Investigation of The Presence of HHV-6 In Patients With Immune Deficiency

T Kurukuyu, N Ardic, M Ozyurt

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Abstract

In this study, HHV-6 presence was investigated in immunosupressed individuals. Forty-five (45) renal-transplante-patients, 35 chemotherapy-patients and 60 healthy control group were included in the study. Anti-HHV-6 IgM and IgG by indirect immunoflourescence technique, HHV-6 DNA by polymerase chin reaction, anti-CMV IgM and IgG by microparticule enzyme immunoassay were analyzed. The HHV-6 DNA positivity rates in the renal transplant group, chemotherapy group and the control group were respectively 20%, 11.4% and 1.6%; whereas anti-HHV-G IgM rates were 17.7%, 14.2% and 3.3%; and the anti-HHV-6 IgG rates were 95.5%, 88.5% and 91.6% respectively. Anti-CMV IgM was found to be 11%, 14.2% and 10% in the same groups and anti-CMV IgG rates were 91%, 91.4% and 86%. In the renal transplant group, HHV-6 DNA and anti-HHV-6 IgM positivity was statistically significant both alone (p=0.002 and p=0.04 respectively) or together (p=0.001). In the chemotherapy group anti-HHV-6 IgM alone (p=0.036) and anti-HHV-6 IgM together with HHV-6 DNA (p=0.007) positivity was significant; HHV-6 DNA positivity alone (p=0.099) was not significant. Conclusively, HHV-6 may be related with some malignant diseases and may play a role in the rejection in some renal transplanted patients.

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INTRODUCTION

Infectious agents belonging to Herpesvirideae family, are still an important cause of morbidity and mortality in most of the immunosupressed patients. Especially the ability of the virus to stay latent, to be able to reactivate in the latter stages and the ability to cause recurrent infections play a central role in mortality and morbidity [1, 2].

Human beings are the primary hosts for especially eight of the herpesviruses (HSV 1-8). Among these, Cytomegalovirus, belonging to betaherpesvirinae subfamily, is known now for years as one of the most important causes of post-transplantation infections. Similarly, it is also stated that the human herpesvirus-6 (HHV-6) within the roseolavirus species belonginging to the same subfamily, may play an important role in the infections observed in immunocompromised patients [3, 4].

HHV-6 was observed for the first time in the blood lymphocytes of a patient with lymphoproliferative disease [5]. It has two variants genetically closely related to each other, however, difference in terms of biological, molecular and epidemiological features. Among these, HHV-6A has not yet been determined to be related with a specific disease; whereas HHV-6B is known to be the primary cause of exanthem subitum, also known as roseola infantum [1]. Normally, this patients diagnosed and treated for this disease recovers without any complications or defects [6]. As it is most commonly observed in childhood, it is seldomly identified in adults; however, the progression is more severe. Various clinical conditions such as fever, rash, pneumonia, encephalitis, hepatitis and myelosupression may be related to herpesvirus. After reactivation of HHV-6 effects on CMV infection, the findings lead to occasional infections, graft disfunction and rejection may also be seen as the indirect effects [4, 7, 8].

The virus replicates in the organisms' T and B lymphocytes, megacariocytes, NK cells, glioblastoma cells and oropharynx; stays latent in the T cells and reactivates into conditions of immune breakdown [9]. Chemotherapy applied to cancer patients also breaks the immune system and sets the base for superinfections [6].

During the thirth-seventh days of primary infection in children, neutralizer antibodies are formed, in the second

week antibodies in the form of IgM peak and stay elevated for two months, after the second week, IgG type antibodies start to form and stay elevated for a lifetime in 95% of humans [10]. Maternal antibodies protect the newborn until the second month. Usually 10% of the maternal antibodies stay until the 6th month. Frequently, the superinfection develops from then on. Generally, HHV-6 related infections are most commonly observed in the 12^{nd}-18^{th} months of lifetime. In different studies, seropositivity has been stated in 13^{th} month between 64-85% of cases [11].

HHV-6 is found commonly all over the world. While it is not quite clear how the virus passes, saliva is stated to be a possible factor [1, 12].

In this study, the presence of HHV-6 was investigated in immunosupressed patients having gone through renal transplantation and chemotherapy.

MATERIAL AND METHODS

Forty-five (45) renal transplanted patients and 35 chemotherapy patients diagnosed with malign diseases were included in the study as the immunosupressed group, together with 60 healthy volunteers as the control group. The patients aged 20-50 years (average 35) in the renal transplant group, 22-67 years (average 44.5) in the chemotherapy group and 45-65 years (average 55) in the control group.

