into subsections with concise, descriptive titles.

Discussion. This section should relate results to previous work and interpret them. It can outline hypotheses based on the work reported. It must not repeat parts of the Introduction or recapitulate the Results section. The Discussion section may also be divided into subsections with concise descriptive titles. The Results and Discussion sections may be combined, and subsections used.

Methods (from January 2010, it will be **compulsory** for the Methods section to be included **after the Discussion**, and before any Acknowledgements and the References, in papers submitted to JGV).

This section should give sufficient information to allow others to repeat the work. It may contain subsections. Established methods should be described very briefly (for instance, with a reference) and novel methods should be given in greater detail. Suppliers of chemicals, biological materials and equipment should be indicated if this may affect the results. For bioreagents (e.g. virus strains, antibodies, cell lines), the source (culture collection, company or colleagues) must be documented adequately. Suppliers' addresses should **not** be given unless this is considered essential for a particular reason.

Acknowledgements. This optional short section should follow the **Methods**.

References. References in the text should be cited thus: two authors, Banks & Davies (2002) or (Banks & Davies, 2002); three or more authors, Banks *et al.* (2002) or (Banks *et al.*, 2002). References to papers by the same author(s) in the same year should be distinguished in the **text** and in the **References list** by the letters a, b, etc. (e.g. 2002a, or 2002a, b). Multiple citations in the text may be given in either alphabetical or chronological order, but the usage should be consistent throughout the paper.

In the References section, papers should be listed alphabetically by first author; papers with three or more authors should be listed in chronological order after any other papers by the first author. References must include the title of the paper, as

well as both initial and final page numbers. Titles of journals should be abbreviated according to the system used by Medline. No stops should be used after abbreviated words. References to books should include year of publication, title, edition, editor(s) (if any), place of publication and publisher, in that order. When the reference is to a particular part of a book, the inclusive page numbers and, if appropriate, the title of the article or chapter, must be given.

For papers with 11 or more authors, the names of the first nine only should be given; the remainder should be represented by '& other authors' (e.g. Banks, A., Davies, C., Franks, E., Smith, J., Brown, D., Garcia, F., Elliott, H. K., Jones, P. M., Evans. G. & other authors).

Example of a journal reference:

Hannoun, C., Norder, H. & Lindh, M. (2000). An aberrant genotype revealed in recombinant hepatitis B virus strains from Vietnam. *J Gen Virol* **81**, 2267–2272.

Example of a book chapter reference:

Kann, M. & Gerlich, W. H. (1998). Hepatitis B. In *Topley and Wilson's Microbiology and Microbial Infections*, 9th edn, pp. 745–774. Edited by L. Collier, A. Balows & M. Sussman. London: Arnold.

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EndNote output style
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Only papers that have been **accepted** for publication but not yet published can be cited as 'in press' in the References list, and they must be accompanied by the name of the journal. Any such papers must be included as supplementary files with the online submission. References to papers not yet accepted should be cited in the text as **unpublished results**, giving the initials and surnames of **all** authors. Such papers do not appear in the list of references. References to 'manuscript submitted' or 'manuscript in preparation' are not permitted. Permission must be secured for all personal communications that are cited in the text.

Tables. These should be employed selectively and should be generally comprehensible without reference to the text. Each Table should have a concise title; additional material should be given in a legend or footnotes as appropriate, but these should be brief and should not contain experimental detail that could be included in the text. The footnote symbols are *, †, ‡, §, ||, ¶ and #, to be used in that order.

Figures. This section gives general guidance on preparing figures for publication.The online submission guidelines give specific advice on preparing figures for inclusion in a PDF file for submission, where file sizes need to be kept below 1 MB. Figures must be designed for both clarity and economy of space. Where lettering is included, a single font should be used throughout (sans-serif fonts, such as Helvetica or Arial, are preferred for clarity). Legends must not repeat the Methods section. Parts of a

single figure should be designated (a), (b), etc., and labelled as such on the figure. Figures should be designed to fit into either one or two columns on the journal page. Maximum printed sizes (width × height), including lettering, are 84 × 235 mm or 176 × 235 mm.

Line drawings. These should be of a quality suitable for direct reproduction and approximately twice the size that they will appear. Line drawings should be submitted as (i) original drawings in black ink on white paper; (ii) glossy photographs; or (iii) high-quality computer-generated figures. Symbol sizes and line thicknesses must be chosen for clear visibility after reduction. Symbols should be defined in the legend, rather than on the axes. Where possible, the same point symbols should be used for comparable variables in different figures. The preferred symbols are ○, ●, □, ■, △, ▲, ▽and ▼. With computer-generated figures, special care should be taken over the use of shading and hatching. Tints (shading made up of fine dots) can make file sizes very large, and they often do not reproduce well when printed in the journal. The use of tints should therefore be avoided if possible. If tints are used they should have a screen value of 100 lines per inch or lower.

