

# BKV QPCR detection and infection monitoring in renal transplant recipients

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## SUMMARY

BKV associated nephropathy (BKVAN) is a cause of renal dysfunction and loss of the graft in transplants. Viral primary infection is usually inapparent and then BKV establishes latency in kidneys. Reactivation occurs in immunocompromised conditions in renal transplant recipients who can develop a subclinical nephritis and eventually a BKV-associated interstitial nephritis or a BKVAN.

In this study, we searched for BKV copies in urine and plasma of renal transplants by quantitative assay (QPCR). Results showed that in several patients clearance of viremia is associated with persistent viruria, suggesting that both specimens are necessary to correctly monitor a BKVAN.

**KEY WORDS:** BKV, BKVAN, QPCR, Viremia, Viruria

BK virus (BKV) and JC virus (JCV) are the two polyomavirus species most commonly implicated in human disease. BKV was isolated for the first time in 1971 from the urine of an immunocompromised renal transplant patient. JCV was also recovered in 1971 from the brain of a patient with Hodgkin's disease and progressive multifocal leukoencephalopathy (PML). Both BKV and JCV circulate in a large proportion of the population worldwide. BK infects young children and the seroprevalence is 70%-80% in adults (Fioriti *et al.*, 2005). Moreover, since the presence of BKV DNA was observed in a high percentage of maternal and fetal materials, a vertical transmission has been also considered (Pietropaolo *et al.*, 1998;

Knowles, 2006). Primary infection is usually subclinical and is followed by viral latency in the urogenital tract (Randhawa *et al.*, 2006). Reactivation occurs in immunocompromised hosts such as renal transplant recipients who can develop persistent graft dysfunction (5%) and can lose their graft (about one-half). Currently BK virus associated nephropathy (BKVAN) is increasing in this setting, probably due to the introduction of new, potent immunosuppressive drugs (Vera-Sempere *et al.*, 2005). BKV disease typically manifests as an elevation in serum creatinine and is always preceded by clinically silent viruria, thus allowing the identification of patients at risk for BKVAN (Ginepri *et al.*, 2003). Onset of viruria following primary BKV infection has been observed between 10 days and 6 weeks following transplantation. In contrast, onset of viruria for patients who were BKV seropositive prior to transplant (infection is due to either reactivation of their own infection or reinfection with a new strain of BKV), has been documented between 5 weeks and 17 months fol-

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lowing the transplant (Nickeleit *et al.*, 1999). Several investigators have begun to define risk factors for BKV disease among renal transplant recipients. The serologic status of the donor and the recipient appears to be a predictor of BKV infection, but it is not currently clear whether it influences the development of BKV nephritis. Tubular injury could be a factor promoting viral replication in an immunocompromised state induced by tacrolimus or mycophenolate-mofetil. The load of dormant BKV in the grafted organ is likely to be another important risk factor: no dormant virus, no re-activation and most likely, no BKVAN (Van Gorder *et al.*, 1999). As a consequence, BKVAN potentially develops in grafts with high loads of pre-existing latent BK virus under immunosuppression when tubular injury gives the opportunity for productive and massive viral proliferation. From initial small 'hot spots' in the medulla, viral replication might spread to the cortex with severe consequences for graft function and outcome (Hirsch *et al.*, 2002; Randhawa *et al.*, 2002; Nickeleit *et al.*, 2003). BKVAN diagnosis is very difficult since this disease is often misdiagnosed as acute rejection or drug toxicity. In fact, when BKVAN is suspected, a definitive diagnosis requires renal biopsy, therefore morphological recognition of BKV in renal tissue is sometimes difficult because viral cytopathic changes can be confused with rejection or coexist with a true rejection. For this reason, auxiliary techniques, such as immunohistochemistry, in situ hybridization, electron microscopy, and also polymerase chain reaction (PCR) are often necessary (Mannon, 2004). For efficient early diagnosis of BK infection, various molecular approaches are recommended because they are non-invasive tests and represent a way to monitor the clinical course of infection (Nickeleit *et al.*, 2000; Tong *et al.*, 2004). Moreover these techniques can optimize the timing of renal allograft biopsies facilitating morphological diagnosis and the assessment of therapeutic efficacy. An inexpensive screening tool is the examination of a Papanicolaou-stained urine sediment for the presence of decoy cells. These are epithelial cells with enlarged nuclei and large basophilic ground-glass intranuclear viral inclusions (Kipp *et al.*, 2005). Nevertheless this technique is not to be considered a specific morphological marker of BKV disease. Detection of

