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Monica Mischitelli, Daniela Fioriti, Elena Anzivino, Anna Bellizzi, Valentina Barucca, Renzo Boldorini, Umberto Miglio, Simona Sica, Federica Sorà, Silvia de Matteis, et al.

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## VIRAL INFECTION IN BONE MARROW TRANSPLANTS: IS JC VIRUS INVOLVED?

**Running head: JC virus infection in bone marrow transplants**

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**Keywords: JC virus infection, Bone marrow transplants, Haemorrhagic cystitis; Quantitative PCR; Sequencing analysis.**

**ABSTRACT**

Haemorrhagic cystitis is characterized by haematuria due to inflammation of the bladder. In bone marrow transplants, this disease is linked to the infection by human polyomavirus BK, whereas the role of the human polyomavirus JC is unclear. The transcriptional control regions of both viruses contain important cellular transcription factor binding sites that undergo rearrangement process generating suitable variants that could be more active for viral replication and for the onset of haemorrhagic cystitis.

In this study urine obtained from seven patients with bone marrow transplant were examined. Polyomavirus genomes were quantified by PCR and viral loads were compared. The transcriptional regions of both viruses were amplified and sequenced to determine the presence of variants. Subtypes of polyomaviruses were determined by amplification and sequencing of the viral protein 1 region.

The results showed that 4 of 7 patients were positive for BK DNA, 2 of 7 patients had BK and JC DNA and 1 of 7 had JC DNA. Positive samples were amplified and sequenced successively for transcriptional regions. The viral archetype was always found in both viruses. Finally, typing showed that BK virus subtype I infected patients with BK, whereas JC virus genotype IA and genotype 1B were found in patients infected with JC.

The data suggest that new and different approaches are required to improve the morbidity and mortality caused by polyoma-associated haemorrhagic cystitis, since it known that BK virus is involved in the onset of haemorrhagic cystitis, whereas the role of JC virus should be investigated further.

## INTRODUCTION

Haemorrhagic cystitis is characterized by painful haematuria due to haemorrhagic inflammation of the bladder mucosa. It is a distinct clinical disease that has been associated with various predisposing factors [DeVries and Freiha, 1990] and it is an important cause of morbidity and occasional mortality in patients undergoing bone marrow transplantation [Sencer et al., 1993]. The clinical features of haemorrhagic cystitis vary from microscopic haematuria to severe haemorrhage in the bladder leading to clot retention and renal failure. Cystitis can be classified into two types: early onset haemorrhagic cystitis, which occurs within 48–72 hours of a conditioning regimen, and a later onset haemorrhagic cystitis, which occurs beyond 72 hours from the preparative regimen. The incidence of haemorrhagic cystitis varies from 7% to 68% [Santos et al., 1983; Brugieres et al., 1989; Sencer et al., 1993; Russel et al., 1994; Yang et al., 1994; Bedi et al., 1995; Leung et al., 2001; Xu et al., 2007].

Numerous causes, such as chemical immunosuppression and irradiation, cytopenia and viral infections, can contribute to the onset of haemorrhagic cystitis. Infection or reactivation of the human polyomavirus BK is common and represents a consistent risk factor for the development of this disease. It has been associated with a later onset haemorrhagic cystitis in patients undergoing allogeneic haematopoietic stem cell transplantation [Reploeg et al., 2001; Leung et al., 2005; Giraud et al., 2006; Giraud et al., 2008]. On the other hand, the role of the human polyomavirus JC is still poorly understood.

The two human polyomaviruses BK and JC belongs to the *Polyomaviridae*, a family of small non-enveloped icosahedral DNA viruses, which infect human population worldwide [Imperiale, 2001].

The BK viral genome can be divided into an early region (regulatory proteins), a late region (viral capsid proteins and the agnoprotein) and a transcriptional control region, which contains the origin of replication and sequences for transcriptional cell factors involved in early and late viral transcription [Moens and Van Ghelue, 2005]. These binding sites undergo deletion and

