



# Human polyomaviruses DNA detection in peripheral blood leukocytes from immunocompetent and immunocompromised individuals

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Peripheral blood leukocytes from immunocompetent and immunocompromised individuals were analyzed for human polyomavirus BK and JC DNA presence. A nested polymerase chain reaction which amplify the transcriptional control region of the genome of both viruses was employed. The immunocompromised patients included bone marrow transplantation recipients and AIDS patients. BKV sequences were detectable in 52.8–62.5% of the individuals included in this study, whereas the percentage of individuals with JCV sequences in peripheral blood lymphocytes varied from 38.8% to 50%. The frequency of reactivations of BKV and JCV were also determined by detection of shedding in urine of viral DNA. The highest frequency of reactivations of either BKV or JCV was demonstrable in the group of bone marrow transplantation recipients, but reactivations occurred also in immunocompetent individuals. JCV sequences amplified from urine samples showed a restriction pattern similar to the archetype one, whereas sequences obtained from lymphocytes showed rearranged pattern as well as archetype pattern. Finally all JCV sequences from cerebrospinal fluid seemed to be rearranged. These observations suggest that peripheral blood lymphocytes have a fundamental role in the persistence of polyomaviruses infection and in the dissemination at least of JCV within the organism allowing that rearranged variants, better adapted to grow in brain tissue, emerge.

**Keywords:** BKV; JCV; genomic variants; sites of persistence

## Introduction

The human polyomaviruses BK (BKV) and JC (JCV) infect more than 60% of human population worldwide as can be inferred by serological surveys (Gardner, 1973; Padgett and Walker, 1973). Both viruses, after primary infection, early in childhood, often persist and reactivations may occur under different clinical conditions, such as in pregnant women, in immunocompromised patients and in immunocompetent elderly individuals. BKV and JCV infections occur independently, but concomitant infections and simultaneous persistence of both viruses may sometimes be observed.

Persistence of BKV and JCV was first and frequently demonstrated in the kidney (Gardner *et*

*al*, 1971; Chester *et al*, 1983; Dörries, 1984; Tominaga *et al*, 1992); thus BKV and JCV shedding in urine is the most common and easy to detect evidence of reactivations (Arthur and Shah, 1989). More recently BKV and JCV sequences have also been detected in normal brain tissue, thus brain now has to be included among the sites of persistence (White *et al*, 1992; Elsner and Dörries, 1992).

Primary infections and reactivations of BKV and JCV are usually asymptomatic; reactivations in particular host conditions may cause serious, but rare, diseases. BKV reactivations are associated with hemorrhagic cystitis in bone marrow transplantation patients, in presence of other factors (Arthur *et al*, 1986; Azzi *et al*, 1994). Moreover BKV DNA has been detected in human brain and pancreatic islets tumours and in Kaposi's sarcoma in AIDS patients (Monini *et al*, 1996).

JCV reactivations may cause the progressive multifocal leukoencephalopathy (PML) in patients with severe immunological impairment. This disease, once rare, is now frequently observed in AIDS patients (Berger *et al*, 1987).

So far, several aspects of JCV and BKV natural infection, like the route of transmission, the mechanism of induction of reactivations, the outcome of JCV infection leading to PML, are not yet clearly understood.

Studies concerning BKV and JCV infections are in remarkable progress because of the development of rapid and sensitive methods for the detection of BKV and JCV DNA such as DNA hybridization assays with specific, labelled, probes and most of all the polymerase chain reaction (PCR). Several investigations have been carried out with the aim to identify cells which might harbor persistently BKV and JCV genomes and contribute to their dissemination to target organs. Low copy number of JCV genome has been detected in liver, lung, lymph nodes and spleen of some PML patients in whom large amounts of JCV DNA was detected in brain (Grinnell *et al*, 1983). In addition, JCV-infected mononuclear cells from bone marrow have been detected in PML patients and B-cells showed to harbor JCV DNA (Houff *et al*, 1988). Subsequently, PCR allowed detection of JCV DNA in peripheral blood lymphocytes (PBL) from patients with PML (89.5%) and from HIV infected patients (38%) but not from individuals of the immunocompetent control group (Tornatore *et al*, 1992). Other authors report the demonstration of BKV, but not of JCV, DNA in PBL of 2/82 HIV infected patients (Sundsford *et al*, 1994). More recently the presence of BKV and JCV DNA in PBL from immunocompetent individuals has been reported (Dörries *et al*, 1994).