Sequence data and schematic diagrams. Diagrams of nucleotide and amino acid sequences and schematic diagrams of, for example, genome structure and organization, should be prepared 'camera-ready' with a good-quality printer. The layout should be designed to fit the journal page economically, i.e. to fill either the full width of the page (176 mm) or a single column (84 mm). The height of the characters should be about 1.5–2 mm (or 6–8 point). For printing sequence data at full-page width with this size of type, a layout with 80–100 nucleotides per line is appropriate (or 60–70 if there are spaces between the codons). For a single-column layout, 50–60 nucleotides is about right. The lines of sequence should be as close as is consistent with clarity. Use of the single-letter amino acid code is preferred. See below for guidelines on reporting sequence data.

Photographs. These should be well-contrasted and approximately final size (maximum width 176 mm). For photomicrographs, magnification should be shown by a bar marker.

Digitally generated images. It is usually possible to obtain better reproduction from graphics files than from hard-copy printouts made from these files. The resolution should be at least 300 d.p.i. The preferred file format is TIFF or EPS, but a considerable range of file types generated by various graphics programs can, in principle, be handled; authors should seek detailed guidance from the Editorial Office. In certain cases it may, however, be necessary for appropriate hard-copy images to be supplied.

Where images of gels, autoradiograms etc. have been digitally generated or processed, either directly or after being scanned, the hardware and software used must be stated.

Colour photographs. Colour figures are reproduced free of charge if the use of colour is considered by the Editors to be necessary for scientific reasons. Graphics files for colour images should, if possible, be supplied as CMYK, not RGB files.

Supplementary data

Online data that usefully supplement the contents of a paper may be referred to either by including the URL of the relevant website in the paper or by attaching supplementary data files (e.g. tables, figures, sequences, video etc.) to the published paper in JGV Online. In the latter case, the material should be supplied as a supplementary file for peer review along with the paper: authors should contact the Editorial Office for details.

Processing of papers

Reviewing and scientific editing

During the online submission process, authors may select up to two Editors who would be appropriate to handle their paper. To do this, the relevant Editor(s) should be selected from the drop-down list. If you are unsure which Editor would be most appropriate, the fields should be left blank (reading 'Suggest Editor...'). You may also exclude up to two Editors who you do not wish to handle your paper. If you do not select an Editor, the Editorial Office will select an appropriate Editor for you. You will then be sent an acknowledgement stating the Editor's name and contact details, together with the paper's reference number. Any subsequent queries should be directed to the Editor. A list of the Editors' interests and contact details is available here.

After preliminary examination to establish that a paper appears to be a competent submission within the scope of the Journal, the Editor will assign it to two or more independent reviewers for review online. These reviewers will review the paper for originality and significance of the work described and judge its acceptability for publication. There are three possible recommendations: accept, conditional accept (revise) and reject. Conditional accept implies that the manuscript requires modifications that could be carried out within 6 weeks. The reviewers may also make critical comments and, where necessary, suggest improvements or additional experiments that could be done in support of the findings. However, it is the Editor who makes the final decision as to the acceptability of the paper.

Papers are reviewed as quickly as possible, but authors should not usually expect to receive a decision in less than 6 weeks.

Electronic files for accepted papers

Authors will be asked to upload a word-processor file, plus separate electronic files of figures, when they submit the revised version of their paper. Details of any extra files or information needed will be sent by the Editorial Office when acceptance is confirmed (see also online submission guidelines for extensive additional information). Advice on file types for figures is given above.

Copy-editing

Once accepted, a paper is copy-edited by the Editorial Office staff in preparation for printing. This is done by making use of a word-processor file uploaded to

Bench>Press by the author when the revised manuscript is submitted. Papers are copy-edited to the extent necessary to achieve clarity of expression and to conform with the Journal's conventions and standard scientific nomenclature. No changes of substance are made without the authors' consent.

Proofs

Proofs are e-mailed as a PDF file. The PDF file should be printed and corrections marked on the paper copy. No attempt should be made to edit the PDF file electronically. The corrected proof should be sent to the Editorial Office within 3 days (by airmail from outside Europe). If necessary, corrected pages may be sent by fax (+44 118 988 1834) to the Editorial Office or minor corrections may be communicated by e-mail (jgv@sgm.ac.uk), but the corrected proof should still be mailed. A copy of the proof should be retained by the authors. Extensive changes at the proof stage are not permitted. Authors may be charged for correction of their non-typographical errors.

Offprints

Twenty-five offprints are provided free of charge. Additional copies may be ordered at prices given on the order form that accompanies the proofs. The order form should be completed and returned to the Editorial Office with the proof, irrespective of whether additional offprints are required. Later orders are subject to increased prices. Click here to download an Offprint Order form.

Charges

There are no handling or page charges.

Ethics of human and animal work

A paper describing any experimental work with humans should include a statement that the Ethics Committee of the institution in which the work was done has approved it, and that the subjects gave informed consent to the work.