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2  
3 enhancement process that could generate variants that could offer advantages to the virus in its host  
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5 [Sharma et al., 2007].  
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8 Four antigenic subtypes of BK virus (I–IV) have been characterised by genomic subtyping and  
9  
10 serological reactivity [Jin et al., 1995]. The association of a particular BK virus subtype with  
11  
12 clinical features is controversial, although some investigators demonstrated that BK virus subtype I  
13  
14 was the predominant in urine samples taken from bone marrow transplants affected by  
15  
16 haemorrhagic cystitis [Bogdanovic et al., 1998; Fioriti et al., 2005].  
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19 Like BK virus, the JC viral genome is divided into three regions: the early, the late and the  
20  
21 transcriptional control region which includes the origin of replication and contains binding sites for  
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23 transcriptional cell factors involved in viral early and late transcription [Frisque et al., 1984; Kim et  
24  
25 al., 2001]. Like BK virus, deletion and enhancement process could give rise to more active variants  
26  
27 with altered tissue tropism and pathogenic capability [Vaz et al., 2000; Pietropaolo et al., 2003;  
28  
29 Mischitelli et al., 2005]. The analysis of the viral protein 1 gene allowed to distinguish 8 major  
30  
31 genotypes and numerous subtypes of JC virus [Kmieciak et al., 2008]. Types I and IV predominate  
32  
33 in Europe and in the USA [Jobes et al., 1998; Stoner et al., 2000; Agostini et al., 2001; Pagani et al.,  
34  
35 2003].  
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40 Primary polyomavirus infection is usually asymptomatic and is acquired during childhood probably  
41  
42 via the upper respiratory tract and other ways [Brown et al., 1984; Sundsfjord et al., 1994;  
43  
44 Pietropaolo et al., 1998; Zambrano et al., 2002; Ahsan and Shah, 2006; Knowles, 2006]. Following  
45  
46 primary infection, these viruses disseminate and establish lifelong persistence especially in the  
47  
48 kidney [Imperiale, 2000; Rabenau et al., 2002]. Reactivation may occur in conditions of  
49  
50 immunosuppression such as following bone marrow and kidney transplantations [Drachenberg et  
51  
52 al., 2007; Boldorini et al., 2008; Dropulic and Jones, 2008]. JC virus is the etiologic agent of a rare  
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54 neurodegenerative disease named Progressive Multifocal Leukoencephalopathy, whereas BK virus  
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56 causes nephropathy in kidney transplant patients and it is associated with haemorrhagic cystitis in  
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3 allogeneic bone marrow transplants [Khalili et al., 2006; Mischitelli et al., 2007; Giraud et al.,  
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5  
6 2008].

7  
8 About 50–100% patients with a bone marrow transplant develops BK viraemia, nevertheless only 5–  
9  
10 40% progress to haemorrhagic cystitis indicating that a specific subtype, genome mutations and  
11  
12 graft versus host disease may contribute to its development [Bogdanovic et al., 1996; Azzi et al.,  
13  
14 1999; Leung et al., 2001; Carr et al., 2006; Ikegaya et al., 2006; Giraud et al., 2008]. Nevertheless,  
15  
16 very few studies have been carried out linking haemorrhagic cystitis with polyomavirus infection  
17  
18 and some studies pointed to a significant correlation between the pathogenesis and shedding of  
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20 polyomavirus in urine, whereas data from other studies showed no such relationship [Rabenau et  
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22 al., 2002; Bogdanovic et al., 2004; Erard et al., 2005; Fioriti et al., 2005; Giraud et al., 2006].  
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26  
27 In order to assess better the association of BK and/or JC viruses infection with the onset of  
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29 haemorrhagic cystitis or progression, urine taken from patients with bone marrow transplants and  
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31 immunocompetent individuals was analyzed. BK virus and JC virus genomes were quantified and  
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33 viral loads were compared to determine whether high peak urine viral loads were linked to the onset  
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35 of haemorrhagic cystitis and with variation of immunosuppressive therapy. In addition, BK virus  
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37 and JC virus transcriptional control regions were amplified and sequenced to determine whether if  
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39 mutations in transcription factor binding sites were associated with a more aggressive pathology.  
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41 Finally, the BK and JC viruses were subtyped to determine whether a particular subtype was  
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43 circulating most commonly in bone marrow transplants.  
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## MATERIALS AND METHODS

### *Patients and clinical specimens*

This study was based on seven Caucasian patients (two males and five females) who underwent allogeneic bone marrow transplantation and developed post-transplant haemorrhagic cystitis. The average age of the patients was 35 for females (30-45) and 32 for males (24-39). They were admitted to “Policlinico Gemelli” Hospital of Rome with a different diagnosis of haematopoietic malignancies, none had a history of urinary tract infection, coagulopathy, pelvic irradiation or evidence of microscopic haematuria prior to bone marrow transplantation. All patients received allogeneic bone marrow transplants. One of these patients received a bone marrow transplant from a relative and six from matched unrelated donors. The seven patients developed haemorrhagic cystitis at an average of 33 days after bone marrow transplantation. The clinical data of the patients are shown in Table 1. Seven healthy individuals were recruited as controls.