With the aim to evaluate the role of BKV and JCV presence in PBL as well as its diagnostic and prognostic implications we used a very sensitive nested PCR for the detection of BKV and JCV DNA in PBL and in urine samples from either immunocompromised and immunocompetent individuals. Two groups of immunocompromised patients, due to bone marrow transplantation (BMT) and AIDS, have been studied to evaluate if other factors, in addition to immunodepression, may contribute to polyomaviruses reactivation and dissemination.

Moreover, a preliminary characterization, by restriction pattern analysis, of JCV transcriptional control region amplified from different clinical specimens has been performed.

## Results

BKV and JCV DNA has been detected by a nested PCR in PBL of either immunocompromised and immunocompetent individuals as Table 1 shows.

Both BKV and JCV DNA have been detected in PBL of AIDS patients a little more frequently than in PBL of blood donors without significant differences. The data in Table 1 were obtained from BMT recipients at 30 days after the transplant; moreover PBL have also been available for BKV and JCV DNA detection 60 days after the transplant from 18 of the 33 BMT recipients included in this study: BKV and JCV DNA have been detected respectively in 15 (83%) and in nine (50%) of these 18 patients. Simultaneous infections by either BKV and JCV have been detected in PBL from five blood donors, eight BMT patients and 13 AIDS patients.

The data in Table 2 show the detection of BKV and JCV DNA by the nested PCR in urine samples from immunocompromised (AIDS and BMT patients at variable times after transplantation) and from immunocompetent individuals. At this stage no significant differences emerged between healthy individuals and AIDS patients. A significantly higher frequency of BKV DNA was detected in urine samples from BMT patients as compared to two other groups; moreover, in BMT recipients JCV shedding in urine was significantly higher than in healthy individuals.

The high variability of JCV transcription control region is well documented (Martin *et al*, 1985). At this level two main patterns have been identified: an 'archetype' one, characteristic of the urine isolates and a 'rearranged' type, including the MAD-1 prototype sequences, characteristic of PML isolates (Yogo *et al*, 1990; Ault and Stoner, 1993).

We have analyzed the restriction pattern, with the endonucleases SacI and SphI, of JCV transcriptional control region amplified from urines and from PBL from both immunocompetent and immunodepressed individuals; in addition JCV control regions amplified by the nested PCR from CSF of AIDS patients with PML have also been

**Table 1** Detection of JCV and BKV DNA by a nested PCR in PBL of immunocompromised and immunocompetent individuals

Individuals	Total No	BKV-No	DNA %	JCV-No	DNA %
Blood donors	36	19	52.8	14	38.8
BMT patients <sup>a</sup>	33	19	57.5	3	10.0
AIDS patients	32	20	62.5	15	46.8

<sup>a</sup>At 30 days after transplant

**Table 2** BKV and JCV urinary shedding in immunocompromised and in immunocompetent individuals

Individuals	Total No	BKV-No	DNA %	JCV-No	DNA %
Healthy	30	5	16.6	7	23.3
BMT patients	30	23	76.6	16	53.3
AIDS patients	30	12	40	11	36.6

analyzed. The 'archetype' region should have one cutting site for *SacI* and one for *SphI*, whereas in the corresponding region of MAD-1 there are two cutting sites for *SacI* and no cutting site for *SphI* (Markovitz *et al*, 1991). By sequencing, Ault and Stoner (1993) detected one rearranged variant from PML brain in which the duplication of *SacI* was not included. However, it seems that most rearranged strains have a restriction pattern MAD-1 like.

