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Detection of human herpesvirus-7 by qualitative nested-PCR: comparison between healthy individuals and liver transplant recipients

Detecção de herpesvirus humano-7 por *nested*-PCR qualitativo: comparação entre indivíduos sadios e receptores de transplante hepático

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ABSTRACT

Diagnosis of human herpesvirus-7 active infection in transplant patients has proved difficult, because this virus is ubiquitous and can cause persistent infections in the host. The significance of viral DNA detected in leukocytes by PCR is unclear and cross-reaction in serological tests may occur. This study aimed to evaluate nested-PCR to detect human herpesvirus-7 active infection in liver transplant recipients compared to healthy individuals. Human herpesvirus-7 nested-PCR was performed on leukocytes and sera of 53 healthy volunteers and sera of 29 liver transplant recipients. In healthy volunteers, human herpesvirus-7 was detected in 28.3% of leukocytes and 0% of serum. Human herpesvirus-7 was detected in sera of 48.2% of the liver transplant recipients. Nested-PCR on DNA extracted from leukocytes detected latent infection and the study suggests that nested-PCR performed on serum could be useful to detect human herpesvirus-7 active infection in liver transplant recipients.

Key-words: Human herpesvirus-7. Healthy individuals. Nested-polymerase chain reaction. Liver transplantation.

RESUMO

Diagnóstico da infecção ativa pelo herpesvirus humano-7 é difícil devido ao fato deste vírus ser ubíquo e poder causar infecção persistente no hospedeiro. O significado da detecção do DNA viral por reação em cadeia da polimerase não é claro e, reações cruzadas podem ocorrer em testes sorológicos. O objetivo deste estudo foi avaliar a *nested*-PCR para detectar infecção ativa pelo herpesvirus-7 em receptores hepáticos comparando com indivíduos sadios. *Nested*-PCR para herpesvirus-7 foi realizado em leucócitos e soro de 53 voluntários sadios e em soro de 29 receptores hepáticos. Nos voluntários sadios, herpesvirus-7 foi detectado em 28,3% de leucócitos e 0% de soro. herpesvirus-7 foi detectado em soro de 48,2% de receptores hepáticos. *Nested*-PCR em DNA extraído de leucócitos detectou infecção latente e o estudo sugere que *nested*-PCR realizada em soro poderia ser útil para detectar infecção ativa por herpesvirus-7 em receptores de fígado.

Palavras-chaves: Herpesvirus humano-7. Indivíduos sadios. *Nested*-reação em cadeia da polimerase. Transplante hepático.

Human herpesvirus-7 (HHV-7) was first isolated by Frenkel *et al*[5] from activated CD4$^+$ peripheral blood T cells of a healthy individual. It is a member of the *betaherpesvirinae* subfamily of the *Betaherpesviridae* (DNA virus). Both, HHV-7 and HHV-6 (Human herpesvirus 6) primary infections cause common febrile infectious syndromes of early childhood, known as *exanthem subitum* and roseola[16]. Investigations conducted in the United States[8] and Mexico[11] presented HHV-7 seroprevalence rates of 65% and 98%, respectively. In Brazil, Freitas *et al*[4] found a HHV-7 seroprevalence rate of 93.3% in individuals > 10 years of age.

Similar to other betaherpesviruses, HHV-7 frequently remains latent in the host and can reactivate during immunosuppression following organ transplantation[9]. The most well-known member of the betaherpesviruses is human cytomegalovirus (HCMV) and it is considered an important cause of morbidity and mortality in solid organ and bone marrow transplantation. Although the role and impact of HCMV infection on the posttransplant course is well characterized, the role of HHV-7 in transplant patients remains unclear[6,9].

Diagnosis of HHV-7 active infection in transplant patients has proved difficult, because this virus is ubiquitous and can

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cause persistent infections in the host. The significance of viral DNA detected in peripheral blood leukocytes by polymerase chain reaction (PCR) is therefore unclear[2]. The interpretation of serological results is complicated by the fact that cross-reaction with other herpesviruses may occur[14]. Several methods and different biological materials have been proposed to detect HHV-7 active infection. Nested-PCR using DNA extracted from either serum or plasma could be used to detect only active infection[9,12], but some authors have suggested the use of quantitative PCR, such as Quantitative Competitive PCR[7], and Real-Time PCR[13].

The aim of this study was to evaluate the efficacy of nested-PCR to detect and to monitor HHV-7 active infection in liver transplant recipients compared to healthy individuals.

MATERIAL AND METHODS

Healthy volunteers. Peripheral blood samples were obtained from 53 adult healthy volunteers (23 men and 30 women), median age 22 years-old (range 18 to 42). This cohort was represented by undergraduate and graduate students who presented no history of systemic disease, infectious or noninfectious chronic diseases, autoimmune syndromes and had not used drug therapy. Individuals who presented fever, rash, arthralgia and others signs of infections were excluded from the study. Ethylenediamine tetraacetic acid (EDTA)-treated blood samples were used to DNA extraction from peripheral blood leukocytes. Serum from each blood sample was also obtained and then frozen (-20ºC) until testing.