Specimens were collected when haematuria was evident or when the symptoms were of haemorrhagic cystitis. Two patients were also infected with BK virus and JC virus and had recidivant haemorrhagic cystitis. Serial urine specimens were collected from these two patients from the onset of the symptoms to determine whether high peak urine viral loads were associated with haemorrhagic cystitis episodes and with variation of immunosuppressive therapy.

Investigations were made on fresh uncentrifuged urine.

Informed consent was obtained from each patient and each volunteer and the study design was approved by the medical ethics committee of the “Policlinico Gemelli” Hospital of Rome.

### *Diagnosis and treatment.*

Haemorrhagic cystitis was diagnosed by urine culture and PCR to distinguish bacterial disease from viral etiology. The treatment is shown in Table 2.



### *DNA extraction*

One millilitre of urine was incubated in lysis buffer and proteinase K (200 mg/ml). DNA extraction was performed by the DNeasy® Tissue Kit (QIAGEN S.p.A., Milan, Italy), according to the manufacturer's instructions.

### *PCR analysis*

Urine was examined using quantitative assays for quantitation of BK virus and JC virus DNA. The specimens with BK and/or JC DNA were then analyzed using qualitative assays for the detection of viral transcriptional control regions and for virus typing.

### *BK virus quantitative PCR*

The assay was performed using 7300 Real Time PCR System (AB Applied Biosystems, 850 Lincoln Centre Drive Foster City, CA 94404 USA). PCR amplifications were run in a reaction volume of 20 µl (optimized mix including forward and reverse primers and hydrolysis probes) containing 5 µl of DNA sample. 50 ng/µL of total purified DNA was used.

Thermal cycling was initiated with a first denaturation step of 10 min at 95°C, followed by 45 cycles of 95°C for 15 s, 60°C for 1 min, 72°C for 1 min, at the end of which, fluorescence was read.

Amplification data were analyzed with software provided by the manufacturer. Standard curves for the quantitation of the viral genome were constructed using serial dilutions of a plasmid containing the target sequences (Large T Antigen). Concentrations of the plasmid ranged from  $10^2$  to  $10^5$  copies of target plasmid. All samples were tested in triplicate and the number of viral copies in each sample was calculated from the standard curve. The results were expressed as copies of viral DNA per milliliter (c/ml) of sample. Standard precautions to prevent contamination were followed. In each run non-template control lanes were included. The assay can to detect about 10 molecules of target sequences in 5 µl of DNA.

### *JC virus quantitative PCR*

The assay was performed using 7300 Real Time PCR System (AB Applied Biosystems, 850 Lincoln Centre Drive Foster City, CA 94404 USA). PCR amplifications were run in a reaction volume of 40  $\mu$ l containing 10  $\mu$ l of the DNA sample, reaction mix for quantitative amplification, forward and reverse primers and finally SYBR GREEN I probes. 50 ng/ $\mu$ L of total purified DNA was used.

Thermal cycling was initiated with a first denaturation step of 2 min at 94°C, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, 77°C for 15 s at the end of which, fluorescence was read. Dissociation stage was characterized by a temperature curve with an initial step at 65°C and a final step at 95°C. The temperature increment was 0.3°C every 10 s. The amplification data were analyzed with a specific software provided by the manufacturer. Standard curves for viral genome quantification were constructed using serial dilutions of a plasmid containing target sequences (Viral Capsid Protein 2). The plasmid concentrations ranged from  $10^2$  to  $10^5$  plasmid copies of the target. All samples were tested in triplicate and the number of viral copies in each sample was calculated from the standard curve. PCR specificity was guaranteed by a dissociation stage characterized by curves with melting point of 79°C $\pm$ 1°C, corresponding to a 132bp fragment. These data were expressed as c/ml of sample. Standard precautions to prevent contamination were followed. In each run non-template control lanes were included. The assay allows to detect about 20 molecules of target sequences in 10  $\mu$ l of DNA.

