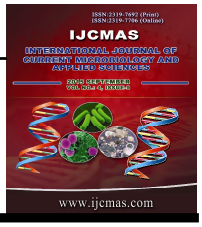
Int.J.Curr.Microbiol.App.Sci (2015) 4(9): 689-695

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 9 (2015) pp. 689-695**
http://www.ijcmas.com



Original Research Article

Are Human Polyomaviruses (BKV) Transmitted by Blood Transfusion: Data from LUX-PCR Analysis

Zlatko Kalvatchev[1], Iliya Tsekov[1], Georgi Toshev[2] and Radka Argirova[3]*

[1]Laboratory of Clinical Microbiology and Virology, UMBAL Sofiamed, Sofia, Bulgaria,
[2]Laboratory of Virology, Biological Faculty, Sofia University "St. Kl. Ohridsky", Sofia,
[3]Immunology and Molecular Diagnostic,Tokuda Hospital, Sofia, Bulgaria
**Corresponding author*

Keywords

BK virus, Polyomavirus, Blood, Horizontal Transmission

A B S T R A C T

Human polyomavirus BK (BKV) is ubiquitous viral agent that infects great proportion of the human population. After primary infection it establishes latency, predominantly in the cells of the uro-genital system, but also lymphoid and nervous cells are discussed to carry BKV DNA. That is why lymphocytes obtained from 122 healthy blood donors are examined for BKV sequences by the sensitive technique LUX real-time PCR. Results demonstrate BKV DNAS 14 out of 122 patients (11.5%). We discuss the possible role of the human leukocytes for establishment of persistent BKV infection or supporting its active form.

Introduction

The Polyomaviridae family includes ubiquitous viruses with specific host spectra. Human polyomavirus BK (BKV) was discovered in 1971 and shortly after that became evident that around 80% of adults had anti-BKV antibodies. This raised important questions connected with its mode of transmission and pathogenesis. Nowadays they are still unclear (Sessa, 2008). Following primary infection, which is generally asymptomatic, the virus persists lifelong in the majority of population. It establishes latent infection principally in uroepithelial cells but also B lymphocytes and tissues (spleen, brain) might be involved. Under different immune alterations BKV can be reactivated, and depending on the level of immunosuppression, the symptoms vary from asymptomatic viruria and viremia to serious dysfunction of the affected organ (Hirsch, 2003).

The main site of BKV latency in healthy subjects is the kidney but in the recent years lymphoid cells are also proposed as carriers of BKV DNA. The first evidence of an interaction between BKV and hematopoetic cells was given by studies on the stimulatory effect of BKV infection on lymphocyte cultures and on BKV receptors on the surface of peripheral blood cells. This data suggested the participation of lymphoid cells in the pathogenesis of BKV infection.

Consequently, BKV sequences have been often detected in blood cells from healthy and immunocompromised people by sensitive molecular techniques. Nevertheless, the data on the prevalence of BKV DNA in peripheral lymphocytes is highly discordant and contradictory. Blood BKV positivity varies from 0 to 90% (Dorries, 1994; Dolei, 2000). Besides, the detection of BKV sequences in lymphocytes has disputable significance for the pathogenesis- yet it is not clear whether these cells are persistently or transiently infected, had integrated or episomal copies of viral DNA.

We tried by the help of sensitive molecular technique –Light Upon Extension real-time polymerase chain reaction (LUX real-time PCR) to detect BKV sequences and study the prevalence of the infection in peripheral lymphocytes from healthy blood donors. Moreover, this technique allows fast detection of even few viral genome copies and the obtained data is easily interpreted.

Materials and Methods

Donor group and blood collection

Blood samples from 122 healthy donors (19-63 years) were collected randomly at the Center of Hematology and Blood Transfusion, Military Medical Academy-Sofia. Blood was taken via radial vein puncture in sterile vacutainers (Becton-Dickinson, K2E, 3 ml) supplied with EDTA as anticoagulant.

