

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Prevalence study of emerging new human polyomaviruses and human herpesvirus 6A

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**PREVALENCE STUDY OF EMERGING NEW HUMAN POLYOMAVIRUSES AND
HUMAN HERPESVIRUS 6A**

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INTRODUCTION

Recently more and more virus sequences have been identified due to the development of sequencing technology. Since these viruses were observed during the research of unknown virus sequences and not as causative agents, there is not enough information about the prevalence and pathogenesis of these viruses yet. In order to understand it, we need to study the portal entries and transmission, the site of replication and the pathogenetical roles of the virus infection.

In the recent years a number of new polyomaviruses have been developed.. Examination of these viruses may be important, because they can cause serious disease after the asymptomatic first infection such as BK and JC polyomaviruses. It has already been proven that viruses that can establish latent infection (for example BK and JC polyomavirus) the primary infection is often asymptomatic, but reactivation of the virus due to the malfunction of the immune system can result in serious disorders. Immunosuppressive therapy for the treatment in organ transplantation and immunological changes during pregnancy can lead to higher susceptibility to infections, reactivation of latent infections or predict higher risk for reinfection. Therefore the aim of this study was to examine whether these unknown polyomaviruses (WU, KI and human polyomavirus 9) can cause disease, to determine the prevalence of the viral infections and to investigate the portal entries and transmission of the viruses.

Higher risk for diseases due to human cytomegalovirus (HCMV) infection is well-known in case of organ transplant recipients and pregnant women. Diagnosis of HCMV infection is a routine method in Hungary. Nevertheless the diagnosis and therapy for human herpesvirus 6A and 6B (HHV-6A and HHV-6B) infections are part of the routine clinical procedure in other countries, it is not the case at the University of Debrecen and in Hungary. Therefore the aim of this study was to examine the frequency and symptoms of these infections in pregnant women and in patients receiving immunosuppressive therapy, and whether it is necessary to introduce the examination of these viruses to the routine clinical diagnosis.

REVIEW OF THE LITERATURE

Herpesviruses

Herpesviridae family, that can establish latent infection, is divided into three subfamilies: Alpha-, Beta- and Gammaherpesvirinae. In this study we examined the infections caused by human herpesvirus 5 (usually called as human cytomegalovirus) and human herpesvirus 6A and 6B which are the members of Betaherpesvirinae.

Human herpesvirus 6A and 6B

HHV-6 was first isolated from a blood sample from a patient suffering from lymphoproliferative disorder in 1986. HHV-6 has been spread all over the world, seropositivity exceeds 95%, although it is important to note that most of these publications did not differentiate between HHV-6A and HHV-6B. Primary HHV-6 infection (almost exclusively HHV-6B) occurs in early childhood, usually between the age of 6 months and 3 years. The date of primary HHV-6A infection is not clear yet, but more likely it happens after the HHV-6B infection, although there are publications about primary HHV-6A infection in children. HHV-6B DNA was detected mainly in saliva and in the cells of salivary glands, indicating that the virus spreads via saliva. Vertical, transplacental transmission is also possible. Transmission of HHV-6A is not clear yet, since it is not detectable in saliva. Sexual transmission is a possibility because the virus was detected in the genital tract. HHV-6A was detected in several regions of the brain and in the liquor as well. HHV-6 can establish latent infection in different cells and tissues such as mononuclear cells, bone marrow, liver, spleen, kidney. In case of organ transplantation infected organs can lead to HHV-6 reinfection in the recipient. Primary HHV-6A infection is supposedly asymptomatic or happens in a subclinical form. Primary HHV-6B infection at the third of immunocompetent patients can cause a disease called exanthema subitum, that has mild symptoms such as fever and rash but can also be asymptomatic. Reinfection or virus reactivation due to immunosuppression can lead to more serious symptoms like fever, rash, thrombocytopenia, leukopenia, pneumonia, hepatitis, pancreatitis, colitis, encephalitis, meningoenzephalitis, even prolonged bone marrow suppression. HHV-6 is able to integrate into the human chromosomes (ciHHV-6). Chromosomal integration of HHV-6 means that the complete sequence of HH-6 is integrated into the germ cells enabling the vertical transmission via a Mendelian manner.

Human herpesvirus 5 (human cytomegalovirus, HCMV)

Human herpesvirus 5 is also ubiquitous in the human population, seropositivity is about 40% between 6-11 years and exceeds 90% in adults. The virus spreads with different types of excretion of the infected person for example saliva, urine, faces, blood, secretion of genital tract and breast milk. It is also possible to transfer the virus by organ transplantation. Vertical transmission can occur by transplacental transfer, during delivery, by the secretion of an infected mother and during breastfeed. In healthy individuals the primary infection is nearly or completely asymptomatic or can cause mononucleosis infectiosa. HCMV infection during the first and second trimester can cause congenital disorders. During primary infection not only monocytes and lymphocytes but also epithelial cells are getting infected. After primary infection HCMV can establish latency in numerous tissues and cell types, for example monocytes, macrophages, lymphocytes, salivary glands, bone marrow, renal epithelial cells and in the brain, respectively. Immunosuppression because of organ transplantation or AIDS can result in reactivation of the latent infection. The main location of virus replication during the reactivation are specifically the monocytes, macrophages and the renal epithelial cells. At the same time reinfection by new virus strains may result in active infection. Reactivation and reinfection in immunocompromised patients can cause severe symptoms including pneumonia, gastrointestinal diseases, hepatitis, retinitis, rejection of the transplanted tissue or organ.

