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Betaherpesviruses in Adult Liver Transplant Recipients

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1. Introduction

Liver transplantation similar to other allograft transplants requires the use of immunossupressive therapy to avoid graft rejection in the host. Immunossupressive drugs can also decrease the capacity of the host immune system to response against infectious agents which would not be a problem to immunocompetent persons. Many infectious agents such as bacteria, fungus, protozoa and viruses can cause serious complication in the post-transplant course (Blair & Shimon, 2005).

Several different viruses have been studied at long of the time and these studies have demonstrated that herpesviruses can be important infectious agents and affects the management of the liver transplant recipients (Kotton, 2010).

Herpesviruses belong to the *Herpesviridae* family (Hudnall et al., 2008), and have been isolated eight different types of these viruses (Table 1). The human herpesvirus simples type I and type II (HSV-1 and HSV-2), are usually associated with labial and genital herpes, respectively. However, genital herpes can be a consequence of HSV-1 infection and labial herpes can also be caused by HSV-2. The human herpesvirus type 3 (varicella-zoster) causes chickenpox, especially in children, and re-infection or reactivation, may be the cause of the appearance of zoster. Human herpesvirus type 4 (Epstein-Barr virus) is associated with infectious mononucleosis syndrome, Burkitt's lymphoma and nasopharyngeal carcinoma. The human herpesvirus type 8 is associated with Kaposi's sarcoma, and can cause death in immunosuppressed individuals, especially in acquired immunodeficiency syndrome (HIV/AIDS).

Cytomegalovirus (CMV), Human Herpesvirus 6 (HHV-6) and Human Herpesvirus 7 (HHV-7) are DNA viruses, members of the *betaherpesvirinae* subfamily of the *Betaherpesviridae* (Tong et al., 2000). Cytomegalovirus primary infection causes 'mononucleosis like syndrome' and

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HHV-6 and HHV-7 primary infections cause common febrile infectious syndromes in early childhood, known as *exanthem subitum* and roseola.

Virus	Synonymous	Sub-family	Abbreviation
Human herpesvirus -1	Herpes simplex-1	α	HSV-1/HHV-1
Human herpesvirus -2	Herpes simplex-2	α	HSV-2/HHV-2
Human herpesvirus -3	Varicella-zoster	α	VZV/HHV-3
Human herpesvirus -4	Epstein-Barr	γ	EBV/HSV-4
Human herpesvirus -5	Cytomegalovirus	β	CMV/HHV-5
Human herpesvirus -6	None	β	HHV-6
Human herpesvirus -7	None	β	HHV-7
Human herpesvirus -8	None	γ	KSHV/HHV-8

Table 1. Complete list of the human herpesviruses

Betaherpesviruses are ubiquitous and seropositivity for these viruses can differ dependently of geographical region and other characteristics of the studied cohort. CMV seroprevalence is largely known around the world while HHV-6 and HHV-7 seroprevalences remain less studied. However, is estimated that HHV-6 and HHV-7 prevalences would also be high in the majority of the places.

In immunocompetent individuals, beteherpesviral primary infections are usually self-limiting although some cases of neurological manifestations have been described especially regarding HHV-6 in children (Donati et al., 2003; Matsumoto et al., 2011). It is not clear whether the neurological manifestation is caused by herpesviral brain tissue invasion or is an indirect effect of the infection.

After primary infection, betaherpesviruses remain latent in the host and could reactivate sporadically leading to a transient viremia. Although some syndromes (e.g. chronic fatigue syndrome and multiple sclerosis) have been related to herpesviral reactivation, the role of the viruses in these syndromes remains unclear (Dewhurst, 2004).

CMV, HHV-6 and HHV-7 can more frequently reactivate during immunosuppression following organ transplantation (Tong et al., 2000). CMV infection is known as major infectious complications after transplantation and has been considered an important cause of morbidity and mortality in bone marrow transplantation and solid organ transplant recipients. Although the role and impact of CMV infection on the post-transplant course is well characterized, the other two members of the betaherpesviruses family have been acknowledged only recently.

1.1 Cytomegalovirus

The diseases caused by CMV occur in underdeveloped and developing countries and the prevalence varies from 40 to 60% in the northern hemisphere countries, while in Africa and Latin America rates from 80 to 100% were observed (Suassuna et al., 1995; Costa et al., 1999). About 80% of the population between the late childhood and early adolescence is already infected by CMV (Almeida et al., 2001) and can harbor the virus in several body sites, especially in the salivary glands and different types of leukocytes. The peripheral blood mononuclear cells appear to be the most important site for CMV latency.

There are different variants or genetically distinct strains of CMV and therefore the cross-protective immunity is considered partial (Ishibashi et al., 2006). The possibilities for the occurrence of a new exposure to another CMV strain are numerous. Immunosuppressed patients can be submitted to transfusions of blood components containing latent viruses, they may receive bone marrow or solid organs containing CMV and, in some cases, undergoing dialysis in equipment contaminated with viruses. It is for this reason that has been verified that the rate of cytomegalovirus infection/reinfection in these circumstances can be high (approximately 50%).

In healthy adults, CMV is usually asymptomatic. Some individuals may have symptoms similar to infectious mononucleosis syndrome, such as lymphadenopathy, fever, rash, malaise, arthralgia, hepatomegaly and splenomegaly. In immunocompromised patients, CMV may modulate the immune response and leads to more complex clinical presentation including death, dependently of the situation involved.

The American Society of Transplantation classified the presence of CMV in the body into two situations (Kotton et al., 2010).

