DETECTION OF HUMAN HERPESVIRUSES 6 AND 7 DNA IN THE SALIVA OF RENAL TRANSPLANTED PATIENTS AND HEALTHY INDIVIDUALS FROM RIO DE JANEIRO, BRAZIL

Rebeca Vazquez Novo Martins¹, João José Cossatis², Larissa Alves Afonso¹, Maria da Gloria de Almeida², Silvia Maria Baeta Cavalcanti¹*

¹. Departamento de Microbiologia e Parasitologia, Instituto Biomedico, Universidade Federal Fluminense, Rua Ernani Melo 101, Laboratório 319, Niterói, RJ, Brazil cep24210-130;
². Laboratório de Imunologia Viral, Instituto Oswaldo Cruz, Rio de Janeiro, RJ, Brazil.

ABSTRACT
In this study, we have sought to verify the prevalence of human herpesviruses 6 and 7 (HHV-6, HHV-7) in the saliva of renal transplanted patients from Rio de Janeiro State, Brazil, and compare results with those of healthy subjects, since Roseolovirus DNA detection in body fluids from transplanted patients has been associated with often misdiagnosed chronic symptoms, organ rejection and even death. The studied group was composed by 120 individuals: 60 were renal transplanted patients and 60 were healthy subjects attending the Hospital Universitário Pedro Ernesto, for odontological follow-up. Saliva specimens were submitted to a multiplex nested polymerase chain reaction (PCR) to detect the presence of HHV-6A, HHV-6B and HHV-7. The total Roseolovirus DNA prevalence was 56.7% for transplanted patients and 23.3% for healthy individuals (p<0.001). For immunosuppressed patients, the PCR detected a HHV-6A prevalence of 16.7% in transplanted, HHV-6B in 26.6% and HHV-7 DNA was revealed in 13.3% of the studied cases. In healthy subjects, HHV-6A was found in 5% of the samples, HHV-6B in 6.7% and HHV-7 in 11.7%. Multiple infections were observed in 12/60 (20%) individuals. No co-infection was demonstrated for healthy subjects, reinforcing the idea that immunosuppression can favor reactivation and possibly transactivation among herpesviruses (P<0.001). Statistically significant differences were recorded for HHV6A and B infections in transplanted patients, when compared with healthy individuals (p<0.05). No statistically significant differences were observed regarding HHV-7 infection. Clinical symptoms and laboratorial findings were not specifically associated with patients shedding any of the studied viruses. Our results showed relevant differences in Roseolovirus prevalence among the two studied groups, suggesting a potential role for those viruses in disturbing host homeostasis that can compromise life quality. Although PCR methodology proved to be a useful tool for Roseolovirus detection, the standardization of samples and procedures is necessary to evaluate possible a pathogenic behavior among different agents in order to analyze their role in the post-transplant scenario.

Keywords: Roseolovirus – HHV6 – HHV7 – Renal transplant - healthy adults - saliva

INTRODUCTION
The β human herpesviruses-6 and -7 (HHV-6 and HHV-7) are members of Roseolovirus genus, characterized by tropism for T lymphocytes (Jarret et al. 1990; Wyatt & Frenkel 1992). These genetically related viruses were discovered, respectively, by Salahuddin et al. (1986) and Frenkel et al. (1990). Infection occurs frequently without symptoms in early childhood but both viruses can cause exanthem subitum, a classical illness characterized by spiking fever and rash in young children (Yamanish et al. 1988). Primary infection has also been associated with neurological symptoms, such as seizures (Ward & Gray 1994). HHV-6 is further classified into variant A and variant B. Along with exanthem subitum, HHV-6B has also been related to diverse neoplasia, chronic fatigue syndrome, multiple sclerosis, among other human idiopathic disorders (Ward, 2005). On the other hand, no human disease has been clearly associated to HHV-6A (Tanaka-Taya et al. 1996). It is important to notice that in young adults and adolescents, HHV-7 has also been associated with pityriasis rosea (Black & Pellet, 1999).