Experiments with animals should be done in accordance with the legal requirements of the relevant local or national authority. Procedures should be such that animals used in experiments do not suffer unnecessarily. Papers should include details of the procedures and anaesthetics used. The Editors will not accept papers where the ethical aspects are, in their opinion, open to doubt.

During the online submission process, authors will be asked to confirm either that their paper complies with these requirements, or that the question is not applicable (i.e. no human or animal experimentation is reported in the paper).

Reagent sharing

By publishing in JGV, authors agree that any reagents such as viruses, plasmids, antibodies and living materials, such as cell lines or bacterial strains, that are mentioned within the article are available without unnecessary delay and at a

reasonable cost to members of the scientific community for non-commercial purposes.

Reproducibility of results

An indication of this should be given. Variability should be indicated statistically wherever possible; when error terms are given, the measure of dispersion and the number of observations should be stated. Statistical techniques used must be specified.

Abbreviations

Abbreviations for units and virus names are dealt with below. The use of abbreviations is permitted in JGV, but they should aid the reader and not simply be a convenience to the author; therefore, their use should be limited. As a general rule, if the abbreviation is used less than three times in the text, it should be removed. All abbreviations should be defined in full and introduced in parentheses at the first mention in both the summary and the main text. For example, 'cells were cultured in Dulbecco's modified Eagle's medium (DMEM).' To download a PDF file listing permitted and compulsory abbreviations, click here.

Quantities, units and symbols

The recommended SI units should be used. Concentrations should be indicated by the use of superscripts, rather than a solidus: e.g. mg ml^{-1} rather than mg/ml. There should be a space between a number and a unit, e.g. 3.2 kb **not** 3.2kb; 37 °C **not** 37°C.

Molecular mass, M_r and K

The mass of a molecule should be specified in one of two ways:

(i) M_r ('relative molecular mass'). This is the ratio of the mass of a molecule to 1/12 of the mass of ^{12}C; it is thus a pure number and has no units.

(ii) Molecular mass (**not** 'molecular weight'). This is the absolute mass of a molecule expressed in daltons (Da); the dalton is defined as 1/12 of the mass of ^{12}C. A protein can be said to have an M_r of 20 000 or a molecular mass of 20 kDa. It is incorrect to express M_r in daltons or to use 'K' to represent an M_r of 1000 or 1 kDa. A number followed by K (e.g. 15K) may be used as a designation of a compound. This designation must be defined when it first appears: e.g. 'the protein with an M_r of 15 000 / molecular mass of 15 kDa (15K protein)'.

Virus nomenclature

Virus names should be given in full in the title of the paper and at their first occurrence in the Summary and in the main body of the text. Where appropriate, a precise strain designation should be included. Names should follow the standard

nomenclature set out by the International Committee on Taxonomy of Viruses. This web page also includes the standard abbreviations for viruses.

For further explanation, click here to download Taxonomy is taxing (PDF file, 19 KB) (adapted from M. H. V. van Regenmortel).

Other nomenclatures

Chemical and biochemical

Authors should follow the recommendations of IUPAC for chemical nomenclature, and those of the Nomenclature Committee of IUBMB and the IUPAC–IUBMB Joint Commission on Biochemical Nomenclature for biochemical nomenclature. A summary of nomenclatural recommendations, with references, is given in the *Biochem J* Instructions for Authors. The recommendations are given in full in *Compendium of Biochemical Nomenclature and Related Documents*, 2nd edn (1992), London: Portland Press.

Enzyme

The system published in *Enzyme Nomenclature* (1992), London & New York: Academic Press, and its supplements is used. Enzyme Commission numbers should be given where appropriate.

Immunology

Immunological terms such as interleukin, interferon etc. should, at first mention, be defined in full, followed by the appropriate abbreviation (note, in particular, the use of hyphens):

Alpha interferon (IFN-α)
Interleukin-1 (IL-1)
Tumour necrosis factor alpha (TNF-α)

Genes and proteins

Authors should be guided by the recommendations of M. Demerec *et al.* (*Genetics* **54**, 61–76, 1966) where these are relevant to virus genetics. Particular care should be taken to distinguish between genes (e.g. *gag*) and the proteins that they encode (e.g. Gag, $p15^{gag}$).

Papers reporting sequence data

Such papers should be accompanied by substantial additional experimentation to characterize the gene(s) and product(s) concerned, and/or substantial comparable analysis. A sequence alone is unlikely to be acceptable.

Papers reporting new sequence data will not be published unless the sequence has an accession number from a recognized nucleotide database. Manuscripts containing sequence data should include, on the title page, the footnote 'The

GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is X00000'.

Comparison of sequence data

When making comparisons between nucleotide or amino acid sequences, it is important to use the correct terminology. 'Homology' has a precise biological meaning of 'having a common evolutionary origin'. When a percentage comparison is made, the terms identity or similarity, as appropriate, must be used.