Polyomaviruses

Before 2007 only two human pathogenic members of Polyomaviridae family were known: BK and JC polyomaviruses. Since then twelve new human polyomaviruses (KI and WU polyomavirus, Merkel cell polyomavirus, human polyomavirus 6, 7, trichodysplasia spinulosa-associated polyomavirus, human polyomavirus 9, 10, Malawi polyomavirus, MX polyomavirus, human polyomavirus 12, Saint Louis polyomavirus) were discovered by modern sequencing technology. Sequence of the third human polyomavirus, KIPyV was described in 2007. The virus was found in samples from children who suffered from acute respiratory symptoms. In the same year the fourth human polyomavirus WUPyV was identified in children who also suffered from acute respiratory symptoms. Human polyomavirus 9 (HPyV9) was discovered in blood sample from renal transplant patient by Scuda and his colleagues in 2011. We started this prevalence study in 2009, when only limited information was available about these viruses. Only 9 different human

polyomaviruses were known during the time period of the examination, therefore the aim of this study was to investigate the WUPyV, KIPyV and HPyV9 viruses.

WU polyomavirus (WUPyV)

Serological studies suggest that WU polyomavirus is widespread, the seropositivity is 44-87% between the age of 1-5 years, while it is 69-98% in adults. These data suggest that primary WUPyV infection occurs in early childhood. Little is known about the transmission, supposedly it happens via fecal-oral route or through the respiratory tract. It is also possible that the virus establishes a latent infection that is reactivated at immunosuppression. In recent years more and more data have been published about the prevalence of the virus: WUPyV was found in respiratory samples, blood, faces, lymphoid tissues, liquor. In case of immunocompetent individuals, the virus was mostly detected in children suffering from respiratory diseases, the frequency of WUPyV was the highest (16.4%) in respiratory samples from Japanese children. Frequency of the virus, just like BK and JC, seems to be the highest in immunocompromised patients (0.9-8.3%). These data suggest that WUPyV causes more serious symptoms in immunocompromised patients compared to the mild infections in immunocompetent individuals.

KI polyomavirus (KIPyV)

Prevalence studies detected KI polyomavirus in respiratory samples from all of the world, the PCR prevalence was 0.5-8%. Seropositivity was 45-94% in children at the age of 5 and 55-100% in adults, suggesting that the virus is widespread in the human population and the primary infection usually occurs in childhood. Several groups studied the prevalence in the human population, and they were able to detect the virus in blood, faces, lymphoid and lung tissue. Although the frequency in the various sample types was different, positive samples had been collected from all over the world. Highest virus frequency (12%) was described in lymphoid tissue in immunocompetent individuals. These data suggest that transmission of KIPyV happens via fecal-oral and/or via the respiratory route, just like WUPyV. Dissemination of the virus probably occurs via blood. The frequency of KIPyV was 1-45% in immunocompromised patients and 0.5-12% in immunocompetent individuals, indicating that immunosuppression may lead to higher susceptibility to infection similar to BK and JC polyomaviruses (BKPyV, JCPyV).

Human polyomavirus 9 (HPyV9)

HPyV9 was first described in blood and urine samples from kidney transplant patients. Seroprevalence was 10.4-18.6% between 1-14 years and 33.7% between the age of 15-19. Seropositivity was 39.4-47% in healthy adults, and exceeds 69.6% at the age of 80. Seropositivity increases with age, indicating that HPyV9 infection can occur any time, in other words non-infected adults are still susceptible to the infection. Immunosuppression may lead to higher susceptibility to HPyV9 infection similarly to the WU and KI polyomavirus infections. Seropositivity was 63-69% in renal and bone marrow transplant patients, which is higher than the healthy control group (41-43%). So far the portal entries, the way of transmission and the site of replication is not clarified yet. The virus was detected in serum, plasma, whole blood and urine samples. It was also found in skin samples from healthy individuals (the prevalence was 0,9%) and in patients who suffered from Merkel-cell carcinoma (in this case the prevalence was 25%). Researchers tried to find the virus DNA, but they could not detect that in any of the examined samples (faces, respiratory samples, nasopharyngeal aspirate, liquor). Since HPyV9 was detected in immunocompromised patients it may cause serious disease similarly to other polyomaviruses only in those patients and the frequency of HPyV9 may also be higher in them.

AIMS

- Our aim was to determine the prevalence of HHV-6A, HHV-6B, HCMV, KIPyV, WUPyV and HPyV9 viruses in healthy adults, pregnant women and renal transplant patients.
- Since the diagnosis or monitoring of HHV-6A and HHV-6B infections are not routine methods in renal transplant patients neither at the University of Debrecen nor in the other regions of Hungary, our aims were
 - to investigate the frequency of these viruses, the presence of infection at different times after transplantation, and to identify the clinical symptoms
 - to examine the association between HCMV and HHV-6A/ HHV-6B infections.
- The aim of the study was to evaluate the prevalence of three new human polyomaviruses KI, WU and HPyV9.
 - We used different samples to investigate the portal entries and transmission of the viruses.
 - We studied whether immunological changes during pregnancy and immunosuppression associated with renal transplantation can lead to higher prevalence of the viruses compared to healthy individuals.
 - We tried to determine the presence and the amount of viruses at different weeks of pregnancy and after renal transplantation.
- We investigated whether there is any association between the virus infection and the observed clinical symptoms.