### *PCR for transcriptional control region of BK virus*

Samples positive for BK virus genome underwent further amplification for detection of the transcriptional region using a nested PCR with BKTT1 and BKTT2 as outer primers and BRP1 and BRP2 (356 bp) as inner primers. The amplification was performed in a reaction volume of 25  $\mu$ l, containing 10 pmol of each primer, 0.2 mM dNTPs, 1.5 mM MgCl<sub>2</sub>, and 2U BioTaq DNA polymerase with an appropriate reaction buffer (Tris-HCl 100 mM, pH 8.3, KCl 500 mM). In the

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3 first step, 5  $\mu$ l of DNA was added to the PCR mixture and, in the second step, 2.5  $\mu$ l of template.  
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5 PCR was run in GeneAmp PCR System 9600 (Perkin-Elmer Cetus, Emeryville, CA). 50 ng/ $\mu$ L of  
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7 total purified DNA was used for the assay.  
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10 The samples were amplified by denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for  
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12 40 s, annealing for 40 s at 55°C in the first step and 50°C in the second step, and extension at 72°C  
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14 for 40 s. The cycles were terminated with a final extension at 72°C for 5 min. All assays included  
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16 positive (purified viral DNA) and negative (all the PCR components except the template) controls  
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18 to exclude false-positive and false-negative results. PCR products were analyzed by 2% agarose gel  
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20 electrophoresis and visualized using ethidium bromide staining [Boldorini et al., 2008].  
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#### 27 *PCR for transcriptional control region of JC virus*

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29 Samples positive for JC virus genome underwent further amplification for detection of the  
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31 transcriptional region using a nested PCR with JRE1 and LP2 as outer primers and RFOR and  
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33 RREV (358 bp) as inner primers. The amplification was performed in a reaction volume of 45  $\mu$ l,  
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35 containing 10 pmol of each primer, 0.2 mM dNTPs, 1.5 mM MgCl<sub>2</sub>, and 2U BioTaq DNA  
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37 polymerase with an appropriate reaction buffer (Tris-HCl 100 mM, pH 8.3, KCl 500 mM). 5  $\mu$ l of  
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39 DNA was added to the PCR mixture at the first step, whereas 2.5  $\mu$ l of template was added in the  
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41 second step of PCR. PCR was run in GeneAmp PCR System 9600 (Perkin-Elmer Cetus,  
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43 Emeryville, CA). 50 ng/ $\mu$ L of total purified DNA was used. The samples were amplified by  
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45 denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 40 s, annealing for 40 s at 61°C  
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47 in the first step and 58°C in the second step, and extension at 72°C for 40 s. The cycles were  
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49 terminated with a final extension at 72°C for 5 min. All assays included positive (purified viral  
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51 DNA) and negative (all the PCR components except the template) controls to exclude false-positive  
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53 and false-negative results. PCR products were analyzed by means of 2% agarose gel electrophoresis  
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55 and visualized using ethidium bromide staining [Boldorini et al., 2008].  
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6 *Sequencing of transcriptional control regions of BK and JC viruses*

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8 PCR products corresponding to transcriptional control regions of BK and JC viruses were purified  
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10 and sequenced. Briefly, amplicons were purified prior sequencing to remove the excess of primers  
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12 with QIAquick PCR purification kit, according to QIAGEN protocol. DNA sequencing was  
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14 performed by automatic DNA sequencer (Applied Biosystem, 850 Lincoln Centre Drive Foster City,  
15  
16 CA 94404 USA, mod. 370 A), according to manufacturer's specifications (Amplicycle Kit, Applied  
17  
18 Biosystem, 850 Lincoln Centre Drive Foster City, CA 94404 USA). Sequences were organised and  
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20 analyzed using homology analysis, multiple alignments and the Genetic Computer Group sequence  
21  
22 analysis software package. In particular the obtained sequences were compared with BK virus  
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24 archetype (Genbank Accession Number V01108) and JC virus archetype (Genbank Accession  
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26 Number J02226).  
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34 *PCR for viral capsid protein 1 of BK and JC viruses*