Liver transplant recipients. Twenty-nine adult liver transplant recipients (20 men and 9 women), median age 47 years-old (range 18 to 66), transplanted at the Liver Transplant Unit (University Hospital, State University of Campinas (UNICAMP), Campinas, São Paulo, Brazil) between 2003 and 2005, were included in this study. The basic immunosuppressive therapy consisted of a combination of steroids, azathioprine and cyclosporine; tracolimus (FK) and mycophenolate mofetil (MMF) were prescribed based for selected patient characteristics and specific protocol studies. Acyclovir and ganciclovir were used as antiviral prophylaxis for *Herpes simplex* and treatment of symptomatic cytomegalovirus infections, respectively.

Peripheral blood was obtained from the patient at the time of transplantation and then weekly for the first month and once a month up to 180 days. Serum from each blood sample was separated by centrifugation. The sera obtained were frozen (-20ºC) until testing. The protocol was designed in accordance with the requirements for research involving human subjects in Brazil and was approved by the Institutional Ethics Committee of the Faculty of Medical Sciences, UNICAMP.

Peripheral blood leukocytes DNA extraction. Briefly, peripheral blood leukocytes (PBL) were obtained by centrifugation after erythrocyte lysis (2.5ml of blood). The PBL pellet ($15x10^5$ cells) was washed with PBS (Phosphate Buffered Saline), lysed and the DNA precipitated by cold ethanol. The resulting DNA pellet was eluted in 50µL of TEB-buffer (Tris-EDTA-Borate).

Serum DNA extraction. Briefly, DNA was extracted from 200µL of serum using a phenol-chloroform protocol after incubation overnight in lysis buffer (containing SDS and proteinase K) at 56ºC followed by DNA precipitation with cold ethanol[8]. The resulting DNA pellet was eluted in 50µL of TEB-buffer.

Human herpesvirus-7 nested-PCR. Five µL of DNA extracted from each sample, as described above, were used in the nested-PCR protocol previously described by Pozo *et al*[15], with some modifications (originally a multiplex-PCR). Amplifications were performed in a Peltier Thermal Cycler (MJ Research, Watertown, MA, USA). The nested-PCR product was analyzed under UV light after electrophoresis in 2% agarose (Gibco-BRL) stained with ethidium bromide. All primer sequences and PCR products were analyzed using the Genbank database before initiating the study. All nested-PCR was performed in duplicate using a second fresh aliquot. PCR with primers for beta-globin gene amplification was performed on PBL samples to detect possible false-negative results, which were not included in this study[1].

Comparison of categorical variables was realized using the Fisher exact and Chi-square tests. A p-value < 0.05 was considered statistically significant.

RESULTS

The base sequence analysis of primers and nested-PCR products showed compatibility with HHV-7 genome, thus confirming primer specificities. Figure 1 shows agarose gel electrophoresis of positive nested-PCR samples.

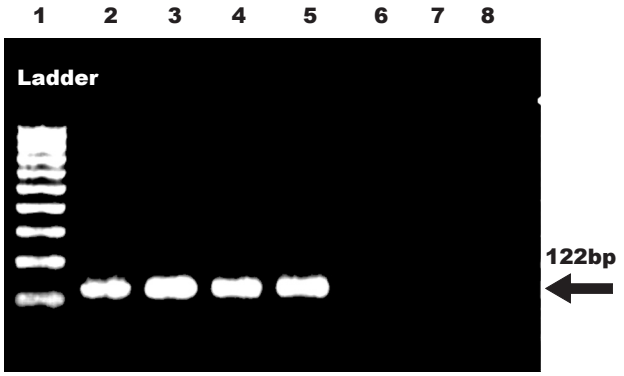

Figure 1 - Agarose gel electrophoresis of human herpesvirus-7 nested-polymerase chain reaction of 4 positive samples, stained with ethidium bromide and photographed under UV Light. Lane 1, denotes molecular weight (Ladder); lanes 2-5, display positive samples; Lanes 6-8, negative samples.

Healthy volunteers. HHV-7 nested-PCR was positive in 15/53 (28.3%) DNA samples extracted from PBL. Of these individuals, 7 (46.6%) were males and 8 (53.3%) were females, a difference that was not statistically significant ($p= 0.49$). Age also had no influence on positive or negative results. None of the samples were positive for DNA extracted from the serum.

Liver transplant recipients. HHV-7 nested-PCR was positive in DNA extracted from serum in 14/29 (48.2%) patients during monitorization, a total of 21/189 (11.3%) samples; the median

time to first HHV-7 detection was 19 days (range 0 to 170). After transplantation, HHV-7 nested-PCR was positive in 8/29 (27.6%) patients and 6/29 (20.7%) were already positive on the day of transplantation. HHV-7 DNA detection after liver transplantation was significantly higher than in the healthy group ($p<0.004$). The kinetics of HHV-7-DNAemia detection during follow-up (pre-transplant to 180 days after liver transplantation) is shown in Figure 2.