-   CMV infection: evidence of CMV replication regardless of symptoms (different from latent CMV).
-   CMV disease: evidence of CMV infection associated with symptoms. CMV disease can be further categorized as a viral syndrome with fever, malaise, leukopenia, thrombocytopenia or as a tissue invasive disease.

Whereas liver transplantation, epidemiological studies demonstrate a high incidence of CMV active infection. Some facts should be considered, for example, the previously infected patients who receive organs from donors with genotypically distinct latent viruses may develop a new infection. In addition, the surgical stress generated by the transplantation procedure may lead to a reactivation of latent CMV (Kotton et al., 2010).

CMV disease is considered one of the most common complications after liver transplant recipients, with significant morbidity and mortality (Thomasini et al., 2007). Studies in the transplant series have shown that higher viral load values correlate with increased risk for development of disease.

Thus, sensitive techniques were described in an attempt to identify earlier individuals who have higher risk to development of CMV disease with the goal to reduce the severity of the cases. The direct detection of the virus by conventional techniques, urine or saliva, is a procedure with limited clinical value. Moreover, it is technically difficult, expensive and provides results only after 3-5 weeks. Culture of CMV in blood or urine has low sensitivity.

Culture of tissue samples is an option for confirmatory diagnosis of invasive diseases, especially in the case of gastrointestinal manifestations, in which, generally molecular and antigen-based diagnosis are negatives (Kotton et al., 2010).

Before liver transplantation, serology for CMV can be used in both the organ donor and the recipient. A quantitative test for anti-CMV IgG should be used in combination with IgM test due to IgG serological tests are more specific than IgM tests. The serology of donor and recipient is the key to predicting the risk of infection. In the case of donor and recipient were sero-negative during the pre transplant, serology should be repeated at the time of transplantation, if there is a significant time between screening and transplantation.

However, recent blood transfusion could present false results in the serological tests. In patients after liver transplantation, serology has no role in diagnosis of active CMV disease.

The detection of CMV antigen matrix (pp65) –antigenemia is a technique highly sensitive, rapid, quantitative, and with significant clinical correlation (van der Bij et al., 1988; Bonon et al., 2005). Patients who present positive results can be submitted to antiviral therapy and the response can be monitored periodically to demonstrate the efficacy of the treatment and the possibility of drug resistance. The limitation may be the definition of a limit of positivity ('cut-off') to start the treatment. Moreover, neutropenia can raise difficulties to perform this technique due to the fact that antigenemia requires a sufficient number of neutrophils to detect CMV viral antigen. CMV causes an abortive replication within neutrophils and leads to uptake of antigen in perinuclear area (Kas-Deelen et al., 2001) which can be detected by use of monoclonal antibodies against pp65-antigen. Either fluorescent or enzyme labelled conjugate can be used to reveal the reaction. However, enzyme labelled conjugate can be revealed by coloured reaction and dispenses the use of ultraviolet microscope. Figure 1 shows positive pp65-antigenemia using enzyme labelled conjugate.
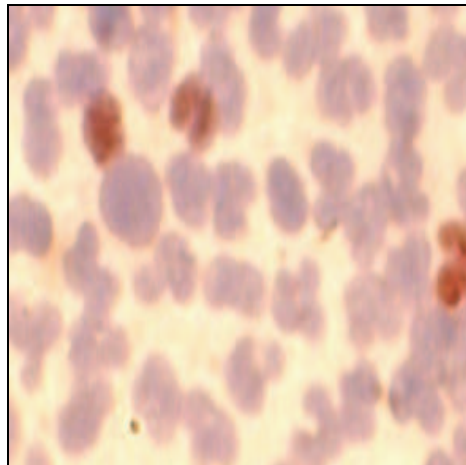

Fig. 1. Nuclei of neutrophils stained in brown indicating positive pp65-atigenemia (counterstained with Harris´s hematoxylin). Mouse C10 and C11 monoclonals atibody against pp65-matrix CMV antigen and rabitt anti-mouse Ig horseradish peroxidase conjugate. The reaction was revealed by hydrogen peroxide and amino-ethyl-carbazole (Sampaio et al., 2011)

Molecular techniques such as Polymerase Chain Reaction (PCR) and Nucleic Acid Sequence Based Amplification (NASBA) had gradually been incorporated in the laboratorial diagnosis of CMV.

Whereas the viral biology, it is necessary to demonstrate the presence of viral mRNA, or portions of the viral genome expressed only in the replicative phase and not in latency. Using PCR the sensitivity of the PCR should be adjusted to detect only significant viral loads which could not easily performed. Unfortunately, due to very high sensitivity, molecular techniques may reveal positive results without relevant clinical features (Thomasini et al., 2007). In patients with higher risk level to progression of the CMV disease, such as liver transplantation, positive results in molecular tests can be an indicative to introduction to the preemptive therapy despite some of these patients not have clinical manifestation.

Antigenemia has been considered to be less sensitive than molecular tests although has significant clinical correlation. In other hand, the molecular techniques are more sensitive, but may be dissociated of clinical manifestation in some situations. Thus, in patients at high risk (liver transplant recipients, CMV sero-negative patients who received organs from sero-positive patients, patients who used mycophenolate or anti-OKT 3) would be benefited whether monitored by molecular techniques or by antigenemia relying on lower 'cut-off' levels.