MATERIALS AND METHODS

Samples and clinical data

Samples from renal transplant patients and non-transplant, healthy individuals

Two hundred EDTA blood samples from 200 renal transplant patients (114 men, 86 women; median age: 46, range: 11-69 years) were taken for investigation of herpesviruses at different times after transplantation (range: 3-7115 days; median: 1270 days) in the Department of Surgery, at University of Debrecen. We used 200 EDTA blood samples from healthy individuals (75 men, 125 women; median age: 39, range: 10-74 years) as controls.

For investigation of polyomaviruses 195 EDTA blood samples from 195 renal transplant patients (82 women, 113 men, median age: 46, range: 7-96 years) were collected at different times after transplantation (range: 3–7108 days, median: 1188 days). For control measurements, 200 blood samples from 200 healthy individuals (75 men, 125 women, median age: 39, range: 10–74 years) were taken. 50 urine samples from 50 transplant patients were also collected (range: 5–6230 days, median: 141 days). 36 urine samples from healthy people (17 men, 19 women, range: 21-58 years) were used as controls. 90 upper respiratory tract samples using throat swabs from 90 renal transplant patients were obtained 18-6230 days after transplantation (median: 1177 days).

The Regional and Institutional Ethics Committee of University of Debrecen approved all of the studies.

Samples from healthy pregnant and non-pregnant women

Urine, EDTA blood samples and throat swab samples were collected on the same day from 100 healthy pregnant women (age: 17-42 years, median: 32 years; pregnancy 5-39 weeks, median: 26 weeks) and from 100 non-pregnant women (age: 18-44 years, median: 32 years) in the Department of Obstetrics and Gynecology, at the University of Debrecen. All samples were collected at the same time. Samples from pregnant women were collected in all trimesters: from the first trimester 28 samples, from the second trimester 27 samples and from the third trimester 45 samples had been collected. Control samples were taken from healthy, non-pregnant, fertility exam visitor women. The study was approved by Regional and Institutional Ethics Committee of University of Debrecen.

Nucleic acid extraction

Immediately after collection plasma and blood cells were separated by centrifugation (10 min, 1500 g). Red blood cells were removed from cell pellet by lysis.

Nucleic acid was isolated from 1.5×10^6 cells, 200 μ L plasma, 200 μ L urine specimen and throat swab samples using High Pure Viral Nucleic Acid Kit (Roche, Switzerland) according to the manufacturer's instructions. The effectiveness of DNA and RNA isolation and DNA amplification was controlled by β -globin PCR, meanwhile the amplification of RNA was controlled by GAPDH mRNA RT-PCR.

Detection of viruses

Nested PCR for HHV-6

To detect HHV-6 DNA was used nested PCR technique which is able to differentiate between HHV-6A and HHV-6B viruses based on the different length of PCR amplicons. Primers were specific for the U90 gene of the immediate early region . DNA extracts from HHV-6A GS and HHV-6B Z29 virus suspensions were used as positive controls. Viruses were determined according to the length of amplicons after electrophoresis of second-round PCR products (in 1.5 % agarose gel stained with ethidium-bromide in TBE buffer). Our results were evaluated in BioRad GelDoc gel documentation system.

Real-time PCR for quantitative detection of HHV-6

Quantitative detection of HHV-6 DNA were carried out with real-time PCR using TaqMan probe. Absolute quantification was performed with a plasmid containing U65-66 HHV-6A and 6B genome regions. Forward primer and TaqMan probe were the same at the two different viruses, but the reverse primer was different.

In samples from pregnant and non-pregnant women quantitative HHV-6 real-time PCR was carried out with HHV-6 ELITE MGB Kit which contains specific primers and probes for the ORF 13 region of HHV-6 and β -globin gene.

HHV-6 RT-PCR

To distinguish between latent and active HHV-6 infection, reverse transcription PCR (RT-PCR) was performed. To transcribe RNA into cDNA we used High Capacity cDNA Reverse Transcription Kit after DNaseI treatment of nucleic acid from white blood cells. Primers of HHV-6 RT-PCR were specific for U79/80 gene of HHV-6 genome sequence.

WUKI nested PCR

To detect WUPyV and KIPyV polyomaviruses nested PCR were used amplifying both WUPyV and KIPyV DNA, This method does not differentiate between them. Primers of WUKI PCR were specific for the VP2 region of the virus genome sequence. Plasmids containing the genome of WUPyV and KIPyV were used as positive controls. To identify viruses PCR products were visualized by electrophoresis in 1.5% agarose gel containing ethidium bromide. The amplified PCR products were prepared from the agarose gel, purified with QIAquick Gel Extraction Kit and sequenced by using ABI PRISM 3100 Genetic Analyzer.