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36 In order to amplify genome region corresponding to viral capsid protein 1 of BK virus, a nested  
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38 PCR was performed using VP1-7 and VP1-2R as outer primers, and 327-1 and 327-2 as inner  
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40 primers (327 bp), reported by Jin et al., 1993. The amplification was performed in a reaction  
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42 volume of 20  $\mu$ L containing 10 pmol/ $\mu$ L of each primer, 0.2 mM dNTPs , 1.5 mM MgCl<sub>2</sub>, 2U  
43  
44 BioTaq DNA polymerase and 10x Bionline NH<sub>4</sub> buffer. In the first step, 5  $\mu$ L of DNA was added to  
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46 the PCR mixture and, in the second step, 2.5  $\mu$ L of template. The nested PCR was performed using  
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48 an Eppendorf Mastercycler gradient PCR System. 50 ng/ $\mu$ L of DNA was used. The samples were  
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50 amplified by means of denaturation at 95°C for 5 min, followed by 35 cycles (30 cycles for the  
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52 inner PCR), at 95°C for 40 s, annealing at 55°C for 40 s and extension at 72°C for 40 s. The cycles  
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54 were terminated with a final extension at 72°C for 5 min.  
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60 Single step PCR was used to amplify the genome region corresponding to viral capsid protein 1 of  
JC virus, using JLP15 and JLP16 primers (215 bp) reported by Pagani et al. 2003. The amplification

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3 was performed in a reaction volume of 20  $\mu$ L containing 25 pmol/ $\mu$ L of each primer, 0.4 mM  
4 dNTPs , 2.5 mM MgCl<sub>2</sub>, 2U BioTaq DNA polymerase, 10x Bioline NH<sub>4</sub> buffer and 5  $\mu$ L of DNA.  
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8 50 ng/ $\mu$ L of DNA was used.  
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10 After initial denaturation at 95°C for 9 min, followed by 40 cycles at 95°C for 40 s, annealing at  
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12 63°C for 40 s and extension at 72°C for 40 s; the amplification protocol was concluded with a final  
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14 extension at 72°C for 7 min. PCR products were analyzed using 2% agarose gel electrophoresis and  
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16 visualized using ethidium bromide staining.  
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#### 19 20 21 22 *Direct DNA sequencing for viral genotyping.* 23

24 PCR products corresponding to the viral capsid protein 1 region of both viruses were separated by  
25  
26 electrophoresis on 3% agarose gel. The fragment corresponding to each sample (327 bp for BK  
27  
28 virus and 215 bp for JC virus) was excised, extracted and purified using a commercial kit (PCR  
29  
30 clean-up gel extraction, NucleoSpin Macherey-Nagel, 52355 Düren, Germany).  
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34 A cycle sequencing PCR reaction was performed as previously described [Boldorini et al., 2008]. In  
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36 order to avoid Taq polymerase mistakes [Jin et al., 1993], the sequence was analyzed twice for each  
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38 sample. The sequences were read manually, compared with the prototypes (Dunlop strain for BK  
39  
40 virus and Mad-1 strain for JC virus) and classified into known genotypes [Jin et al., 1993; Agostini  
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42 et al., 2001]. The known genotypes are distinguished on the basis of specific polymorphisms within  
43  
44 the portion of the viral protein 1 region spanning nucleotides 1744-1812 (BK virus) and nucleotides  
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46 1735-1902 (JC virus). The presence of single mutations was also noted.  
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## RESULTS

In this study, urine from seven Caucasian patients who developed post-transplant haemorrhagic cystitis was analyzed.

Quantitative results showed that 4 of 7 patients had BK DNA, 2 of 7 patients had BK and JC DNA, whereas only one patient had JC DNA. Patient no.1 developed haemorrhagic cystitis 60 days after transplantation when, BK virus quantitative assay showed  $65 \times 10^8$  DNA c/ml, whereas JC virus quantitative PCR revealed that the specimen was suitable for analysis, but it did not have viral DNA (Tab.3). In patient no.2,  $15 \times 10^7$  c/ml of BK virus DNA was found, whereas the urine did not have JC viral DNA (Tab.3). The BK virus quantitative assay performed on the urine of patient no.3 revealed that there were  $33 \times 10^7$  c/ml of viral DNA, but in the some specimen JC viral DNA was not found (Tab.3). In urine obtained from patient no.4, the quantitative PCR revealed the presence of  $14 \times 10^7$  c/ml of BK virus DNA whereas JC DNA was not found (Tab.3).