The study was conducted with blood samples taken into test tubes with EDTA. After sampling, the plasma was isolated after 4 minutes of centrifuge in 3500 g and was kept in 20°C until the study started.

In the samples, the presence of HHV-6 DNA with anti-HHV-6 IgM and IgG antibodies was analyzed; as well as anti-CMV IgM and IgG antibodies taking into consideration the cross reactions.

Anti-HHV-6 IgM and IgG antibodies were investigated with indirect immunofluorescence technique (IIFT) using readily made slides (Euroimmun/Germany) covered with infected lymphocytes. With this purpose, the slides were kept under –70°C and the reagents in the kit were kept under +4°C.

Test procedures were realized with 1/10 scan titration in parallel with the directions of the manufacturer. Sera were also applied for absorption in order to eliminate abnormal positivity. Evaluations were made under immunofluorescence in 10x40 magnification. Bright green samples showing fluorescent pigmentation, in granular shapes were evaluated as positive.

HHV-6 DNA presence was investigated with nested polimerase chain reaction (PCR), determining the 246 bp region common for HHV-6A and 6B.

In the first round of PCR P1 (5'-AGT CAT CAC GAT CGC CGT GCT ATC-3') and P2 (5'-TAT CTA GCG CAA TCG CTA TGT CG-') primers, in the second round P3 (5'-TCG ACT CTC ACC CTA CTG AAC GAG-3') and P4 (5'-TGA CTA GAG AGC GAC AAA TTG GAG-3') primers were used.

Extraction was done using the phenol-chloroform method. In DNA amplification, in the first and the second rounds, 50µl mixture was prepared per the sample.

For the first round, the 35,375 µl distilled water (H2O) not containing DNAse-RNAse, was titrated to a final of a 50µl mixture with 3,0 µl of 25mM MgCl2, 1,0 µl of 2mM dNTP mixture, 0.25 µl of Primer1, 0.25 µl of Primer2, 0.125 µl of 5U/µl Taq DNA polimerase, 5.0 µl of 10xTaq DNA polimerase buffer and 5.0 µl of the extracted sample DNA.

For the second round, a mixture was prepared using 38,25 l, 3,0 l, 1,0 l, 0.25 l, 0.25 l, 0.25 l, 5.0 l of these substances, respectively and 2 l was added from the model DNA from the first round.

The mixtures were placed in the thermocycler (BioRad/Italy) and the same protocol was applied for both rounds. In summary, it was 1 cycle in 4 minutes under 95°C, 35 cycles per 40 minutes each under 95°C, 58°C and 72°C and the PCR product was kept under 4°C.

In order to determine the amplified PCR products, the agarose gel electrophoresis method was applied. The bands produced were evaluated in UV transilluminator in 312 nm wavelength. The presence of 246 bp size HHV-6 DNA specific bands were assessed positive compared with a size marker and was photographed. Distilled water was used in the study as the negative control.

The presence of anti-CMV IgM and IgG type antibodies in the study group was investigated using the microparticule enzyme immunoassay (MEIA) method (AXSYM/ABBOTT Labs, IL/USA) and with 1/100 dilution in line with the manufacturer's instructions.

The study results were entered into a computer program (SPSS 11.0) and were evaluated statistically using the chi-square method with $p<0.05$ as the accepted standard for statistical significant of the results.

RESULTS

In 9 (20%) of the renal transplant (RT) patients, HHV-6 DNA was positive, while anti-HHV-6 IgM was found positive in 8 (17.7%) and HHV-6 IgG in 43 (95.5%) in those samples. Also, in 41 (91%) patients, anti-CMV IgG was present and in five (11%) patients, IgM was found to be positive (Table 1). In all of the 9 HHV-6 DNA positive renal transplant patients, anti-HHV-6 IgG was present and in five of them IgM seropositivity was detected. In two of the five patients with anti-HHV-6 IgM and HHV-6 DNA positivity, anti-CMV IgM and IgG positivities were detected. In three of the patients out of eight with anti-HHV-6 IgM positive, HHV-6 DNA and anti-CMV IgM were negative. While HHV-6 IgM, IgG and HHV-6 DNA were positive in two of the patients out of eight (17.7%), anti-CMV IgM positivity was also detected in one of them.