Blood preparation

Freshly collected peripheral blood was initially left at 4ºC for approximately half and an hour, until the natural deposition of the cellular components at the bottom of the tube. The result was stratification of the obtained blood into clearly visible plasma and lower layer of forming elements. The plasma was then carefully removed and the cells were used for lymphocyte separation. This was performed by the use of the ficoll containing density gradient reagent Lymphoflot (Biotest, Cat. No. 824012 Germany). In general two milliliters volume/volume Hanks diluted blood were laid over equal quantity of Lymphoflot and for further operations the manufacturer's instructions were followed. After centrifugation at 2500 rpm, the lymphocytes accumulate as opalescent ring between the adjacent layers of plasma and Lymphoflot captivated red blood cells. Following its removal, they were suspended in 500 μl Hanks solution, pelleted at slow centrifugation (1000 rpm) and kept at -70°C until DNA preparation.

DNA extraction and LUX Real-time PCR

The extraction of DNA was performed by the use of in-house modification of the phenol/chloroform/iso-amyl (Invitrogen, Cat No 15593-049, USA) method applicable for leukocytes. DNA was precipitated by absolute ethanol and 3M sodium acetate, pelleted and rehydrated with 30-50 μl ultra pure water. All the extraction procedures were held along with the proper extraction controls.

BKV sequences in peripheral blood lymphocytes were detected by LUX real-time PCR optimized in the Laboratory of Molecular Virology, National Center of Infectious and Parasitic Diseases (Slavov S., et al., 2008).

The primer set BKLUX-1/BKLUX-2 designed for the genomic region of the small t antigen and labeled with 6-carboxyfluorescein (FAM) was used for amplification of BKV sequences in leukocytic DNA. The features of these primers are given in table 1.

The optimized PCR mixture consisted of the following components: 5 μl 10X PCR buffer; 2.5 mM $MgCl_2$ (Abgene, USA); 1.25U of thermostable Taq DNA polymerase (Invitrogen, USA); 200 μM of each of the four nucleotides (USB, USA); FAM-labeled LUX primer and corresponding unlabeled primer (400 nM each, final concentration), 1 μl ROX reference dye (Invitrogen, USA) and 5 μl (~5 ng) leukocytic DNA up to a final volume of 50 μl. MJ Research Opticon (Bio-Rad, USA) was programmed for the reaction protocol: initial denaturation at 95°C for 10 min, followed by 50 cycles consisting of denatiration at 95°C (15s) and one-step of annealing/elongation at 60°C (1 min). Fluorescence spectra were recorded during the annealing/elongation phase of each PCR cycle. The cycle threshold was calculated as the cycle number at which the reaction became exponential.

To identify the specific PCR products generated at the presence of LUX-primers, Tm analysis was performed by increasing the temperature from 60 to 95°C at a transition rate of 0.1°C/s. The software calculates the Tm, i.e. the rate of change of fluorescence (Tm=dI/dT) is displayed as a function of temperature.

BKV DNA positive control was obtained from the sera of patient (Lab. N 251) who has developed hemorrhagic cystitis due to reactivated virus and has been previously identified as viruric and viremic. The negative control consisted of ultra pure PCR water.

Statistical analysis

The statistical calculations and the estimation of frequencies and mean values were carried out using the statistical software package SPSS 9.0 (SPSS Inc.).

Results and Discussion

LUX real-time PCR showed to be sensitive and specific tool for the detection of BKV sequences in DNA from peripheral blood leukocytes. At the end of the reaction the samples were subjected to melting curve analysis and the estimated melting temperature of the amplicons was about 78°C corresponding to that of the positive BKV DNA control. No background non-specific fluorescence signal was observed. The amplification plot and melting curve analysis of BKV LUX real-time PCR are described in figure 1.

Our survey included 122 healthy individuals, visiting a transfusion centre for blood-donation. The group consisted of 95 men (77.8%) and 27 women (22.2%) with mean age 37. 9951 yrs (19-63 years). Their clinical records revealed that they were absent of systemic or chronic diseases. LUX real-time PCR for the detection of BKV sequences showed positive increase of the fluorescence signal in 14 out of 122 patients (11.5%).