In patient no.5, JC DNA quantification showed a number of viral DNA copies of  $63 \times 10^7$  c/ml, whereas BK DNA was not found (Tab.3). The quantitative assays for patients no.6 and no.7 showed that these subjects were co-infected with JC and BK viral DNA. For patient no.6, the BK virus DNA copies were  $12 \times 10^8$  c/ml in the urine collected on 12-22-2006, whereas JC virus DNA copies were  $9 \times 10^5$  c/ml. During the serial collection of specimens, corresponding to a recidivant haemorrhagic cystitis, the BK virus DNA average was  $52 \times 10^7$  c/ml, whereas the JC virus DNA average was  $6 \times 10^6$  c/ml. In the sample analyzed during the follow-up after remission (07-24-2007),  $9 \times 10^5$  c/ml of BK virus DNA were detected, whereas the JC virus DNA copies were  $10^4$  c/ml. For patient no.7, in the urine collected on 03-07-2007, the assays quantified  $5 \times 10^3$  c/ml of BK virus DNA and  $4 \times 10^6$  c/ml of JC virus DNA. In the specimens collected during recidive, a BK virus DNA average of  $6 \times 10^4$  c/ml was detected, whereas the JC virus DNA average was  $3 \times 10^6$  c/ml. In the urine collected on 09-03-2007 (follow-up after remission), the number of copies of BK and JC viruses DNA decreased to  $10^3$  c/ml for BK virus and  $10^4$  c/ml for JC virus (Table 3). No control subject was found positive (data not shown).

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3 Samples with viral DNA were amplified and sequenced successively for viral transcriptional control  
4 regions. Homology analysis, multiple alignments and the Genetic Computer Group sequence  
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6 analysis always revealed the presence of the BK and JC viral archetypes in all sequences obtained  
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8 from the specimens of all patients.  
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12 The antigenic subtyping of BK and JC viruses revealed that BK virus type I, corresponding to the  
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14 MM strain, was found in patients infected with BK including those co-infected. JC virus genotype  
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16 IA was found in all samples obtained from co-infected patients, whereas in the patient infected only  
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18 with JC virus, genotype IB was detected (Tab.3).  
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## DISCUSSION

Haemorrhagic cystitis is a distinct clinical disorder of multiple etiologies. It is characterized by painful haematuria due to haemorrhagic inflammation of the bladder mucosa [Leung et al., 2005].

The disease that occurs before the graft is mostly transient and self-limiting, whereas after the graft, it is severe and sometimes life threatening [Leung et al., 2005].

About 50% of bone marrow transplants show BK viraemia within 2 months of transplantation; it is similar in allogeneic (range 46–53%) and autologous (range 39–54%) transplants [Bedi et al., 1995; Azzi et al., 1999; Dropulic and Jones, 2008]. Although the association of BK virus infection with haemorrhagic cystitis in bone marrow recipients was first demonstrated two decades ago, the role of JC virus remains obscure. However, it is known that bone marrow recipients are susceptible to the infection. Boubenider et al. [1999] demonstrated that BK and JC viruses reactivate in 55% and 6.7% of bone marrow patients, respectively. BK virus infection or reactivation alone is not sufficient to cause the disease. Nevertheless, the transcriptional control region rearrangements could strengthen the virus tropism and generate more suitable variants that could be more active for viral replication and for the onset or development of haemorrhagic cystitis. A particular BK virus subtype in patients with a bone marrow transplant, could be involved in the onset of haemorrhagic cystitis.

JC viral infection occurs in <10% of patients with bone marrow transplant in whom progressive multifocal leukoencephalopathy is a rare complication [Przepiorka et al., 1997; Yasuda et al., 2008].

Like BK virus, JC transcriptional control region variants could alter viral tissue tropism and increase pathogenic capability, even if the genomic structures isolated from urine samples are highly conserved in healthy and immunocompromised individuals and they always appear very similar to that of the archetype [Vaz et al., 2000; Pietropaolo et al., 2003]. Finally it is suggested that different genotypes of JC virus would act differently in immunocompromised individuals as in the case of progressive multifocal leukoencephalopathy [Ferrante et al., 2003].