Despite being members of Herpesviridae family, limited information is available concerning HHV-6
and HHV-7 excretion from body sites during and after infection. Latency and persistence have been described in the salivary glands, peripheral blood mononuclear cells (PBMC) and central nervous system (Braun et al. 1997). Previous studies have also suggested that both viruses can be detected in saliva throughout life (Hidaka et al. 1993). Caserta et al (2001) demonstrated that viral reactivation occurs in immunocompromised patients and is associated with fever, rash, encephalitis, bone marrow suppression and graft rejection. Severe complications have also been described, leading to the suggestion that Roseoloviruses are cofactors promoting syndromes varying from encephalitis to pneumonitis on post-transplant patients and even death (Ward 2005).

Healthy adults can shed this virus intermittently, behaving as the epidemiological source of infection among human populations (Ward 2005). In a previous study to evaluate the saliva of healthy adults in the state of Rio de Janeiro, the prevalence of both HHV-6 and 7 ranged from 9.8% to 12.6% (Magalhães et al. 2010). Nevertheless, little is known concerning Roseolovirus shedding in post-transplant patients, especially in long-term ones, among whom indirect effects such as persistent fever, anemia, chronic fatigue syndrome among others are frequent and can compromise life quality. Although the Cytomegalovirus (CMV) remains the most relevant virus in post-transplant patients, several authors have suggested that their pathogenic role are observed from 2 to 12 months after transplantation, contributing to organ rejection and death (Razonable, 2008). Our study, therefore, sought to determine the prevalence of Roseolovirus DNA detection in long-term renal transplanted-patients, comparing the results with those found in a group of healthy subjects, in order to contribute to the recognition of possible viral effects in these immunosuppressed patients.

**MATERIAL AND METHODS**

**Study design**

A cross-sectional study was conducted in Hospital Universitário Pedro Ernesto (HUPE), Universidade do Estado do Rio de Janeiro, a large tertiary public hospital in Rio de Janeiro, Brazil. The studied group was composed by 60 renal transplanted patients, matched by age with a group of 60 healthy individuals residing in Rio de Janeiro, attending at HUPE for annual odontological follow-up between May, 2006 and May, 2010. All the patients presented an average posttransplant time of 36 months, ranging from 22 to 44 months, thus being considered long-term survivors (Soucie et al. 1999). The average age of healthy participants was 44.6 years, ranging from 34 to 66 years old. This study was approved by the Ethics Committee from HUPE/UERJ (protocol 138-2005).

**Polymerase Chain Reaction to detect HHV6A, HHV6B and HHV7**

Non-stimulated saliva samples were collected and stored at -20°C until DNA extraction. A multiplex nested PCR assay was used in order to detect HHV-6A, B and HHV-7 as previously described by Magalhães et al. 2010. Briefly, DNA was extracted from 500 μl of whole saliva using Invisorb DNA mini kit (Invitek, Germany). Amplification was carried out in 50μl reaction mixture (1X PCR buffer, 200μM dNTPs, 1.5mM MgCl2, 50pmol of each primer, 0.25U unit of Taq polymerase platinum, and 10μl of sample). The mixture was subjected to 30 amplification cycles of denaturing at 90°C for 1min, annealing at 62oC for 2min and extension at 72oC for 3min. After the first round, 2μl of the amplicon was used as template for the second round of PCR, using the same conditions except for the inner primers used. Polymerase chain reaction products were analyzed on 1.5% agarose gel with ethidium bromide staining for visualization of DNA under ultraviolet light. HHV-6A generated 195bp fragments, HHV-6B 423bp and HHV-7 generated a 140bp amplicon. The technique presented a sensitivity of 100 copies/50μL for both HHV-6A and HHV-7 genomes and of 10 copies/ 50μL for HHV-6B.

**Statistical analysis**

A data bank was generated and data were analyzed using EPInfo 2004 statistical software package (Center for Disease Control and Prevention, Atlanta, EUA, 2004). Frequencies were compared through Chi-square tests with Yates correction. The significance level of tests (p) was set at 0.05.