Figure 1 shows the pattern of digestion with *SacI* of some of our JCV control regions, examined on agarose Metaphor. The results of the restriction analysis are summarized in Table 3. JCV sequences amplified from urines showed a pattern similar to the 'archetype'. In one urine isolate from an AIDS patient there was a deletion of about 40 bp, although the restriction pattern was that reported for the archetype. 11/25 JCV sequences detected in PBL and all the isolates (6/6) from CSF analyzed showed a rearranged pattern like; the rearranged types had the same number of cutting sites characteristic of the MAD-1 strain but a polymorphism in the length of the restriction fragments was evident (Figure 1).

## Discussion

The results of this study demonstrate that BKV and JCV DNA is frequently present in peripheral blood



**Figure 1** Electrophoretic pattern of various JCV control region sequences, after digestion with the restriction enzyme *SacI*. The sequences were amplified by nested PCR from urine, PBL and CSF of different individuals. Lane 1 and 8: molecular weight markers IX (Boehringer); lane 2: archetype-like pattern of JCV control region amplified from an urine sample; lane 3: archetype-type pattern of JCV control region amplified from a PBL sample; lane 4: rearranged-type pattern of JCV control region amplified from a CSF sample, different from the MAD-1 one; lane 5: rearranged-type pattern of JCV control region amplified from another CSF sample, similar to the MAD-1 pattern (lane 6); lane 7: uncleaved control region of MAD-1. The arrows indicate the presence of two co-migrating bands.

**Table 3** Transcriptional control region variants detected in urines, PBL and CSF from immunocompetent and immunocompromised individuals by restriction pattern analysis

Specimen	Individuals	No	Archetype	Rearranged type
Urine	Healthy	4	4	0
	BMT patients	4	4	0
	AIDS patients	4	4	0
PBL	Healthy	5	2	3
	BMT patients	7	4	3
	AIDS patients	13	8	5
CSF	PML patients	6	0	6

lymphocytes of immunocompetent and of immunocompromised subjects. 52.8% to 62.5% of the individuals of all the groups tested have BKV DNA in PBL. The percentage of individuals carrying JCV DNA in PBL, from 38.8% to 50%, was lower in comparison with BKV DNA in all the groups studied, perhaps in relationship to a lower diffusion of JCV in the population.

We detected a lower percentage of positive immunocompetent subjects in comparison with the results reported by Dörries *et al* (1994) who demonstrated BKV DNA presence in PBL from 94.4% and JCV DNA presence in PBL from 83.3% of health subjects. As regards JCV DNA detection our percentage of PBL positive AIDS patients was only a little higher than that reported in a group of HIV-1 seropositive men with varying degrees of immunodeficiency (38%) by Tornatore *et al* (1992), who, on the other hand, failed to detect JCV DNA in a group of immunocompetent patients with Parkinson's disease. Such discrepancies could be due to the different methods used in these studies such as DNA extraction procedures, PCR primers, PCR conditions, PCR detection methods, which may affect the sensitivity of the reaction. It is possible that few polyomavirus genome equivalents are present in a low number of lymphocytes of healthy subjects and that PBL infected number and/or the genome copy number per cell increases in the setting of severe disimmunity and mostly in PML patients. The constitutive expression in B lymphocytes of AIDS patients of a B-cell domain B-binding protein, transactivating viral transcriptions, could have an important role at this purpose (Rieckmann *et al*, 1994). The use of quantitative PCR assays should give useful indications concerning the amount of infected cells and viral load per cell in subjects with different clinical conditions.

BKV and JCV DNA presence in PBL from BMT recipients has been first assessed in the present study: the percentage of BMT recipients with detectable levels of BKV and/or JCV DNA in PBL is very low if assessed 30 days after the transplant but rises to 83% and to 50% respectively in BMT



patients controlled 2 to 3 months after the transplant.