More recently, real-time PCR has been considered faster, very sensitive and provides more accurate discrimination than other molecular techniques. However, the establishment of the 'cut-off' levels to discriminate between significant viral load and transient viremia is also necessary. Moreover, real-time PCR has been considered expensive and requires specialized staff. Either plasma or whole blood specimens can provide diagnosis and prognostic information regarding CMV disease. Qualitative PCR is an option for surveillance if this technique is the unique available option. The diagnosis of tissue invasive CMV disease, should be confirmed by immunohistochemistry or *in situ* DNA hybridization The decision regarding which test to use will depend on many factors including available resources, technical staff, patient population, volume of samples tested and cost (Kotton et al., 2010).

The gold standard for treatment of CMV is intravenous ganciclovir, although oral valganciclovir is non inferior in nonlife-threatening disease. In patients with life-threatening CMV disease and in children, intravenous ganciclovir still the preferred drug, because data on the effect of oral treatment are limited. The treatment should be monitored weekly by viral loads and treating must continue until one or two consecutive negative samples are obtained, but not shorter than 2 weeks (Kotton et al., 2010).

Universal prophylaxis involves the administration of antiviral drugs to overall of patients or a subset of "at risk" patients. Antiviral administration are usually started in the immediate or very early post transplant period and continued about 3 to 6 months. Several antivirals have been used, including acyclovir, valacyclovir intravenous ganciclovir, oral ganciclovir and valganciclovir. In the preemptive therapy, laboratory monitoring detects asymptomatic viral replication and antiviral therapy is initiated to prevent the progression to clinical disease. One of the major concerns with preemptive therapy is that it may not prevent the indirect effects on graft and patient survival.

Dosing of antiviral medications should be based on standard recommended dosing algorithms (for patients with normal creatinine clearance: valganciclovir 900 mg once a day, intravenous ganciclovir 5 mg/kg once a day, or oral ganciclovir 1,000 mg three times a day) and carefully adjusted for renal function.

Drug resistance in some CMV strain had been reported and this fact must be considered in non-responsive patients. Some studies have focused in genotyping of CMV which could indicate strains presenting resistance to conventional treatment.

1.2 Human Herpesvirus 6

In Brazil serological prevalence surveys conducted in North and Southeast regions show that antibodies against HHV-6 were present in 90% of the individuals among the studied population (0-40 years-old) with occurrence of the primary infection in the first years of life

(Freitas et al., 1997; Linhares et al., 1991). Reactivation of latent HHV-6 is common after liver transplantation, possibly induced and facilitated by allograft rejection and immunosuppressive therapy (Abdel et al., 2009; Griffths et al., 2000). HHV-6 may affect the success of the transplant procedure which is observed clinical findings as: fever, neutropenia, nervous central system manifestations or other visceral involvements (DesJardin et al., 2001). In addition, HHV-6 viremia is an independently significant predictive factor for invasive fungal infections and is associated with late mortality in liver transplantation recipients (Rogers et al., 2000). On the other hand, the rejection of the transplanted organ can also be enhanced when the patient is co-infected with CMV (Lautenschlager et al., 2000; Humar et al., 2000).

The expression of different cellular antigens can be dramatically altered in HHV-6-infected tissues which the viral infection can induce to CD4 up regulation and CD3 down modulation in the T cells. HHV-6 can severely affect the physiology of secondary lymphoid organs through direct infection of T lymphocytes and modulation of key membrane receptors and chemokines (Grivel et al., 2003). Since the effects of HHV-6 in cellular immune system it could be affect the response against other infectious agents or facilitate the mechanism of graft rejection in the host.

The diagnosis of reactivation or new infection by HHV-6 is not made easily. Serological techniques are available but the contribution of a positive result is limited by the high prevalence of infection in adults, as mentioned above (Freitas et al., 1997; Linhares et al., 1991). The report of specific HHV-6 IgM in sera or a four-fold rise in IgG antibodies can be used as diagnostic criteria, but is not as sensitive as desired. Moreover, the interpretation of serological results is complicated by the fact that both primary and secondary infections with other herpes viruses may be associated with a concurrent antibody response to HHV-6 (Osman et al., 1997). In addition, the presence of residual IgM against HHV-6 in the bloodstream can complicate the interpretation of the serological tests (Peigo et al., 2009).

Antigenemia techniques to detect HHV-6 in blood have been described in the literature (Sampaio et al., 2011; Lautenschlager et al., 2002). Similar to pp65-atigenemia used for CMV, monoclonals antibodies against specific HHV-6 protein could be use with the purpose to detect only active infections. The antigenemia could be an alternative to molecular techniques because is a quantitative method and requires relatively few apparatus. HHV-6 antigenemia, different from CMV, requires the use of purified lymphocytes to detect antigen. Positivity in peripheral monocytes occurs occasionally although lymphocytes are more frequently positive.

Although the use of HHV-6 antigenemia could be promissory, the technique still needs improvements and establishment of 'cut-off' values to clinical use. Moreover, the sensitivity and specificity to detect HHV-6 active infection have been not completely studied.

The techniques based on nuclei acid amplification are also available for the diagnosis of HHV-6 (Secchiero et al., 1995). However, the results obtained are controversial, because it depends on the PCR's method employed (Shibata et al., 1992; Demmler et al., 1998).

Since HHV-6 disease can be established, infection can be treated with intravenous ganciclovir, foscarnet, or cidofovir and this should be complemented by a reduction in immunosuppression (Razonable & Lautenschlager, 2010). The efficacy of acyclovir against HHV-6 infection seems to be lower than others. Moreover, foscarnet and cidofovir could be

more effective than ganciclovir against HHV-6 infection of astroglioma cells. Although ganciclovir and cidofovir are therapeutic options, on the basis of *in vitro* data and limited clinical experience reported in the literature, foscarnet is probably the preferred treatment for HHV-6 associated encephalitis.