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3 Given this, urine specimens obtained from bone marrow transplanted and immunocompetent  
4 individuals were analyzed. BK virus and JC virus genomes were quantified and viral loads were  
5 compared to understand if high urine viral loads were correlated to haemorrhagic cystitis episodes.  
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10 In addition BK virus and JC virus transcriptional control regions were amplified and sequenced to  
11 investigate if mutations in transcription factor binding sites were associated with a more aggressive  
12 pathology. Finally, the BK and JC subtypes were determined to establish whether a particular  
13 subtype circulated more commonly in patients with bone marrow transplant.  
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20 Quantitative assays showed that four of seven patients were positive for BKV DNA, 2 of 7 patients  
21 were BK and JC co-infected, whereas only one patient was positive for JC virus DNA. In particular,  
22 it was observed that a high viral load in urine was related to haemorrhagic cystitis episodes in all  
23 patients. Interestingly, in serial urine specimens collected from co-infected patients, it was found  
24 that when BK virus replication increased, the JC virus replication decreased. Conversely, when the  
25 number of copies of JC DNA increased, BK viral DNA decreased. This suggests that both  
26 Polyomaviruses may contribute to the pathogenesis or development of haemorrhagic cystitis. It may  
27 be that JC virus involvement in transplant diseases is underestimated since the detection of this  
28 virus is not required in the diagnostic approach.  
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41 The sequencing of the transcriptional control regions of both viruses, revealed that no particular  
42 mutations or structures could be associated with a more strong viral replication and disease onset or  
43 recidive. Probably, in the genitourinary tract, viral replication did not require specific transcriptional  
44 cell factors, so the host proteins may be sufficient to allow virus replication. Therefore, as  
45 demonstrated by Gosert et colleagues, the study of functional proteins in permissive cells could help  
46 to understand the possible associations between virus replication and the specific subset of trans-  
47 acting factors [Gosert et al., 2008].  
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58 According to the literature, BK virus typing showed that subtype I is found most commonly in  
59 infected individuals [Zheng et al., 2007; Krumbholz et al., 2008]. This study confirmed previous  
60 results that demonstrated how subtype I was predominant in urine samples obtained from patients

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3 with bone marrow transplants and haemorrhagic cystitis [Fioriti et al., 2005]. The epidemiology of  
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5 BK virus shows that genotype I is the most common BK virus circulating in Europe and the genome  
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7 detected most frequently in the Italian population [Stoner et al., 2000; Agostini et al., 2001; Pagani  
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9 et al., 2003; Rossi et al., 2007]. In these seven patients, the JC genotype IA was detected in co-  
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11 infected individuals, whereas in patient n.5 the genotype IB was found. Although there are few  
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13 patients, it may be that this diversity could be used as a tool to outline the origin of infection and the  
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15 selection of a particular JC genotype in patients co-infected with BK virus.  
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19 However, since haemorrhagic cystitis pathology is complex, it is essential to consider many  
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21 cofactors in its development, such as the conditioning regimen, gender, the donor source and the  
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23 histocompatibility between recipient and donor patients. Regarding polyomavirus infection, it is  
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25 important to evaluate the dynamics of the infection, the timing and the frequency of monitoring and  
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27 how BK and JC viruses are implicated in renal disease in patients with bone marrow transplant.  
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29 Additional work is required to understand whether the JC virus acts only as a cofactor or it plays a  
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31 role in the onset or development of haemorrhagic cystitis.  
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39 **Acknowledgments:** This work was partially supported by Italian MIUR grants.  
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**Table 1: Clinical data of BMT patients**

Pz	Clinical history	BMT	HC onset	Polyomavirus infection	Other viral infections*	Other complications	Outcome
F1	AML	MUD 03-18-05	60 days after BMT	BKV	AdV, CMV, EBV	LPD-EBV related	dead
F2	NHL	MUD March 2008	10 days after BMT	BKV	none	Numerous bacterial infections	dead
F3	AML	MUD 05-05-07	20 days after BMT	BKV	EBV	/	dead
M4	ALL T cells	BMT from relative 05-09-07	60 days after BMT	BKV	CMV	Acute GVHD	dead
M5	ALL	MUD 05-10-05	30 days after BMT	<u>ICV</u>	EBV	/	dead
F6	ALL PH+	MUD 11-21-06	30 days after BMT	BKV, <u>ICV</u>	CMV, EBV	/	remission: dasatinib administration; DLI
M7	M3-AML	MUD 02-16-07	20 days after BMT	BKV, <u>ICV</u>	CMV, EBV	/	M3-AML recidivant

**Legend:** ALL PH+ = Acute Lymphoblastic Leukemia Chromosome Philadelphia Positive; M3-AML = M3 Acute Myelocytic Leukemia; AML = Acute Myelocytic Leukemia; NHL = Non Hodgkin's Lymphoma; MUD = Match Unrelated Donors; LPD = LinfoProliferative Disorders; GVHD = Graft Versus Host Disease; DLI: Donor Leukocyte Infusions.