**RESULTS**

Among the 60 renal transplant patients, Roseoloviruses were detected in 56.7% (34/60) of the saliva samples. HHV-6A DNA was demonstrated in 16.7% (10/60), HHV-6B in 26.6% (16/60) and HHV-7 in 13.3% (8/60). These 60 patients were diagnosed to be in good general health conditions, with no apparent infection, according to the medical records, although every one of them presented at least one clinical symptom, such as fever, anemia, fatigue, non-specific loss of weight. When the average age of each group was analyzed, no statistical difference was found (transplanted patients : 47.2 and healthy subjects : 43.1).

Among the healthy subjects, PCR results showed a total Roseolovirus prevalence of 23.3% (14/60). HHV-6A was detected in of 5% (3/60), HHV-6B in 6.7% (4/60), and HHV-7 in 11.7% (7/60) of the studied cases. Multiple infections were demonstrated in 20% of patients (12/60) but were not detected in healthy individuals. After statistical methods were applied, no specific clinical symptoms were statistically related to viral DNA detection. Nevertheless, relevant differences were revealed concerning HHV-6A and B, with higher frequencies in transplanted patients when compared to...
healthy subjects (HHV-6A, p<0.05, HHV-6B, p<0.001). No difference was detected among groups regarding HHV-7 DNA detection.

Table 1: Prevalence of HHV6A, HHV6B and HHV7 infection* among transplanted patients and healthy subjects

<table>
<thead>
<tr>
<th>Groups</th>
<th>HHV6A (%)</th>
<th>HHV6B (%)</th>
<th>HHV7 (%)</th>
<th>Roseolovirus's Infection Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transplanted patients</td>
<td>10 (16.7%)</td>
<td>16 (26.6%)</td>
<td>8 (13.3%)</td>
<td>34/60 (56.7%)</td>
</tr>
<tr>
<td>Healthy individuals</td>
<td>03 (5%)</td>
<td>4 (6.7%)</td>
<td>7 (11.7%)</td>
<td>14/60 (23.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>13 (10.8%)</td>
<td>20 (16.7%)</td>
<td>15 (12.5%)</td>
<td>48/120 (40%)</td>
</tr>
</tbody>
</table>

*Identified by multiplex nested PCR in saliva
Co-infections in renal transplanted patients: 6A + 6B = 5 (8.3%); 6A + 7 = 3 (5%); 6B + 7 = 4 (6.7%). Relevant statistical differences were shown for HHV6A (p<0.05) and HHV6B (p<0.001) in transplanted patients in relation to healthy subjects.

No difference were detected for HHV7 (p>0.05).

**DISCUSSION**

In the last few decades the application of molecular biology techniques have led to an understanding of several pathologies associated with infectious agents in transplant recipient patients, among whom symptoms are usually different and possibly more severe than those in immunocompetent subjects (Ljungman, 2002).

As viruses remain the most significant and elusive pathogens infecting patients after solid-organ transplantation (Fischer, 2008), a role for herpesvirus was suggested. Hence, this evaluation of the presence of Roseoloviruses, a recognized group involved in pathologies occurring in transplant recipients, which can result in harm to the organ or even its failure, possibly leading to death.

We found HHV6-A showing a statistically significant prevalence in patients, when compared to healthy subjects (p<0.05). It is worth noting that HHV6-A has been rarely found in post-transplant syndromes. Nevertheless, Martins et al (2009) described the detection of HHV-6A in an acute hepatitis syndrome in a renal transplanted patient.

As expected, we found a higher statistical relevance concerning HHV-6B detection in transplanted patients, as compared with healthy individuals (p<0.001). HHV6-B has been commonly found and also associated with a greater pathogenic potential among these patients (Ward, 2005). In our study, we have also noticed a dominance of HHV6-B over HHV6-A, in accordance to the findings presented by Ward (2005). It is interesting to notice that a previous study from our group showed the opposite profile in healthy subjects, in which these agents are often shed without clinical signs (Magalhães et al. 2010). Nevertheless, due to the small sample studied, divergences were expected.