This observation could suggest that the lymphocytes depletion required for the transplant in BMT recipients also involves the clearance of both BKV and JCV from blood. Both viruses however persist in the kidney and are frequently reactivated, as evidenced by urinary shedding. Such reactivations may contribute to PBL reinfection. As an additional hypothesis, owing to the fact that JCV has also been detected in bone marrow (Houff, 1988), JCV could be transmitted to the recipient by the bone marrow of the donor.

Our results confirm that shedding of the virus in urine as a consequence of reactivation is not caused exclusively by immunodepression, in fact it may also occur in healthy individuals, although the frequency of this phenomenon increases mostly in BMT patients. Moreover, as previously reported, our data suggest that BKV reactivations in the urinary tract are more dependent on immunodepression than JCV reactivations (Markowitz *et al*, 1993).

The restriction analysis of JCV sequences detected in urine samples shows a similar pattern, 'archetype-like', among the various isolates, clearly distinguishable from the rearranged variants detected in all the isolates from CSF analyzed here and in a number of isolates from PBL. The restriction fragment length polymorphism observed among the rearranged variants either from PBL or from CSF demonstrates the presence of different variants in PBL as well as in CSF from different subjects. A higher similarity of the archetype sequences from urine isolates in comparison with the rearranged types from brain has already been reported in literature (Iida *et al*, 1993); this observation could suggest that the archetype is the form of the virus spread in nature whereas different rearrangements occur within the host. This hypothesis has been reported the first time by Yogo *et al* (1990). Furthermore, Ault and Stoner (1993), analyzing JCV control region sequences, showed that control regions from brain isolates lack archetype control region sequences, suggesting a one-way rearrangement from archetype to PML-type.

The demonstration that rearranged variants are already detectable at the PBL level allows us to hypothesize that PBL may play a fundamental role in the dissemination of the polyomavirus infection within the organism to the final target sites not only as virus carriers but also allowing that rearranged variants, better adapted to grow in brain tissue, emerge. In fact, it has been demonstrated that similar intracellular factors regulating JCV expression are present in B lymphocytes as well as in glial cells (Rieckmann *et al*, 1994).

Deeper studies concerning the amount of viral DNA and the genomic variants detectable in PBL

from subjects with different clinical conditions could give useful indications as regards the pathogenesis of PML and the possibility of monitoring of JCV infection in PML patients by testing lymphocytes, as alternative to the CSF analysis.

## Materials and methods

### *Patients and samples*

Blood samples for PBL analysis were drawn from 32 AIDS patients (T4 lymphocytes count <200/mm<sup>3</sup>) receiving care at the Infectious Disease Unit of the Careggi Hospital in Florence, from 33 BMT recipients at the BMT Unit of the Careggi Hospital and from 36 blood donors (attending to the Transfusional Center of the Careggi Hospital) as immunocompetent control group.

Urine samples were collected from 30 AIDS patients, 30 BMT recipients and 30 immunocompetent adult individuals. Moreover in patients with pathological and magnetic resonance imaging evidence of PML, receiving care at the Infectious Disease Units at the Careggi Hospital and at the Santa Maria Annunziata Hospital in Florence, JCV DNA detection has been carried out on CSF.

PBL were isolated from 10–15 ml of peripheral blood by density centrifugation on Ficoll-Hypaque gradient (Hystopaque, Sigma). Lymphocytes were resuspended in 500 µl of TNE buffer and frozen at –20°C. After thawing, PBL were lysed by addition of Proteinase K and SDS to final concentration of 200 µg/ml and 20% respectively. 1 µg of DNA, extracted by the phenol-chloroform method, was added to the PCR mixture.