Patients could be treated with intravenous ganciclovir (2.5 mg/kg daily) for 3-6 weeks, cidofovir 5mg/kg once weekly for 2 consecutive weeks or intravenous foscarnet (40 mg/kg every 12 hours) for 3-4 weeks (Vinnard et al., 2009). However, in patients with renal failure, dose must be adjusted to avoid toxicity in the patients. In addition, viral monitoring is necessary (by PCR or antigenemia) to avoid interruption of the treatment prior disappearance of viremia.

Similar to CMV, some strains of HHV-6 can present drug resistance and the strategies described above could also not have efficacy.

1.3 Human Herpesvirus 7

HHV-7 has been isolated from T-CD4+ cells purified from peripheral blood mononuclear cells of a healthy individual by Frenkel et al. (1990). HHV-7 like other betaherpesviruses remains latent or at low level of viral replication after primary infection or can reactivate during immunosuppressed states (Ihira et al., 2001). HHV-7 shares many properties of HHV-6, suggesting that the factors that control their reactivation or increased viral replication in immunosuppressed patients may be similar (Mendez et al., 2001).

HHV-7 infects most specifically, T-CD4+ cells, which could result in cytotoxicity and mmunomodulatory activities (Secchiero et al., 2001). It has also been demonstrated that the down modulation of human leukocyte antigen (HLA) and beta-2-microglobulin expression by HHV-7 is linked to viral replication and is not merely the consequence of the interaction of virions with the cell surface. Infected cells can therefore efficiently escape from host immune pressure that might explain the persistence of HHV-7–positive cells in several types of tumors and chronic infectious diseases (Mirandola, 2006). Although HHV-7 has restricted tropism to CD4+ cells, it should be noted that HHV-7–infected T-CD4+ cells kill uninfected T-CD8+ cells *in vitro*. Moreover, HLA class I and beta-2-microglobulin are also down modulated in the T-CD8+ cells on the presence of HHV-7–infected leukocytes *in vitro* (Secchiero et al., 2001). Similar to HHV-6, HHV-7 infection could modulate the host immune system enhancing the risk to graft rejection and other type of infections.

Although in liver transplant recipients HHV-6 has been related to clinical consequences (Feldstein et al., 2003), the specific clinical syndrome spectrum of HHV-7 remains not clear (Ihira et al., 2001; Mendez et al., 2001). Several methods and different biological materials have been proposed to detect HHV-7 infection. Serological assays presents the same problems reported to HHV-6 and interpretation of these test are frequently difficult. Nested polymerase chain reaction (nested-PCR) using DNA extracted from either serum or plasma could detect only HHV-7 active infection (Ihira et al., 2001; Feldstein et al., 2003).

In our center, we found that nested-PCR carried out in DNA extracted from sera did not detect latent HHV-7 in a healthy cohort (Thomasini et al., 2008). In addition, positive IgM anti-HHV-7 and/or significant increase in IgG anti-HHV-7 titers were correlated with

positive nested-PCR for HHV-7 in adult liver transplant recipients (Peigo et al., 2009). However, many technical and clinical aspects remain to be clarified regarding these tests.

Antigenemia can be performed to detected HHV-7 antigen in peripheral lymphocytes using similar technique describe to HHV-6 (Sampaio et al., 2011; Lautenschlager et al., 2002). HHV-7 antigen can be detected mainly in lymphocytes probably in T-CD4+ lymphocytes.

The majority of HHV-7 infections do not require antiviral medication, but the severe complications could be treated with ganciclovir and its derivates or foscarnet and cidofovir (Ongrádi et al., 2010).

There a few reports in the literature regarding treatment against HHV-7 infection probably due to fact that HHV-7 commonly causes not remarkable clinical outcomes. However, studies have demonstrated that treatment based on ganclclovir or valganciclovir following the same protocol used to CMV can be effective against concomitant HHV-6 and HHV-7 infection after lung and heart-lung transplantation (Lehto et al., 2007). Thus, the same protocol could hypothetically be used against HHV-7 in liver transplant patients.

2. Experience of the State University of Campinas regarding betaherpesviruses in liver transplantation

The aim of this study was to detect and to monitor CMV, HHV-6 and HHV-7 active infections in adult liver transplant recipients using nested-PCR and to describe the clinical aspects related to betaherpesviruses in these patients.

2.1 Materials and methods

Twenty-nine adult liver transplant patients (20 man and 9 woman), median age of 47 years (range 18 to 66), transplanted at the Liver Transplant Unit (University Hospital, State University of Campinas – Sao Paulo – Brazil) were included in this study.

The basic immunosuppressive therapy consisted of cyclosporine (0.4 mg/kg/d), methylpredinisolone (1.0 g first month, 20 mg at 30 days decreasing to 5 mg/mo to 90 days), azathioprine (100mg/d). Mycophenolate mofetil (100 mg/d) and tracolimus (FK) (0.1 mg/kg/d) were prescribed based on selected patient's characteristics and specific protocol studies. Acyclovir (5 mg/kg per day for 2 months) was employed as antiviral prophylaxis to *Herpes simplex*.