The obtained results could be interpreted in several ways. We found a middle level of infection with BKV (11.5%) in blood leukocytes. The data obtained by multiple surveys is disputable as the percentage varies between 0 and 90% (Sundsfjord, 1994, Dorries, 1994). The divergence of tropism of BKV for circulating cells is even higher in the different compartments of the host. This might be due to several reasons. First of all, the technical differences are important. The sensitivity of the assays is crucial, as it is estimated that the amount of BKV DNA in leukocytes is very low. For example the previous studies employed single or nested PCRs, and not real-time viral detection (Dolei, 2000). Despite all, our LUX real-time PCR assay was highly sensitive and specific and information for

positive amplification signal was obtained at every stage of the PCR process. And at the same time we did not find high number of infected individuals (14 out of 122/11.5%). This outlines the second, most probable reason- differences in the study group. The generally low percentage of BKV in the leukocytes is probably due to the individual susceptibility of blood cell subpopulations to BKV or the half-life of the infected cell type. Another very important reason, which might not be ignored, is the state of individual infection at the time of sampling (latent, reactivated).

The discussion on the role of BKV infection in lymphocytes is very difficult. On one hand it must be distinguished whether lymphoid cells carry BKV persistent or reactivated infection. BKV DNA is demonstrated in the lymphoid tissue of oropharynx and tonsils. In the lymphocytes of these tissues the BKV genomes were of full length, in low copy numbers and episomal state. This data supports the fact that the BKV infection in lymphocytes is persistent. Our real-time PCR results showed that the increase of fluorescence compared to the positive DNA control was at the final protocol cycles, which hypothetically corresponds to low BKV DNA copy number. We were not able to explore the physical state of the BKV DNA and we detected single gene (genome region of small t antigen). That is why we suppose that in our study group the infection is persistent.

This is also supported by the observation that high number of rearranged polyomavirus sequences is reported in lymphocytes, but in plasma they were archetypal. This is not connected to recombinase activity of the lymphocytes because a consensus recombination signal is not found in BKV NCCR (Sharma, 2007). That is why, leukocytes have fundamental role for the polyomavirus persistence and its dissemination allowing rearranged variants, which are better adapted to grow in different tissues (Azzi 1996). Moreover, monocytes from peripheral blood are able to bind and uptake BKV, but are shown not express viral proteins. This also suggests the viral persistence, as most probably monocytes take the rearranged virions and are responsible for their spread through the body and the protection from the immune surveillance as this shows the natural mechanism of the infection (Portolani, 1985). So the prevalence of BKV DNA in lymphocytes of healthy individuals reveals that they most probably present viral latency site, permitting the establishment of viral persistence in the affected organs or a vehicle for the spread of the infection to different tissues (Degener, 1997).

On the other hand, viral expression products (early mRNA) have been detected in lymphocytes from healthy blood donors that were positive for BKV DNA. This contrarily of the above said, is an evidence of active BKV infection in lymphoid cells. It is also demonstrated that with increase of the age the presence of some genome parts (VP1 region) of BKV genome decreases, suggesting that part of the genome may be lost probably by accumulation of BKV defects or negative immune selection of BKV-infected lymphocytes.