Real-time PCR for WUPyV and KIPyV

To determine WUPyV and KIPyV viral load real-time PCR with TaqMan probe was used. Primers were specific for VP1 region of the virus genom sequences. For absolute quantification serial dilutions of KIPyV and WUPyV plasmids were used.

HPyV9 nested PCR

HPyV9 PCR was carried out with diagnostic primers published by discoverers, which were specific for VP1 region of HPyV9 genom. Plasmids containing the genome of HPyV9 were used as positive controls. To identify the virus amplified PCR products were prepared from agarose gel, purified with QIAquick Gel Extraction Kit and sequenced by using ABI PRISM 3100 Genetic Analyzer.

HCMV pp65 antigenaemia

HCMV active infection was examined by pp65-antigenaemia using CINAKit according to the manufacturer's instructions as a routine diagnostic procedure. These results were used also at the analysis of our date.

Statistical analysis

Chi square, Fisher's exact test and Mann-Whitney U test were applied for statistical analysis. Difference was considered significant if p value was less then 0.05.

RESULTS

Human herpesvirus 6 infection

Examination of renal transplant patients and healthy adults

Frequency of active HHV-6 infections was 4.5% (8 HHV-6A and 1 HHV-6B positive) among renal transplant patients. In healthy adults HHV-6 viraemia was not detected. Based on Fisher's exact test the frequency of HHV-6 viraemia was significantly higher in renal transplant patients compared to healthy adults (9/200 vs. 0/200; $p=0,004$).

The level of HHV-6 DNA in plasma samples from renal transplant patients ranged from 5×10^2 to 6×10^5 genome equivalent/mL (GEq/mL), while the copy number of HHV-6 genome was below the detection limit (less than 250 GEq/mL) in the plasma samples from healthy adults. Viral load was 5.1×10^2 - 2.1×10^6 GEq/ 1.5×10^6 in white blood cells from renal transplant patients and 5.8×10^3 GEq/ 1.5×10^6 cells in healthy adults. In those patients who had ciHHV-6, the ratio of viral genome to human genome was 1:1 consequently the viral load was over 1 million GEq/mL blood. In one renal transplant patient (0.5% of the examined patients) viral load was 2.1×10^6 GEq/ 1.5×10^6 cells, 6.2×10^3 GEq/mL in plasma, suggesting the presence of ciHHV-6.

HHV-6A was 6% (12/200) and HHV-6B latency was 2% (4/200) among renal transplant patients. HHV-6 latency was revealed in the healthy control group, the frequency of HHV-6B was 9% (18/200) while HHA-6A had 1% (2/200). There was no significant difference between the prevalence of latent HHV-6 infection in the two examined groups (16/200 vs. 20/200; $p>0.005$). Statistical analysis revealed that frequency of HHV-6B latency was significantly higher than HHV-6A latency in both renal transplant patients (18/20 vs. 2/20; $p=0.01$), and in healthy adults (18/20 vs. 2/20; $p=0.0001$). At the same time frequency of HHV-6A was significantly higher in viraemia than during latency in renal transplant patients (8/9 vs. 4/16; $p=0.0036$). There was no significant difference between the time of sample taking after the transplantation of HHV-6 positive (21 days-13,1 years; median 6,2 years) and negative patients (3 days-19.6 years; median 3.3 years). Statistical analysis did not reveal significant differences between the age of HHV-6 positive (17.9-61.7 years, median 45.4 years) and negative patients.

Among the examined patients, 31 did not have any clinical symptoms at the time of sample collection, others had fever, respiratory and gastrointestinal symptoms. Statistical

analysis of HHV-6 viraemia and clinical symptoms did not reveal any association between the presence of the virus and the observed clinical symptoms.

Examination of pregnant and non pregnant women

HHV-6A DNA was detected by HHV-6 nested PCR in the plasma samples and white blood cells from pregnant and non-pregnant women at the same time, but HHV-6 RT-PCR did not show any sign of active virus infection. Frequency of HHV-6A was 1% (1/100) in pregnant and 3% (3/100) in non-pregnant women, respectively. Level of HHV-6 DNA was 2.03×10^2 GEq/mL in the plasma sample, while 5.07×10^5 GEq/ 1.5×10^6 cells were detected in white blood cells from pregnant women. Viral load was $<250 - 2.55 \times 10^3$ GEq/mL in the plasma samples and $<250 - 1.6 \times 10^5$ GEq/ 1.5×10^6 cells in white blood cells from non-pregnant women. Although the level of HHV-6 DNA was high in the white blood cells, the ratio of viral genome to human genome (0.08-0.33 GEq/1 white blood cell) did not reveal the presence of ciHHV-6.

Frequency of latent HHV-6B infection was 14% (14/100) and 2% (2/100) for HHV-6A in white blood cells from pregnant women. In non-pregnant women HHV-6B and HHV-6A latency was 15% and 4% in white blood cells, while one of the leukocyte samples from non-pregnant women carried both HHV-6A and HHV-6B DNA. Latency was found in all trimesters. Frequency of HHV-6B latency was significantly higher than HHV-6A latency both in pregnant (14 vs. 2; $p=0.0035$) and non-pregnant women (15 vs. 4; $p=0.014$). There was no significant difference in the frequency of HHV-6 latency between pregnant and non-pregnant women (16/100 vs. 19/100; $p>0.005$).