Figure 1

Table 1: Collective data from RT, CT and the control group

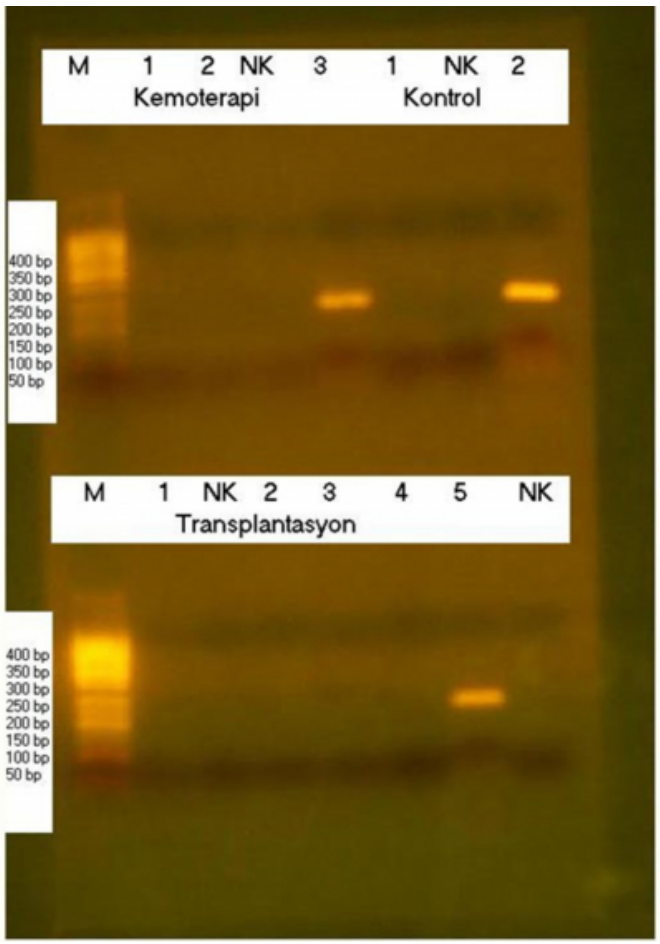
Study group	HHV-6 DNA n (%)	HHV-6 IgM n (%)	HHV-6 IgG n (%)	CMV IgM n (%)	CMV IgG n (%)
RT (n=45)	9 (20)	8 (17.7)	43 (95.5)	5 (11)	41 (91)
CT (n=35)	4 (11.4)	5 (14.2)	31 (88.5)	5 (14.2)	32 (91.4)
Control group (n=60)	1 (1.6)	2 (3.3)	55 (91.6)	6 (10)	52 (86)

*RT: Patients with renal transplant , CT: Patients receiving chemotherapy

While IgM and IgG positivities were detected together in five (14.2) and anti-HHV-6 IgG was detected in 31 (88.5%) of the chemotherapy (CT) patients, HHV-6 DNA was found positive in four (11.4%). In 32 (91.4%) of the patients, anti-CMV IgG and in five (14.2%) anti-CMV IgM and IgG together were found to be positive (Table 1). While HHV-6 DNA was negative in three of the patients out of five with anti-HHV-6 IgM positive, anti-CMV IgM positivity was detected in two of these.

Figure 2

Figure 1: The pattern of product from some patients' and control group sera is shown in agarose gel electrophoresis.



M: Molecular Size Marker, NK: Negative control, Numbers: Patients' samples, Transplantasyon: Patients with renal transplant, Kemoterapi: Patients receiving chemotherapy, Kontrol: Control group.

In the control group, 55 people (91.6%) were anti-HHV-6 IgG positive and two people (3.3%) were IgM positive, while one person (1.6%) was found to be HHV-6 DNA positive. Anti-CMV IgG was positive in 52 people (86%) in the control group and IgM in six (10%).

Figure 3

Table 2: Other results from HHV-6 DNA positive outcomes

	HHV-6 IgM	HHV-6 IgG	CMV IgM	CMV IgG
RT (n=9)	5	9	3	9
CT (n=4)	2	4	1	3
Control group (n=1)	1	1	1	1

In all of the nine renal transplant patients with HV-6 DNA positive, anti-HHV-6 IgG and anti-CMV IgG antibodies were also positive. In all of the four chemotherapy patients with HHV-6 DNA positive, anti-HHV-6 IgG and in three of

them anti-CMV IgM were positive. Anti-HHV-6 IgM was positive in five patients of the renal transplant group and two patients of the chemotherapy group. Anti-CMV IgM was positive in three patients and one patient of those groups, respectively. All of the required parameters were positive in one person in the control group (Table 2).