We detected similar prevalence rates for HHV7 among the studied groups (p>0.5). Similar results were previously described by Caserta et al (2001) who speculates on the true role of these viruses as potential pathogens. Nevertheless, Razonable et al (2003) described a patient showing solely HHV-7 DNA and having developed a CMV-like syndrome. In addition, our results have pointed out much lower prevalence rates than those described in the literature, which can also be attributed to the size of the studied sample (Hidaka et al. 1993; Ward, 2005). Studies dating back to the nineties may also have overestimated prevalence rates due to the fact that, at that time, the first nested PCR protocols were being developed and their proneness to contamination was not fully recognized.

In our study we also analyzed coinfections and detected them exclusively in transplanted patients (20%), reinforcing the idea that these viruses can transactivate each other. Humar et al (2009) reported similar results, of nearly 20%, but the authors did not found clinical or therapeutic indication for testing HHV-6 and HHV-7. On the other hand, Razonable et al. (2003) found as much as 80% of coinfections studying Betaherpesviruses, and also associated them with viral syndromes and indirect effects. As described by DesJardin et al (1998), HHV6 reactivation may be associated with cytomegalovirus infection and syndromes in kidney transplant recipients at risk for primary cytomegalovirus infection. Our patients were evaluated for the presence of CMV DNA but data showed no difference among non-transplanted and transplanted patients (data not shown), which can be attributed to the fact that CMV plays its pathogenic role in recent transplanted patients but seems to be controlled by these long-term survivors (Ljungman, 2002).

Immunomodulatory effects of herpesviruses would explain their role in pathologies found in transplanted recipients (Ljungman, 2002). Both HHV6 variants can induce production of interleukin-1β and tumor necrosis factor-α, suppress T lymphocyte function due to reduced interleukin-2 synthesis, as well as interferon-α. This immunomodulating effects could create such a state of immunosuppression, triggering several disorders and predisposing the transplant recipient to an opportunistic infection with CMV or to other infectious pathogens (DesJardin et al. 1998).

It is important to emphasize that most of the available literature has reported data associated with the first 6 months post-transplantation. In our study, we analyzed long term survival as well as patients with 3 years of transplant on average. It is among this population that indirect side effects are established. In the first few months post-transplantation antiviral therapy can warrant survival but, after some time, late viral recurrence can induce side effects with high morbidity and even risk of increased mortality rates (Soucie et al. 1999; Ljungman, 2002).

The chosen clinical specimen was the whole
saliva: a non-invasive easy-to-collect sample. Zerr et al. (2000) proposed the whole saliva usefulness as an appropriate specimen for genomic analysis, since throat swabs or blood can contain lymphocytes harboring latent viruses. Despite the advantages of molecular biology methodologies, it is important to keep in mind their key limitations concerning the fact that detection of a viral genome on a biologic sample are not always accomplished by the recognition of clinical syndromes. Despite its limitations, the PCR is still the most suitable technique for HHV-6 detection, since a rapid diagnosis is essential to determine a proper drug regimen. Its usefulness has been recently described after HHV6 DNA detection that has led to a successful therapy using ganciclovir (Martins et al. 2009). Quantitative assays may assist in understanding the clinical relevance of such infections, allowing for differentiation between latent processes and active infections (Humar et al. 2009). Yoshikawa et al. (2003) proposed a reverse transcription PCR for the detection of RNA transcripts and distinguish between active and latent HHV6 infection. Future studies using these procedures may help us define the risk of disease associated with active infection.

It is well known that HHV-6 and HHV-7 primary infections occur in early childhood and causes febrile diseases associated with cutaneous rash (exanthem subitum) (Caselli & Di Luca, 2007). Several have authors pointed out that both HHV-6 and HHV-7 are highly prevalent in the healthy population and can be intermittently shed in saliva usually without clinical symptoms. These features have contributed to the notion that HHV-6 and HHV-7 are more or less “harmless” viruses. Nevertheless, it is important to notice that recently, different studies have showed HHV-6 and 7 as probably capable of causing severe diseases and even death in transplant recipients (Ljungman, 2002; Caselli & Di Luca, 2007).

Given the degree of uncertainty surrounding these emerging viruses, we reinforce the need for further studies. The standardization of samples and procedures is also necessary in order to evaluate possible interactions among different agents and to analyze their role in the post-transplant scenario.

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REFERENCES