No routine CMV prophylaxis was used and ganciclovir (5mg/kg/d) for 6 weeks was administrated as treatment for symptomatic CMV patients. High doses of methylpredinisolone were used as antirejection treatment. Patient's characteristics related to age, sex and underlying liver disease were summarized in Table 2.

Peripheral blood was obtained from patients at the time of transplantation, as well as weekly for the first month and once a month to 180 days. Ethylenediamine tetraacetic acid (EDTA)-treated blood samples were used to DNA extraction from peripheral blood leukocytes (PBL) and serum (from without anticoagulant tube) of each blood sample was also separated by centrifugation. The obtained sera were then frozen (-20°C) until testing. The protocol was designed on accordance with the requirements for research involving human subjects in Brazil, and it was approved by the Institutional Ethics Committee.

2.1.1 CMV serological assay

Anti-CMV IgG and IgM were tested in sera of the donors and patients before transplantation. Assays were carried out using ELISA-Commercial Kits (Sorin Diagnostics, Saluggia, Italy) following manufacture's instructions.

Patient´s Characteristics	
Median age (years)	47 (range: 18-66)
Sex (male/female)	20/9
Diagnosis of underlying liver disease	
Hepatitis C	15
Alcoholic liver disease	3
Hepatitis B	2
Hepatitis C and alcohol	2
Cryptogenic cirrhosis	2
Hepatitis B and alcohol	1
Primary biliary cirrhosis	1
Autoimmune hepatitis	1
Primary sclerosing cholangitis	1
Hemochromatosis and alcohol	1

Table 2. Demographic characteristics of the patients studied

2.1.2 HHV-6 and -7 serological assays

IgG and IgM antibodies against HHV-6 and HHV-7 were tested in sera of the donors and patients before transplantation by an indirect immunofluorescent assay. The standard HHV-6 and HHV-7 antigens were prepared from viral culture of each virus (cord blood mononuclear cells infected by only one virus) and absence of cross-infectivity was confirmed by immunological or molecular methods. Infected cells were coated onto wells of immunofluorescence slides, air dried, and then fixed (cold methanol-acetone). The wells were covered with serial dilutions of patients' sera (starting from a 1:10 dilution) and incubated for 1 h at 37°C. For IgM detection, a single dilution of 1:20 of each sample was carried out. Slides were washed 3 times with PBS, wells were covered with anti-human IgG or IgM fluorescent conjugate diluted PBS/Evans's blue (Biomerieux Inc., Lyon, France), and then incubated for 1 h at 37°C.The slides were washed 3 times with PBS, buffered glycerin mounted, and immediately observed under an ultraviolet (UV) photo microscope (Leica DM2000,Wetzlar, Germany). All the samples were pre-treated with RFAb-sorbant (Hoescht-Behring, Kanata, Ontario, Canada) to avoid interference of IgG and rheumatoid factor in the IgM immunofuorescent assay (Ihira et al., 2001; Ablashi et al., 1998). The antibody titer was defined as the reciprocal of the serum dilution showing specific fluorescence.

2.1.3 Peripheral blood leukocyte (PBL) DNA extraction

Briefly, PBL were lysed after separation following protocol previously described (Bonon et al., 2005). PBL DNA was precipitated with cold ethanol and then eluted in 50μL of TE-buffer (10mM Tris, 1mM EDTA) and stored frozen (-20°C) until PCR analysis.

2.1.4 Serum DNA extraction

Briefly, DNA was extracted from 200 μL of serum using a phenol-chloroform protocol after incubation overnight in lysis buffer (10mM Tris-HCl pH 8.0, 10 mM EDTA, 10 mM NaCl, 0.2% dodecyl sodium sulfate and 100 μg proteinase K) at 56°C followed by DNA precipitation with cold ethanol. The resulting DNA pellet was eluted in 50μL of TE-buffer (10mM Tris, 1mM EDTA) and stored frozen (-20°C) until PCR analysis.

2.1.5 CMV nested-PCR

Five microliters of DNA extracted from PBL, as described above, were used in the nested-PCR using reaction mixture containing specific primers to CMV following protocol previously described (Shibata et al.,1992; Demmler et al.,1998).

2.1.6 HHV-6 and HHV-7 nested-PCR

Nested-PCR was carried out for each virus using 5 μL of DNA, extracted from serum as described above. Primers and protocol used to HHV-6 nested-PCR were previously described by Secchiero et al. (1995). Primers and protocol used to HHV-7 nested-PCR were previously described by Pozo et al. (1999) with some modifications (originally a multiplex-PCR).

Amplifications were carried out on a Peltier Thermal Cycler - MJ Research (Watertown–MA-USA).This The nested-PCR product was analyzed under UV light after electrophoresis in 2% agarose (Gibco-BRL) stained with ethidium bromide. All nested-PCR was carried out in duplicate using a second fresh aliquot. Polymerase chain reaction for beta-globin gene was carried out to detect contamination of serum with leukocytes and false negative results from incorrect DNA extraction from PBL.

Positive and negative controls for each virus were included systematically. Genomes amplifications using the referred primers results in DNA fragments containing 159, 258 and 122 base pairs of CMV, HHV-6 and HHV-7, respectively. Some nested-PCR products of each virus were sequenced analyzed and compared to the GenBank database using Software ChromasPro® (Thecnelysium Pty Ltd).

2.1.7 Definitions

CMV active infection was defined based on detection of CMV DNA in PBL by nested-PCR. HHV-6 and HHV-7 active infections were also defined based on detection of virus DNA in serum by nested-PCR. Transient viremia was defined when virus DNA was detectable only once or in no-consecutive samples.