In 12 patients (26.7%) of the renal tansplant group and seven (20%) patients of the chemotherapy group, HHV-6 DNA and/or anti-HHV-6 IgM was positive. In both groups in all of the anti-HHV-6 IgM positive patients, anti-HHV-6 IgG was also positive.

In the RT patients, anti-HHV-6 IgM and HHV-6 DNA positivities were assessed to be statistically significant when evaluated alone (p= 0.002 and p=0.04, respectively) or together (p= 0.001). In the CT patients, anti-HHV-6 IgM positivity was significant alone (p=0.036) or anti-HHV-6 IgM and HHV-6 DNA together (p=0.007); whereas HHV-6 DNA positivity alone (p=0.099) was not significant. When the RT and CT patients were compared, there was no significant difference (p= 0.30 for HHV-6 DNA, p= 0.67 for HHV-6 IgM, p= 0.39 for HHV-6 DNA and/or HHV-6 IgM) (See Table-3).

DISCUSSION

It is known that HHV-6 which has a lymphotropical characteristic, is commonly found in the world, is established in organism in the very early stages of life, stays latent and may cause various clinical conditions alone or together with some opportunist pathogens. Immunosuppression caused by miscellaneous reasons, predisposes the patients to viral reactivation or infection. HHV-6, by targeting lymphocytes, natural killer cells and monocytes, may further increase immunosuppression and may cause a more severe clinical episode. The infections caused by the virus may be overlooked due to the fact that the clinical conditions of HHV-6 infections resemble those of other members of the Herpesviridae family, and that the laboratory tests used in differential diagnosis are not commonly used in routine practice.

The antibody screening tests play an important role in serological studies of HHV-6. On the contrary, their clinical value is limited. Due to the high rate of seropositivity in the population, single serum samples are not remarkable in diagnosis. Although it is confirmed by culture and IgG seroconcersion, IgM cannot be a reliable determinant due to the fact that the absolute IgM seroconversion is not observed in many cases and that the IgM response is present in about 5% of the adults. Again, the cross reactions observed between HHV-6 with HHV-7 and CMV, and also the genetic resemblence between the A and B variants of HHV-6 may make it difficult to comment on the serological tests [6].

In our study, five samples out of eight with anti-HHV-6 IgM in the renal transplant group were found to be HHV-6 DNA positive. HV-6 DNA were negative for the other three speciemens. Anti-CMV IgM was also negative for these same three speciemens. These findigs has led us to the assumption of HHV-6 presence in this group. In the chemotherapy group, three patients out of five anti-HHV-6 IgM positive patients HHV-6 DNA was negative and in two of them anti-CMV IgM positivity was detected. Although this does not eliminate the thought of cross reaction between CMV and HHV-6, it may be considered that HHV-6 and CMV may occur alone or together.

The the frequency of HHV-6 reactivation in healthy individuals is not known. However, even if it reactivates, this is thought to be irrelevant with the disease [1]. The virus also having an immunomodulator characteristic, is a sign of increased immunosuppression [13].

The HHV-6 infection appears in different rates in transplant receivers, depending on the degree of immunosuppression, the organ transplanted and the charcteristics of the method used in diagnosis. It is stated that this rate is between 38-66% in renal transplant receivers. It is usually observed within the first post-transplantation month and almost always the HHV-6B variant. This appearance in the early post-transplantation period is also a susidiary criteria to differentiate it from CMV which appears in the latter stages [12, 13].

Ratnamohan et al. [15] have stated in their study that they have not observed serious symptom in renal transplanted patients with HHV-6 reactivation.

Okuno et al. [16] determined anti-HHV-6 IgG in 21 kidney transplant donors and receivers, and a significant increase in titration has been seen in eight of the receivers after transplantation. The virus has also been examined by tissue culture method in the peripheric blood luekocytes of two of eight of those showing signs of rejection, and the virus has been isolated in both. Among the rest, of 9 rejected kidney biopsies, five had HHV-6 antigen in the tubular epithelium. Based on these results, they concluded that HHV-6 may infect the renal tissues and that the infectiond may be related with rejection and/or immunosuppressive treatment.