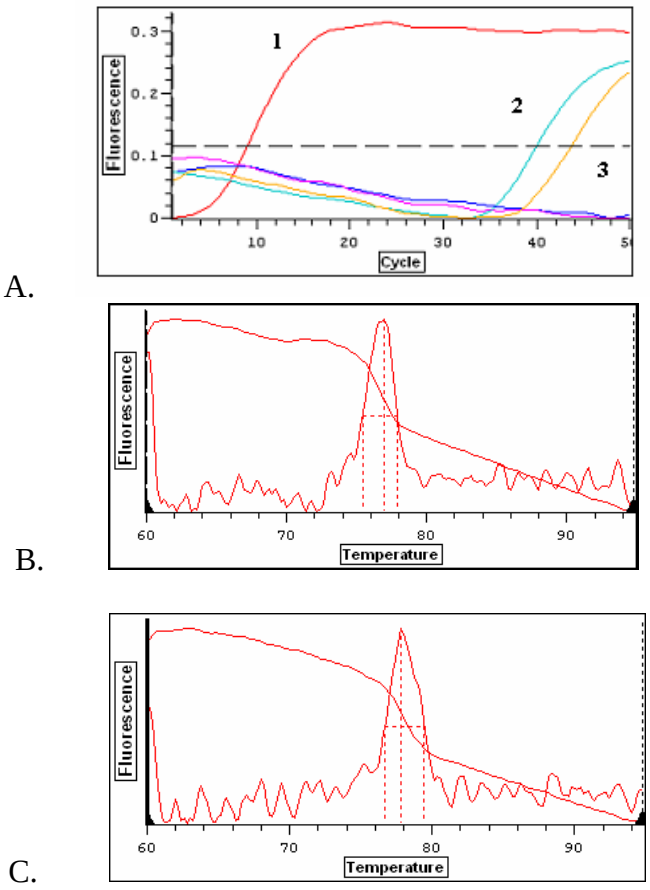
In that connection lymphocytes are not likely to be a site for lifelong virus persistence in immunocompetent individuals. That is why the detection of, for example VP1 sequences might be related to recent or reactivated infection with BKV. That is why a conclusion that blood cells do not harbor biologically active BKV DNA for a long time, after acute infection of reactivation might be considered (Dolei, 2000).

Table.1 Characteristics of the LUX PCR primer set

Name	5'→3' LUX sequences	Position	Ta[1]	Size[2]
BK virus (strain Dunlop); GenBank accession number V0 1108				
BKLUX-1	**cacaca**TCTAAGCCAAACCACTGTGTG-FAM[3]	4708-4728		
BKLUX-2	TTGGATAGATTGCTACTGCATTGA	4735-4759	60°C	51bp

Note: in bold symbols: six nucleotide tail quencher; 1Optimized annealing temperature; 2Size of the amplified PCR product; 3Reporter fluorophore (6-carboxyfluorescein, acronym "FAM")

Figure.1 LUX real-time PCR for the detection of BKV sequences in human peripheral blood leukocytes



A. Amplification plot of the BKV DNA control and the DNA obtained from leukocytes: 1.Positive amplification signal of the BKV DNA control (Ct=10); 2. Positive amplification signals from the DNAs of some leukocytes (Ct=39; Ct=42; generally low copy numbers); 3.Negative amplification signal of negative leukocytic DNAs and the negative control; **B.** Melting curve analysis of the PCR product of the positive BKV DNA control (Tm ~ 78°C); **C.** Melting curve analysis of a positive leukocytic DNA for BKV sequences (~ 78°C)

Apart from that the transmission of BKV, among human population is not clear. Given the upper discussion, the transmission of BKV virions via blood products becomes disputable. If the lymphocytes, contain low copy numbers of BKV DNA in episomal state, the transfusion of blood might play possible role as a factor for transmission. But on the other hand, if lymphocytes contain some only genome regions, which are not able to support active replication, the role of blood is questionable unless during a time of reactivated or recent infection.

It is clear that the detection of BKV DNA in lymphocytes has various aspects and is highly contradictory. But the localization of BKV DNA in lymphocytes by radioactive in-situ hybridization, detection of mRNA and the indirect evidence for BKV susceptibility of monocytes argues for regular blood cell tropism of the host. Another question is whether the BKV sequences in leukocytes are related to persistent of active infection.

Most studies accentuate on the persistent type of BKV infection in lymphoid cells but the observation that the immunosuppressed patients have much higher rate of BKV infection in lymphocytes, reveals that the lymphocytes might help the viremia, which is in contrary with the hypothesis for the involvement of the kidney for the passage of the virus in sera and the persistence of the infection. That is why, more specific studies, must be carried out to distinguish the exact role of the lymphocytes in BKV infection.

Acknowledgments

The authors thank Dr. Rumen Popov, Center of Transfusion Hematology, Military Medical Academy, Sofia, for providing the blood donor samples for the study.

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