HCMV infection of renal transplant patients

Frequency of HCMV infection was 3.5% (7/200) among renal transplant patients shown by detection of pp65 antigen in white blood cells. Simultaneous presence of HCMV and HHV-6A or HHV-6B viraemia was not detected. The frequency of active HHV-6 infection was not significantly different from the frequency of HCMV infection (9/200 vs. 7/200; $p>0.05$).

Statistical analysis did not show significant differences between the age of HCMV positive (32.7-61.6 years; median 55.4 years) and negative (11.2-68.8 years; median 44.8 years) patients. There was no significant difference between the time of sample taking after

the transplantation of HCMV positive (50 days-15,7,6 years; median 124 days) and negative (3 days- 19.6 years; median 3.5 years) patients either. Statistical analysis of HCMV infection and clinical symptoms did not reveal association between the presence of virus and the observed symptoms.

Polyomaviruses

Examination of renal transplant patients and healthy adults

Frequency of WUKI PCR positive plasma samples from renal transplant patients was 3.6% (2/195, 1% KIPyV; 5/195, 2.6% WUPyV). Level of DNA load in plasma samples was less than 250 GEq/mL plasma which is below the detection limit, with the exception of one KIPyV positive sample (2.5×10^2 GEq/mL). WUPyV and KIPyV positivity was significantly higher shortly after the transplantation (8-2122 days, median 24 days vs. 3-7108 days, median 1271 days; $p=0.001$). Statistical analysis did not show correlation between the presence of viruses and the observed clinical symptoms. KIPyV and WUPyV DNA were not detected in plasma samples from healthy individuals.

14% (7/50) of renal transplant patients' urine sample showed PCR positivity for WUKI. Frequency of WUPyV and KIPyV positive samples were 12% (6/50) and 2% (1/50), respectively. Viral loads of two urine samples were 5×10^2 GEq/mL and 1.1×10^3 GEq/mL, but the others were less than 250 GEq/mL urine. In two patients, whose urine samples were positive for WUPyV DNA, WUPyV viremia was also detected. WUPyV and KIPyV DNA were not detected in urine samples from healthy adults. WUPyV and KIPyV positivity was significantly higher shortly after the transplantation (8-58 days, median 30 days) compared to later time points (7-6230 days, median 745 days; $p=0.001$). Statistical analysis did not show association between the presence of viruses and the observed clinical symptoms.

Frequency of WU or KI polyomavirus DNA positive respiratory samples from transplant patients was 10% (6/90, 6.67% for KIPyV and 3/90, 3.33% for WUPyV). Viral loads of KIPyV positive samples were $2.8 \times 10^2 - 3.7 \times 10^5$ GEq/mL and only one out of the WUPyV positive samples had detectable viral load of 6.3×10^2 GEq/mL, the others were less than 250 GEq/mL. The plasma sample of one patient with KIPyV positive respiratory specimen was also positive for WUPyV DNA. Significant difference was found between polyoma-positive (21-822 days, median 101 days) and negative samples (18-6230, median 1177; $p=0.002$) regarding the time after transplantation. All patients with positive respiratory

specimen had acute upper respiratory tract symptoms. Frequency of respiratory symptoms was significantly higher in patients with WUKI PCR positive samples than in those who were WUKI PCR negative.

Examination of pregnant and non-pregnant women

WUPyV DNA was not detected in plasma samples neither from pregnant nor from non-pregnant women. KIPyV was found in two plasma samples (2%) from non-pregnant women, but the viral loads were below the limit of detection by real-time PCR. Neither KIPyV nor WUPyV DNA was detected in the urine and respiratory samples.

HPyV9 DNA was detected in urine, plasma and respiratory samples from both studied groups. Frequency of HPyV in plasma samples from pregnant and non-pregnant women was 2% (2/100) and 6% (6/100), respectively, the difference is not statistically significant (2/100 vs. 6/100; $p > 0.05$). In respiratory samples the frequency of HPyV9 DNA was the same in both studied groups (2/100, 2% and 2/100, 2%). Statistical analysis did not reveal significant difference in the prevalence of HPyV9 between these two groups (2/100 vs. 2/100; $p > 0.05$). The frequency of HPyV9 was 3% (3/100) in urine samples from pregnant women and 2% (2/100) in urine samples from non-pregnant women. The difference is not statistically significant (3/100 vs. 2/100; $p > 0.005$). Two plasma samples from non-pregnant women showed HPyV9 and KIPyV coinfection.