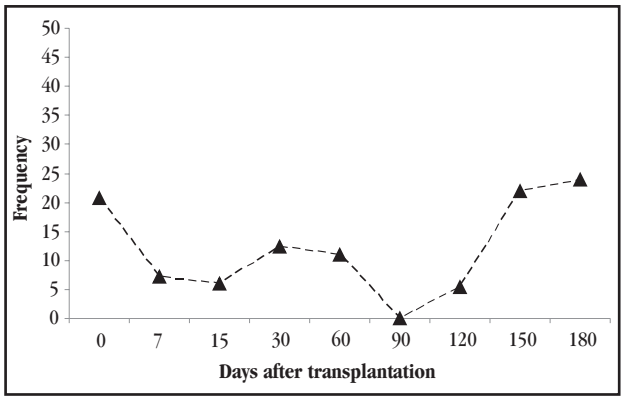

Figure 2 - Kinetics of human herpesvirus-7-DNAemia detection during follow-up (pre-transplant to 180 days after liver transplantation). Triangles indicate the frequency of positive HHV-7 nested-PCR found at each time interval.

DISCUSSION

The primers used showed satisfactory specificities and no amplification of any other herpesviruses was observed (data not shown). The HHV-7 detected in PBL of healthy volunteers by nested-PCR in this study (28.3%) was lower than data reported by others authors. Kozireva *et al*[10] reported HHV-7 DNA detected by PCR in 43.3% of blood donors and Chapenko *et al*[3] described 63.2% of positive PCR in PBL of patients before renal transplantation. However, HHV-7 latent infection is characterized by a low copy number in peripheral blood leukocytes[17], which could be lower than sensitivity of the PCR technique use here, since it detects viral loads of 10-100 copies[5]. The frequency of HHV-7 in the cohort included in this study was not evaluated by other methods, but it might be lower than in populations analyzed in other studies. In addition, the rate of chromosomally integrated HHV-7-DNA and nonintegrated viral DNA persistence after primary infection in host cells of healthy individuals remains poorly studied.

Studies of seroprevalence associated with virus isolation, HHV-7 DNA detection in saliva[18] or nested-PCR in lymphocytes purified by density-gradient could be performed to clarify certain hypotheses and other primers should be studied. No difference was found among the individuals regarding gender and age, probably because all the volunteers were adults.

None of the serum samples were positive by HHV-7 nested-PCR (free cell samples) in healthy volunteers, suggesting that nested-PCR in serum could be a useful marker to detect HHV-7 active infection. Nested-PCR in PBL probably detected latent infection in healthy volunteers. Free viral DNA is not usually found in the plasma or serum of individuals with latent infection[17]. The cohort enrolled in this study consisted of individuals who presented no signs of any infectious or noninfectious syndromes which you would expect not to find in individuals with HHV-7 active infection.

Of the 29 liver transplant recipients, 8 (27.6%) were positive for HHV-7 by nested-PCR in serum after transplantation, showing that HHV-7 active infection was significantly higher than in the healthy group. During the entire follow up, HHV-7 nested-PCR was positive in 14/29 (48.2%) patients at any time. In liver transplant recipients, Griffiths *et al*[6] observed positive qualitative PCR in 48% of the patients and Ihira *et al*[9] reported positive PCR in 40% of the patients up to 8 weeks after transplantation. Tong *et al*[16] observed positive HHV-7 PCR in 35.1% of renal transplanted patients.

The difference rates observed in each report depend on PCR sensitivity, type of transplantation, the size of samples used and differences among subjects. However, the rate found in this study was in agreement with other reports. Interesting, 6/29 (20.7%) patients presented positive PCR on the day of transplantation. Considering that this method did not detect latent infection and blood was collected before surgery, this could be explained by multiple blood transfusions before transplantation or reactivation caused by underlying liver disease. Although some syndromes related to HHV-7 in immunocompetent patients have been described[17], further research in the pretransplant period should be performed to evaluate each hypothesis. Since HHV-7 specific clinical syndrome spectrum in liver transplant remains still unclear, positive and negative predictive values for nested-PCR were not conclusive. Unfortunately, viral isolation or Real Time PCR was not realized for comparison with nested-PCR. However, the determination of cutoff values for quantitative PCR to discriminate HHV-7 active latent infection has been considered problematic[2], given that HHV-7 active infection may occur without clinical signs or laboratorial findings[14]. Viral isolation is not commonly used as a diagnostic method; moreover, HHV-7 viral isolation is considered specific but of low sensitivity.

In conclusion, nested-PCR in DNA extracted from PBL detected latent infection. The study suggests that given the populations analyzed and characteristics of the same, nested-PCR performed on serum could be useful to detect HHV-7 active infection in liver transplant recipients.

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