\*: AdV, EBV and CMV infections were monitored according to BMT guidelines and they were recruited from patient's medical case sheets.

Table 2: Patients treatments

Pz	GVHD Prophylaxis	Conditioning regimen	Antiviral therapy	Other therapies
F1	MTX, CYSP	Bu, Cy, ATG	Rib, Gancyclovir, Foscarnet, Rituximab	/
F2	MTX, CYSP	TBI, Cy, Adriamycin	Rib	Antibiotic therapy
F3	MTX, CYSP	Bu, Cy, ATG	Cid, Rib	/
M4	MTX, CYSP	Bu, Cy, AraC	Gancyclovir,	Corticosteroids, MMF, Thalidomide, Alemtuzumab Influximab
M5	MTX, CYSP	Bu, Cy, AraC	Cid, Rib	/
F6	MTX, CYSP	TBI, Cy, ATG	Cid, Rib, Gancyclovir, Rituximab	Antibiotic therapy, Probenecid, Risperidone
M7	MTX, CYSP	Bu, Cy, ATG	Cid, Rib	Antibiotic therapy, Probenecid

**Legend:** MTX: Methotrexate; CYSP: Cyclosporin; TBI = Total Body Irradiation; Cy = Cyclophosphamide; Bu = Busulfan; ATG = Antihuman Thymocyte Globulin; AraC = Cytarabin; Cid= Cidofovir; Rib= Ribavirin; MMF: Mycophenolate Mofetil

**Table 3: Results of quantitative and qualitative assays for BK and JC viruses.**

patients infected by BK virus	BK DNA (copies/ml)	BK transcriptional control region	JC DNA (copies/ml)	JC transcriptional control region	BK virus type	JC virus type
F1	65*10 <sup>8</sup>	archetype	NEG	ND	sub-type I	ND
F2	15*10 <sup>7</sup>	archetype	NEG	ND	sub-type I	ND
F3	33*10 <sup>7</sup>	archetype	NEG	ND	sub-type I	ND
M4	14*10 <sup>7</sup>	archetype	NEG	ND	sub-type I	ND
patient infected by JC virus	BK DNA (copies/ml)	BK transcriptional control region	JC DNA (copies/ml)	JC transcriptional control region	BK virus type	JC virus type
M5	NEG	ND	63*10 <sup>7</sup>	archetype	ND	genotype I B
patients infected by BK and JC viruses	BK DNA (copies/ml)	BK transcriptional control region	JC DNA (copies/ml)	JC transcriptional control region	BK virus type	JC virus type
F6	12*10 <sup>8</sup>	archetype	90*10 <sup>4</sup>	archetype	sub-type I	genotype I A
F6	91*10 <sup>7</sup>	archetype	90*10 <sup>5</sup>	archetype	sub-type I	genotype I A
F6	41*10 <sup>7</sup>	archetype	20*10 <sup>5</sup>	archetype	sub-type I	genotype I A
F6	20*10 <sup>7</sup>	archetype	90*10 <sup>5</sup>	archetype	sub-type I	genotype I A
F6	33*10 <sup>7</sup>	archetype	40*10 <sup>5</sup>	archetype	sub-type I	genotype I A
F6	75*10 <sup>7</sup>	archetype	50*10 <sup>5</sup>	archetype	sub-type I	genotype I A
F6	90*10 <sup>4</sup>	archetype	10*10 <sup>2</sup>	archetype	sub-type I	genotype I A
M7	50*10 <sup>2</sup>	archetype	40*10 <sup>5</sup>	archetype	sub-type I	genotype I A
M7	20*10 <sup>3</sup>	archetype	60*10 <sup>5</sup>	archetype	sub-type I	genotype I A
M7	20*10 <sup>4</sup>	archetype	50*10 <sup>4</sup>	archetype	sub-type I	genotype I A
M7	30*10 <sup>2</sup>	archetype	90*10 <sup>5</sup>	archetype	sub-type I	genotype I A
M7	70*10 <sup>3</sup>	archetype	70*10 <sup>4</sup>	archetype	sub-type I	genotype I A
M7	20*10 <sup>3</sup>	archetype	30*10 <sup>4</sup>	ND	sub-type I	genotype I A
M7	10*10 <sup>2</sup>	archetype	10*10 <sup>2</sup>	archetype	sub-type I	genotype I A

Legend: NEG= negative, ND= not determined F= female M= male