Latent infection, reinfection and reactivation were defined base on criteria proposed by Ljungman et al., 2002. Co-infections were defined when two or more viruses were detected in the same sample.

Symptomatic CMV infection ('CMV disease') was divided into two situations: Tissue-invasive disease and "CMV viral syndrome" (Kotton et al., 2010).

Briefly, Tissue-invasive disease was defined based on symptoms consistent with CMV disease including fever, malaise, myalgia, anorexia and leukopenia accompanied of CMV

active infection and when biopsy proven CMV identification (Taber et al. 2004, Ljungman et al., 2002). "CMV hepatitis" and "CMV gastrointestinal disease" was diagnosed based on criteria proposed by Ljungman *et al.* (2002). "CMV viral syndrome" was defined based on unexplained fever (>37.5°C) for at least 3 days, in combination with at least one of the following features: arthralgia, leukopenia (<3 x10^9/l), thrombocytopenia (<150x10^9/l), liver enzymes elevation (ALT>50 U/l). Asymptomatic CMV infection was defined when CMV active infection occurs without signs, symptoms, or laboratory abnormalities described above.

Clinical symptoms such as fever, encephalitis, interstitial pneumonitis, hepatitis and laboratorial findings as leukopenia and thrombocytopenia were taken into account and CMV, HHV-6 and HHV-7 active infections were compared to these episodes. The laboratorial monitoring of graft function was based on elevation of serum alanine aminotransferase, alkaline phosphatase, gamma-glutamyl transpeptidase and bilirrubins. Rejection episodes were documented based on histopathological analysis of the liver biopsies (Banffs schema).

2.1.8 Statistical analysis

The comparison of categorical variables was performed using Fisher's exact test or chi-squired test and Mann-Withney-U test for continuous variables. A p<0.05 was considered statistically significant.

3. Results

All patients and donors had positives anti-CMV, anti-HHV-6 and anti-HHV-7 IgG before transplantation (D+/R+), indicating that all of them patients were virus reactivations/reinfections. CMV DNA was detected in 20 (68.9%) of 29 patients, median time to first CMV detection was of 50 days (range 7 to181). HHV-6 DNA was detected in 13 (44.8%) of 29 patients, median time to first HHV-6 detection was of 27 days (range 0 to 143). HHV-7 DNA was detected in 14 (48.2%) of 29 patients, median time to first HHV-7 detection was of 19 days (range 0 to 170). Six patients had HHV-7 DNA detectable already at the time of transplantation contrasting with two cases of HHV-6 and none of CMV. IgM against HHV-7 was detected in 100% of these patients who had detectable DNA already at the time of transplantation (P=0.002). Neither patient nor donor had positive IgM against CMV and HHV-6.

The three viruses together were found in 6/29 (20.7%) patients but in none sample at the same time. Co-infections by CMV/HHV-6, CMV/HHV-7 and HHV6/HHV-7 occurred in 5 (17.2%), 2 (6.9%) and 2 (6.9%) of the patients, respectively. Kinetic of the detection for three viruses was shown in Figure 2.

The statistical analysis showed that the detection of CMV, HHV-6 and HHV-7 was independent of one another (P>0.05). Diagrams illustrating positive nested-PCR for any combination of betaherpesviruses were shown in Figure 3.

Among the 20 patients with detectable CMV DNA, 10 (34.4% of total of the patients enrolled in this study) developed symptomatic CMV infections including "CMV viral syndrome" (n=5), CMV hepatitis (n=4) and CMV gastrointestinal disease (n=1). The symptoms have

occurred 16 days (average) after first CMV DNA detection. Considering the patients with no detectable CMV DNA in their blood, none had CMV disease. The relationship between the detection of CMV DNA and symptomatic CMV infection was considered statistically significant (P=0.009). HHV-6 was detected in 50% of the patients with symptomatic CMV infection and in 30% of the patients without symptoms (P=0.32). HHV-7 was also detected in 60% of the symptomatic CMV infection and in 70% of the patients with asymptomatic infection (P=0.50). Of 10 patients who had liver dysfunction, 7 (70%, P=0.006) had symptomatic CMV infection and 2 (20%) had only HHV-6 active infection at the time of dysfunction.
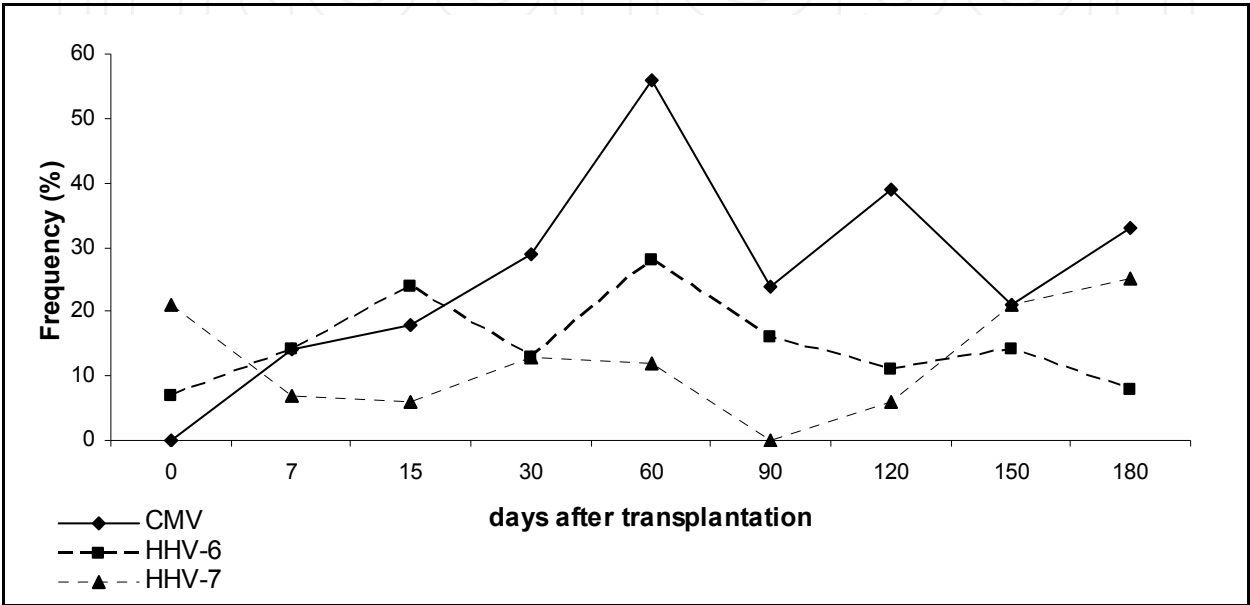
Fig. 2. Kinetic of detection for CMV, HHV-6 and HHV-7 in liver transplant recipients by nested-PCR