DISCUSSION

Both HCMV and HHV-6 may cause severe symptoms in immunosuppressed patients, like this in renal transplant patients, but there are limited information about the importance of complication associated HHV-6 infection and reactivation in Hungary. The aim of this study was to determine the prevalence of HHV-6 viraemia in these patients. The frequency of HHV-6 viraemia was 4.5%, the prevalence of infections with HCMV was 3.5% in renal transplant patients. The frequency of HHV-6 viraemia was not significantly different from the frequency of HCMV infection in renal transplant patients. Based on our results it is suggested that monitoring and diagnosis of HHV-6 infection is needed, because the symptoms caused by HHV-6 and HCMV might be the same. Contrary to some previous publications, simultaneous presence of infection with HCMV and HHV-6A/6B viraemia was not detected in the renal transplant patients, which does not exclude the interaction between these viruses. Statistical analysis of HHV-6 viraemia and clinical symptoms did not reveal association between the presence of the virus and the observed clinical symptoms. At the time of HHV-6 or HCMV infections similar clinical symptoms were observed e.g.: fever, respiratory and gastrointestinal symptoms and the combination of them. A follow up study might help to reveal the consequences of HHV-6A and 6B infection and the interaction of these viruses.

Previous reports noted that HHV-6 viraemia was found in the early (within 2-4 weeks) stage of transplantation, but later after transplantation as well, while HCMV infections were reported between 1-3 months after transplantation. HHV-6A and 6B viraemia were detected in the first month after the transplantation occurred or years later (5-15 years), while HCMV infections was observed after 2-5 weeks following the transplantation or 8 to 15 years later.

HHV-6 is able to establish latency and can integrate into the human chromosomes, hence the correct methods and clinical samples for the detection of active virus replication are essential. To distinguish between latent and active infection, reverse transcription PCR was used. Real-time PCR was used for the absolute quantification of the HHV-6 DNA. Individuals with ciHHV-6 represent more than 5×10^6 GEq/mL HHV-6 DNA in whole blood, and more than 10^3 GEq/mL viral load in the plasma. The prevalence of ciHHV-6 in a renal transplant patient (0.5%) suggested, correspondence with the previously published data (0.2-3%). The application of RT-PCR enabled the confirmation of active infections by the detection of viral mRNA in our study. This method is not considered to be a routine process in the diagnostic laboratory because it is more time-consuming requiring more intensive laboratory attention.

In this study the significantly higher prevalence of HHV-6 viraemia (8 HHV-6A és 1 HHV-6B) was found among the renal transplant patients than among the healthy individuals, which strengthens the concept that immunosuppression due to renal transplantation may result in higher susceptibility to HHV-6, mainly HHV-6A infection and may increase the risk of virus reactivation. In contrast to previous publications in renal transplant patients the HHV-6A viraemia was found to be dominant.

Nevertheless HHV-6 latency was detected both in renal transplant patients and in healthy individuals. In accordance with previous publications latent HHV-6 infection was dominated by HHV-6B and frequencies did not show any difference between the studied patients groups.

Limited information was available about HHV-6 prevalence of pregnant women. We carried out the prevalence study with pregnant women as well who suffered from immunological changes owing to the tolerance of the foetus. The frequency of HHV-6A latency was 2 % in pregnant and 4% in non-pregnant women. The prevalence of HHV-6B latency was 14 % in pregnant and 15 % in non-pregnant control groups. The leukocytes of one non-pregnant woman carried both HHV-6A and HHV-6B at the same time. In accordance with previous publications, the frequency of latent HHV-6B was significantly higher than the frequency of HHV-6A latency both in pregnant and non-pregnant women. Although HHV-6 DNA was found in plasma samples and in HHV-6 positive leukocytes samples at the same time, RT-PCR did not confirm the active HHV-6 infection. Presence of detectable human DNA in plasma samples revealed that viral DNA can originate from physiological lysis of cells. Infection or reactivation shortly before the sample taking neither could be excluded. In one pregnant woman HHV-6 DNA was found only in the plasma and was not present in the leukocytes. In this case HHV-6 might have replicated in another tissue and viral DNA might have been detected in blood because of the release of the virus DNA from the infected tissue into the blood circulation. Quantitative HHV-6 real-time PCR was carried out to determine the viral load in the samples. Although HHV-6 viral loads in one leukocyte sample was high (5.07×10^5 GEq/ 1.5×10^6 cells), the HHV-6 copy number /1 leukocyte (0.08-0.33 GEq/1 leukocyte) did not prove ciHHV-6 unequivocally.

Analysing our data collectively, the frequency of HHV-6 viraemia was significantly higher in renal transplant patients than in pregnant women, although the gender of cohorts were different (114 renal transplanted men and 86 women vs. 100 pregnant women). HHV-6A viraemia was found to be more frequent in renal transplanted patients than the HHV-6B. It is most likely that immunosuppression caused by transplantation may result in higher

susceptibility to HHV-6A infection or reactivation. We have limited knowledge about HHV-6A infection. HHV-6A might be an emerging pathogen and could probably be a more virulent virus than HHV-6B.

We have studied the prevalence of recently discovered human polyomaviruses, which were described in 2007 and in 2011. Limited information were available about these viruses at the time of our study although the number of studies in this topic is increased. Nevertheless the significance of the infection issues of these viruses infections and a lot of important questions about these pathogens are not yet clearly understood.