The urine specimens (10–20 ml) were centrifuged at 1500 × g for 15 min and the supernatants were subjected to treatment with PEG 6000 in order to eliminate PCR inhibitors (Yamaguchi *et al*, 1992): in brief, 50 µl of clarified supernatant were mixed with the same volume of 20% PEG 6000 plus 25 µl of 2 M NaOH and kept at 4°C o.n. before being centrifuged at 15 000 × g for 30 min at 4°C. The pellet was centrifuged again at 6400 × g and then resuspended in 30 µl of double distilled (dd) H<sub>2</sub>O.

300 µl of CSF were mixed with 150 µl of herring sperm DNA (100 µg/ml) and boiled for 10 min; then the DNA was precipitated by addition of two volumes of ethanol and 0.1 volume of 3 M Na acetate. After centrifugation at 7000 × g for 30 min the pellet was resuspended in 30 µl of dd H<sub>2</sub>O (Moret *et al*, 1993). 5 µl of DNA extracted from either urines or CSFs were added to the PCR mixture.

### *Nested PCR for BKV and JCV DNA detection*

A sequence, approximately 700 bp long, including the transcriptional control region and the origin of the replication of BKV and JCV genomes was

amplified in a first reaction by the use of a set of outer primers, BKTT1 and BKTT2 (Sundsford *et al*, 1990); the reaction consisted of 30 cycles, each of 1 min at 94°C, 2 min at 55°C and 3 min at 72°C. 2 µl of the first amplification products were then added to a second PCR mix, containing the set of inner primers JC1 and JC2 specific for JCV DNA or BK1 and BK2 specific for BKV DNA (Markowitz *et al*, 1991). The second amplification was carried out for 40 cycles each of 1 min at 94°C, 1 min at 68°C and 1 min at 72°C with a final extension at 72°C for 10 min. The product of this second amplification was a sequence approximately 300 bp long for both BKV or JCV, corresponding to the transcriptional control region of both viruses. The PCR products were analysed by electrophoresis in 2% agarose gel stained with ethidium bromide (EtBr). Identification of BKV and JCV DNA sequences obtained by the nested PCR was performed by Southern blot hybridization with digoxigenin-ddUTP end labeled oligonucleotide probes: BKP1 (GGAAGGAAAGTG-CATGACTGG) BKV specific and JCP1 (TACC-TAGGGAGCCAACCAGCTGAC) JCV specific (De Santis and Azzi). Duplex polymerase chain reaction for the simultaneous detection of the human polyomavirus BK and JC DNA. Submitted for publication on Molecular and Cellular Probes). Positive samples were further characterized by restriction analysis with the enzyme Bsu36I and SacI, the first one cutting the BKV DNA sequence but not the JCV sequence and the latter *vice versa* (Markowitz *et al*, 1991).

This reaction was able to detect 0.01 fg of viral DNA as resulted by amplification of serial dilutions of positive controls: the plasmid pMAD-1 (ATCC 45027) and the genome sequence of the BKV

Gardner's strain, cloned in pBR322 in our laboratory.

All precautions were used to minimize the risk of contamination; DNA extractions and PCR were conducted in different laboratories and different sets of pipettes were used for each PCR step. Distilled water negative controls were done during extraction procedures as well as during reaction mixture preparation and sample addition to confirm the efficiency of the preventive measures.

#### Restriction pattern analysis

The JCV control regions amplified by the nested PCR from urine samples, from PBL and from CSF have been compared by restriction pattern analysis after separate digestion with the enzymes SacI and SphI (Markowitz *et al*, 1991). The first enzyme, in fact, cut the above mentioned sequence of the variant MAD-1 in three fragments of about 102 bp, 98 bp and 117 bp, whereas the archetype is cleaved in two fragments of about 125 bp and 175 bp. The variant MAD-1 is not cleaved by SphI whereas the archetype is cleaved in two fragments of about 176 bp and 124 bp. One unit of enzyme was added to 5–10 µl of the PCR product and kept at 37°C o.n. The results of the digestion were observed by electrophoresis in 3% agarose Metaphor, stained with EtBr.

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