Of 10 patients that had liver dysfunction, five presented episodes of graft rejection graded as mild. One was related to CMV hepatitis. One had symptomatic CMV/HHV-6 co-infection (CMV hepatitis) 36 days prior rejection and one other had symptomatic CMV/HHV-7 co-infection ("CMV viral syndrome") 45 days prior rejection. Two patients that had co-infection before graft rejection were accompanied with persistent liver dysfunction until rejection episode. Another two patients had only HHV-6 infection accompanied with thrombocytopenia and leukopenia were related with rejection episodes. Unfortunately, we were not able to perform viral antigens detection in liver biopsies. In patients who had liver dysfunction and/or graft rejection, no underlying liver disease (HCV or HBV) were relapsed until end of the monitoring (180 days) and no other infectious agent was found. Only one case of "CMV viral syndrome" was recurrent and occurred after graft rejection.

Two episodes of pneumonitis were related with HHV-6/HHV-7 co-infection. One case of pneumonitis and two of encephalitis were also related with only HHV-6 infection and no others infectious agents were found. However, other tests to detect HHV-6 and HHV-7 in tissue samples might not be performed. In CMV and HHV-6 free patients no symptoms or significant laboratorial findings could be related to HHV-7.

Patients
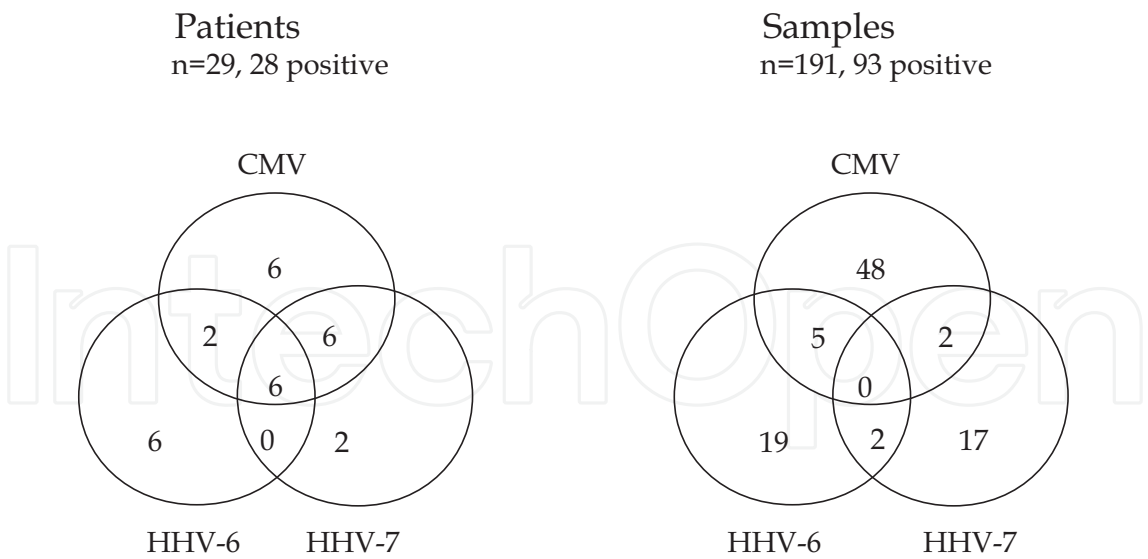n=29, 28 positive

Samples
n=191, 93 positive

Fig. 3. Venn diagrams illustrating the number of patients (n=29) or number of positive samples (n=198) that were positive nested-PCR for any combination of betaherpesviruses

4. Discussion

The high frequency of positive IgG test against CMV observed agrees with previously data indicating a prevalence of 90 to 100% anti-CMV antibodies in Brazilian population (Suassuna et al., 1995; Costa et al., 1999). Previously studies in Brazilian population have also demonstrated high prevalence of HHV-6 and HHV-7 (90 and 84%, respectively).