WU and KI polyomaviruses were detected in respiratory, plasma and urine samples from renal transplant patients, but were not present in plasma and urine samples from healthy individuals. The frequency of WUPyV and KIPyV viraemia was 2.6% and 1% in renal transplant patients and WU virus was more frequent than KIPyV. Significant differences were not found between polyoma-positive and polyoma-negative samples from the aspect of the elapsed time after the transplantation. Both viruses were detected mainly 1-3 months after transplantation. Viral loads were low (≤ 250 GEq/mL) in all plasma samples. Other publisher have found WUPyV and KIPyV with the frequencies of 1.6-8.3% and 2.6-3.2% in plasma samples from immunosuppressed patient, while the prevalences of KI and WU polyomaviruses were 1% and 3.1% in plasma samples from immunocompetent individuals. Based on these data, both viruses might invade the blood circulation and might infect other cells or tissues with the infected blood.

Our study revealed that KI and WU viruses can exist in urine sample, we published first their presence in urine samples. 12% of urine samples from renal transplant patients were WUPyV positive and 2% were KIPyV DNA positive . All of the positive samples were collected within 2 months after the transplantation. The viral loads of these urine samples were low ($\leq 1,1 \times 10^3$ GEq/mL) but at the same time in two patients WUPyV viraemia was also detected. These viruses were not detected in urine specimens of healthy individuals. Even though we only examined a few samples this should still be considered as a remarkable result, since no other research groups have detected the presence of these viruses in the urine before. Our results have been confirmed by other research groups after the publication of our study. Viruria with WUPyV and KIPyV might occur thus urine can also become the source of infection. It is possible that these viruses from their portal entries (for example the gastrointestinal or respiratory tract) might invade the kidney (by blood circulation) infecting the kidney and the cells of the urine tract. Though WUPyV viruria and viraemia was only detected at the same times in two patients they could be detected.

The frequencies of WUPyV and KIPyV were 6.7% and 3.3% in the respiratory samples from renal transplant patients. Statistical analysis revealed that the WUKI PCR positive samples were collected in a significantly earlier stage after the transplantation (within 3 months) than the PCR negative samples. Numerous work groups described both WU and KI polyomaviruses in respiratory samples. The frequency of WUPyV was more than 16.4% in immunocompetent children and less in immunocompetent adults (0.75%). According to seroprevalence studies the seropositivity was 87% at the age of five. It is hypothesized that the primary infection occurs in childhood, and the frequency of primary infection is rare in adults. Our WUPyV prevalence data in immunocompromised patients show correlation with other published data. In case of KIPyV the prevalence was 0.5-3% in immunocompetent individuals and 5.6% in immunosuppressed patients. We examined only 90 respiratory samples from 90 renal transplant patients, and the frequency of WU and KI polyomaviruses were 10%. All of the patients with positive respiratory samples had acute upper respiratory symptoms and a significantly higher frequency compared to the PCR- negative patients. Since none of these samples were tested for any respiratory virus the pathological role and the clinical consequences of the KI and WU polyomavirus infections are not clear.

In this study we detected WU and KI polyomaviruses in blood, urine and respiratory specimen of renal transplant patients, but not in healthy individuals. Our data strengthen the hypothesis that immunosuppression may result in higher susceptibility to infections with WU and KI polyomaviruses. Our aim was to determine the prevalence of these viruses in blood, urine and respiratory samples during pregnancy. Before our examination these viruses were not detected in the urine of pregnant women and neither in foetal tissues. In our study WU and KI was not detected in urine, plasma or respiratory samples from pregnant women. KIPyV was found in two plasma samples from non-pregnant women (2%) but were not present in urine nor respiratory samples. In this study the samples were collected once during pregnancy thus follow-up studies of pregnant women might help to determine the prevalence of these viruses during pregnancy.

HPyV9 was discovered in 2011. Limited number of prevalence publications are available, most of these reported negative results, Based on the seroepidemiological studies the virus is widespread although the seropositivity was only 39.4-47 % and the remarkable part of adult population have susceptibility to infection with the HPyV9. Study on pregnant women was performed soon after the discovery of HPyV9. We have found HPyV9 DNA in all of the examined samples from pregnant women and in the control groups with the frequency of 2-3% in pregnant women and 2-6% in non-pregnant women. There were no

statistically significant differences between the PCR prevalence in respiratory, urine and plasma samples from pregnant and non-pregnant women. It seems that seropositivity increases with age, which might suggest that the HPyV9 infection occurs any time in life in other words the remarkable part of adult population have susceptibility to infection with HPyV9. This may be the reason of the similar frequency of the virus in these two groups. Studied women did not suffer from clinical symptoms.

Our results revealed first the presence of HPyV9 presence in respiratory samples which might suggest the possibility of respiratory virus transmission.