Deborska et al. [13] determined anti-HHV-6 IgG in 91% of patients before transplantation, and anti-HHV-6 IgM in 11%. They observed seroconversion rate of 45% for anti-HHV-6 IgG and 46.6% for anti-HHV-6 IgM in post-transplantation follow-up sera of those patients. In our country, in a study conducted by Yalcin et al. [14], 16 renal transplant patients and 16 controls were evaluated. In the peripheric blood leukocytes of 63% of the renal transplant group and 44% of the control group, HHV-6 DNA was determined. Among those, they have shown HHV-6 DNA in four (80%) of the five patients who developed rejection.

In our study, HHV-6 DNA and anti-HHV-6 IgM positivities were significantly high in the renal transplant group in comparison to the control grup ($p<0.001$). Also, anti-HHV-6 IgM and IgG were positive together with HHV-6 DNA in two (25%) of eight of sera of patients with rejection findings. Beside, in one of those two patients, anti-CMV IgM and CMV IgG positivities were simultaneously present. The HHV-6 DNA and seropositivity were statistically significant in the presence of rejection ($p<0.05$). This finding makes it assumable that the HHV-6 reactivations may set up a possible risk for this group of patients.

While HHV-6 is mentioned to be related with lymphoma in adults, its role in the formation of lymphoma is not yet clear. It may be thought to be related to lymphoproliferative diseases due to the facts that in vitro it binds the antioncogenic P53, transforms human and animal cell series and in vivo it is determined in EBV-negative Burkitt lymphoma patients [17].

Shiramizu et al. [18] have reported that in their studies with PCR, they did not determined HHV-6 DNA in children with Hodgkin's disease. Lyall [19] has investigated anti-HHV-6 IgG and IgM antibodies with IIFT technique in pediatric oncology patients and reported 90% IgG positivity in the control group and patients, while IgM positivity was present in one individual per each of the patient and control groups, and has concluded that HHV-6 does not cause active infection in this group of patients.

Carricart et al. [20] determined 63.5% HHV-6 positivity with IIFT and PCR methods in control group sera, while this rate was 95.5% in the group with neoplasia. In this study conducted on lymphoma/myeloma, leukemia and non-immune solid tumors, they also reported that the viral genome burden was significantly high in comparison to the control group and that this could contribute to the development of lymphoproliferative disease.

In a study conducted on lymphatic tissues, Collot et al. [21] determined HHV-6 DNA at 35.1% in Hodgkin's disease patients, 22.2% in B cell neoplasia, and 23.1% in T or NK cell neoplasia.

Michalek et al. [22] determined 17.4% HHV-6 DNA in peripheric blood of 66 children with cancer, 15.6% in the control group and they did not find any significant difference. After cytotoxic chemotherapy, the rate was 37.1% in patients with fever. Four of 66 patients with serious HHV-6 infection was found, three of which were those having received cytotoxic chemotherapy. Based on those results, they have stated that HHV-6 reactivation was frequent in pediatric cancer patients having received cytotoxic chemotherapy and that this could cause serious complications.

Although in our study the positivities of HHV-6 DNA and anti-HHV-6 IgM were higher in the chemotherapy group, it was not statistically significant compared to the control group ($p>0.05$). On the contrary, HHV-6 DNA and anti-HHV-6 IgM positivity evaluated together was statistically significant ($p< 0.05$).

CONCLUSION

Evaluating findings from our study and those of other investigators, besides the epidemiological importance, immunosuppression may increase the frequency of HHV-6 related primary infection or reactivation in patients. As a result, we believe that more extensive studies need to be conducted to prove the role of HHV-6 in rejection in patients with kidney transplants and its relationship to other malignant diseases.

CORRESPONDENCE TO

Nurittin ARDIC, MD Gulhane Military Medical Academy Haydarpasa Training Hospital Department of Microbiology and Clinical Microbiology 81327 Uskudar/Istanbul-TURKEY Phone: 00 90 216 542 27 97, Fax: 00 90 216 348 78 80 E-mail: nurittinardic@yahoo.com

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Author Information

Tuncay Kurukuyu
Specialist, Department of Bacteriology, Diyarbakir Military Hospital

Nurittin Ardic
Associate Professor, Department of Microbiology and Clinical Microbiology, Gulhane Military Medical Academy, Haydarpasa Training Hospital

Mustafa Ozyurt, Associated Professor
Department of Microbiology and Clinical Microbiology, Gulhane Military Medical Academy, Haydarpasa Training Hospital