CMV, HHV-6 and HHV-7 were frequently detected in patients after liver transplant (68.9%, 44.8% and 48.2%, respectively). Ihira et al. (2001) and Feldstein et al. (2003) suggested that the detection of virus DNA in serum by PCR is a useful marker of HHV-6 and HHV-7 active infection. In adult liver transplant recipients, Griffths et al. (1999) found CMV DNA in 47%, HHV-6 DNA in 32% and HHV-7 DNA in 48% of the patients. Ihira et al. (2001) found HHV-6 DNA in 38% and HHV-7 DNA in 40% of the patients until 8 weeks after liver transplantation. Humar et al. (2000) found CMV DNA in 63.6% and HHV-6 DNA in 54.5% of the liver transplant recipients.

The rate difference in each report depends of the sensitivity of PCR, type of transplantation, immunosuppressive protocol, the size of samples used and differences among subjects. However, the rate found in this study was relatively similar with others reports. Interesting, 6/29 (20.7%) patients had positive detectable HHV-7 DNA at the time of transplantation without symptoms. In addition, IgM against HHV-7 was found in all samples contributing to the hypothesis of true active infection had occurred. Since that this method did not detect latent infection in previously study (Thomasini et al., 2008) and blood was collected before surgery, it could be explained by reactivation caused by underlying liver disease or by transient viremia. Although some syndromes related to HHV-7 in immunocompetent patients have been described (Ward et al., 2005), studies in pre-transplant time should be performed to evaluate each hypothesis.

Ten of twelve (50%) patients who had detectable CMV DNA developed symptomatic CMV infection. The remaining 10 patients without symptoms could be explained by the high sensibility of the PCR, that can detect lower viral load (Tokimatsu et al.,1995), and they were

not treated. However, the statistical analysis showed correlation between detection of CMV DNA and symptomatic CMV infection. Our symptomatic CMV incidence (34.4%) was higher (1.54-fold) than incidence reported by Humar et al. (2000) that reported symptomatic CMV infection in 21.6% of the patients. Similar to this study, Härmä et al. (2006) found 30% of symptomatic CMV infection during 3 first months after transplantation.

We have considered that this higher incidence of CMV infection and symptomatic CMV infection due to high prevalence of CMV in Brazilian population, no routine or preemptive ganciclovir therapy and use of cyclosporine. Humar et al. (2000) have found an independently increasing risk factor for development of CMV disease when patient had D+/R+ CMV serostatus and all patients enrolled in this study were D+/R+. It is conflicting with most reports that suggest higher risk factor when CMV serostatus is D+/R-. Hoppe et al. (2004) had suggests a higher probability of CMV infection among patients treated with cyclosporine compared to tracolimus.

Some cases of pneumonitis and encephalitis were related to HHV-6 active infection or with co-infections HHV-6/HHV-7. Previously reports had suggested association an increase risk of graft rejection associated with CMV (Lautenschlager et al., 1997). Although we have found that symptomatic CMV infection was present in most cases of liver dysfunction and graft rejection, CMV co-infection with HHV-6 or HHV-7 and HHV-6 alone were more likely related with graft rejection than CMV alone. Härmä et al. (2006) had suggested a role of HHV-6 in liver dysfunction and graft rejection (with HHV-6 antigens detected in liver biopsies in same patients). Griffiths et al. (1999) found also association between liver dysfunction and graft rejection with HHV-6 and dysfunction with HHV-7. HHV-6 could either be participating directly in the rejection process or potentially exacerbating the inflammatory response characteristic of rejection (Emery, 2001). However, the fact that the most of patients with HHV-6 active infection were asymptomatic in this study (probably due to transient viremia) turned difficult to establish a relation between liver dysfunction/graft rejections with HHV-6 active infection. In addition, all of the positive patients included in this study have betaherpesviruses reactivation/reinfection and not primary infection. Betaherpesviruses primary infections could have more significant clinical outcomes and this hypothesis should be considered in pediatric liver transplantation which primary infections could be more frequent.

In CMV and HHV-6 free patients no symptoms or significant laboratorial findings could be related to HHV-7. However, the role of the HHV-7 in down regulation of CD4 expression in lymphocytes has been described (Secchiero et al., 1997; Secchiero et al., 1998) and a possible immmunomodulatory effects do not be discarded. Studies regarding CMV, HHV-6 and HHV-7 including determination of viral load with 'cut-off' values for clinical manifestation and detection of viral antigens in liver biopsies as well as evaluation of cellular and humoral immune response could be performed. In this study we have considered qualitative nested-PCR which had limited value for clinical monitoring of the betaherpesvirus.

5. Conclusion

The results described above show that few patients remain free of betaherpesviruses after liver transplantation. Most of the patients with active infection with more than one virus were infected sequentially and not concurrently. Active infection with HHV-6, HHV-7 or CMV might develop independently of one another. Most patients with HHV-6 or HHV-7

active infections were asymptomatic. In few patients, HHV-6 could be associated with some clinical manifestations and episodes of graft dysfunction and rejection. Qualitative nested-PCR was considered of limited value to clinical monitoring of betaherpesviruses.

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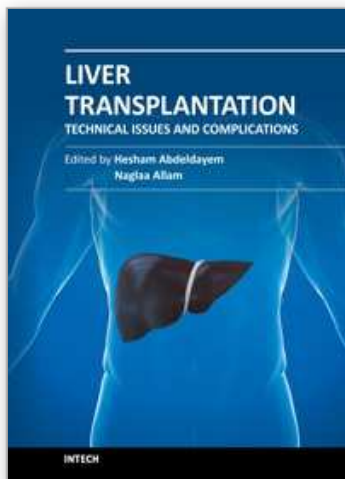
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