We analysed the frequency of WU and KI prevalence in renal transplant patients, pregnant women and healthy individuals (non-pregnant women and non-transplant control group). It is important to note that the gender and the age of the groups were different. Analysing our data collectively, there were no significant differences between the frequencies of KIPyV and WUPyV DNA positivity in renal transplant patients and in pregnant women. However the frequency of WUPyV was significantly higher in the plasma samples from renal transplant patients than in the plasma samples from the control group. Statistical analyses revealed that there were significant differences between the frequencies of WUKI PCR positive urine samples from renal transplant patients and from pregnant women. The frequency of WUPyV was significantly higher in the urine samples from renal transplant patients than in the control group. There were also significant differences between the frequencies of KIPyV positive respiratory samples from renal transplant patients and from pregnant women. Frequency of KIPyV was significantly higher in the respiratory samples from renal transplant patients than from the control group. It is suggested that immunosuppression due to transplantation might result in dysfunction of the immune system which may establish higher susceptibility to KIPyV and WUPyV infections and may lead to the reactivation of these viruses but immunological changes via pregnancy would not. KIPyV DNA was detected mainly in the respiratory samples, while WUPyV DNA was found with higher frequency in the plasma and in the urine samples. Transmission of these viruses are not clarified, but it is suggested that the transmission may occur via respiratory and/or via the urinary tract. It is possible that after the primary infection in childhood with KIPyV, WUPyV and HPyV9, a lifelong persistent infection is established maybe in the renal and in the urinary tract cells. Immunosuppression may lead to the reactivation of these viruses. Our prevalence data suggest that immunosuppression via renal transplantation might result higher susceptibility to WUPyv, KIPyV and HHV-6A, or might lead to the reactivation of these viruses.

SUMMARY

HHV-6 viraemia detected in 4.5 % of renal transplant patients did not differ from the frequency of HCMV infection (3.5%). Contrary to previous publications dominance of HHV-6A was observed. HHV-6 infection was significantly more frequent among renal transplant patients than healthy individuals (0) or pregnant women (1%). Latent HHV-6 infection was dominated by HHV-6B, and frequencies did not show difference between the patients groups studied. Based on our study, immunosuppression due to renal transplantation may result in higher susceptibility to HHV-6A infection. Although statistical analysis did not reveal interaction between HHV-6 viraemia and clinical symptoms in our study, HHV-6A infection may have clinical consequences. Based on our results it is suggested that monitoring, diagnosis of HHV-6 infection is needed, a follow up study may help to reveal the consequences of HHV-6A infection. Chromosomally integrated HHV-6 was observed in a renal transplant patient, the frequency (0.5%) is in accordance with the literature.

Our prevalence data of novel human polyomaviruses also strengthen the hypothesis that immunosuppression might result in higher susceptibility to WU and KI viruses. Neither WUPyV, nor KIPyV was detected in respiratory, urine and blood samples from pregnant women. KIPyV was detected only in two plasma samples (2%) from healthy individuals and not in other sample types. WUPyV was not found in any sample types from healthy individuals. KIPyV and WUPyV were observed in 6.6 % and 3.3 % of respiratory samples from renal transplant patients. Respiratory symptoms were observed in all virus positive patients. We described first the presence of KI (2%) and WU virus (12%) in urine samples. KI and WU viraemia was also found in renal transplant patients (1 and 2.6 %). WUPyV and KIPyV were detected mainly early after renal transplantation. HPyV9 was detected in urine, respiratory and blood samples from both healthy pregnant and non pregnant women, the frequencies were 2-6 %. There was no statistically significant difference of prevalence in any sample types between the two studied groups. This was the first observation of HPyV9 in respiratory sample which suggests the respiratory transmission of this virus. Based on our results it is suggested that WUPyV, KIPyV and HPyV9 from the portal entries (possible the respiratory tract) might invade into blood circulation, might infect kidney and cells of the urine tract by blood and might be secreted by urine.



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List of publications related to the dissertation

1. **Mészáros, B.**, Sápy, T., Gergely, L., Csoma, E.: Prevalence of human herpesvirus 6A and 6B during pregnancy.
Acta Microbiol. Immunol. Hung. Epub ahead of print (2014), p. 1-8, p. 1-8. -
DOI: <http://dx.doi.org/10.1556/AMicr.61.2014.3.5>
IF:0.78 (2013)
2. Csoma, E., Sápy, T., **Mészáros, B.**, Gergely, L.: Novel human polyomaviruses in pregnancy: Higher prevalence of BKPyV, but no WUPyV, KIPyV and HPyV9.
J. Clin. Virol. 55 (3), 262-265, 2012.
DOI: <http://dx.doi.org/10.1016/j.jcv.2012.07.009>
IF:3.287
3. Csoma, E., **Mészáros, B.**, Asztalos, L., Kónya, J., Gergely, L.: Prevalence of WU and KI polyomaviruses in plasma, urine, and respiratory samples from renal transplant patients.
J. Med. Virol. 83 (7), 1275-1278, 2011.
DOI: <http://dx.doi.org/10.1002/jmv.22083>
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- *4. Csoma, E., **Mészáros, B.**, Gáll, T., Asztalos, L., Kónya, J., Gergely, L.: Dominance of variant A in human Herpesvirus 6 viraemia after renal transplantation.
Viol. J. 8, 403, 2011.
DOI: <http://dx.doi.org/10.1186/1743-422X-8-403>
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List of other publications

5. Hernádi, K., Gyöngyösi, E., **Mészáros, B.**, Szakács, L., Szalmás, A., Csoma, E., Mogyorósi, R., Czompa, L., Veress, G., Varga, I., Márton, I.J., Kónya, J.: Elevated Tumor Necrosis Factor-alpha Expression in Periapical Lesions Infected by Epstein-Barr Virus.
J. Endod. 39 (4), 456-460, 2013.
DOI: <http://dx.doi.org/10.1016/j.joen.2012.12.028>.
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