BKV DNA in plasma and in urine of renal transplants, using quantitative PCR (QPCR), is more sensitive and especially more specific than urine cytology, and it also gives information above viral titre that can be related to the onset or progression of a BKVAN (Basse *et al.*, 2007). In fact, depending on the viral load levels in the plasma and/or urine, patients can be grouped into risk levels 1 or 2. 'Presumptive BKVAN risk level 2' is assumed in a patient with BK-virus loads exceeding  $1 \times 10^4$  copies/ml in the plasma and/or  $1 \times 10^7$  copies/ml in the urine (Drachenberg *et al.*, 2004; Hirsch *et al.*, 2005). In these patients, an allograft biopsy is indicated to establish a definitive diagnosis. In this study, we searched for BKV copies in urine and plasma of 67 renal transplants (41 males and 26 females, mean age 45 years) by quantitative assay to establish whether clearance of viremia is associated with absence of viruria and consequently if viremia should be employed as the only index of BKVAN monitoring as suggested in literature (Hirsch *et al.*, 2002). Samples from subjects transplanted at our center ("Umberto I" Hospital, Rome, Italy) were collected during the nine months post-transplantation and when elevation in serum creatinine was higher than >25% of referring value indicated by Clinical Practice Guidelines Committee of the American Society of Transplantation (Kasiske *et al.*, 2000). For all patients informed consent was obtained. Blood samples were collected in EDTA tubes and the plasma fraction was separated. Urines were collected and used without centrifugation. DNA was extracted with the DNeasy tissue kit (Qiagen) for 1 ml of urine or the QIAamp Blood minikit (Qiagen) for 200  $\mu$ l of plasma. QPCR assay was performed using 7300 Real Time PCR System (AB Applied Biosystems). PCR amplifications were run in a reaction volume of 20  $\mu$ l containing 5  $\mu$ l of the DNA sample, Q-Amplimaster (reaction mix for quantitative amplification), Q-Amplimix (forward and reverse primers) and finally Q-Ampliprobe (hydrolysis probes). 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. The amplification data were analyzed with software provided by the manufacturer. Standard curves for the quantification of BKV were constructed using serial dilutions of a plasmid con-

TABLE 1 - Detection of BKV DNA in urine of renal transplants, using QPCR.

Sample	Time of transplantation			
	BKV T=0	BKV T=1	BKV T=2	BKV T=3
Urine	28/67	38/67	29/67	23/67

Legend: t=0: time of transplantation; t=1: three months post-transplantation; t=2: six months post-transplantation; t=3: nine months post-transplantation

Table 2 - Detection of BKV DNA in plasma of renal transplants, using QPCR.

Sample	Time of transplantation			
	BKV T=0	BKV T=1	BKV T=2	BKV T=3
Plasma	12/67	12/67	13/67	13/67

Legend: t=0: time of transplantation; t=1: three months post-transplantation; t=2: six months post-transplantation; t=3: nine months post-transplantation

taining target sequences for BKV (Large T Antigen). The plasmid concentrations ranged from  $10^2$  to  $10^5$  plasmid copies of BKV DNA target. All patient samples were tested in triplicate and the number of BKV copies in each sample was calculated from the standard curve. Data were expressed as copies of viral DNA per milliliter of sample. Standard precautions designed to prevent contamination during QPCR were followed. No template control (NTC) lane was included in each run.

Our results are shown in Table 1 and Table 2. In particular, regarding urine samples, 28 of 67 patients were found positive for BKV DNA at the time of transplantation (T=0), 38 of 67 were found positive three months post-transplantation (T=1), 29 of 67 were found positive six months post-transplantation (T=2) and 23 of 67 were found positive nine months post-transplantation (T=3) (Table 1). The analysis of plasma specimens revealed that BKV DNA was found in 12 of 67 patients at T=0 and T=1 and in 13 of 67 patients at T=2 and T=3 (Table 2). Moreover we observed that viral loads in urine samples correlated with serum creatinine levels whereas viremia was not correlable (data not shown). The results of the molecular approach used in this study showed that for the majority of our patients BKV infection was first detectable in the urine. If viruria persisted, plasma later also became positive. Since positive QPCR-urine cases always preceded

positive QPCR-plasma cases, it is possible to hypothesize that viral replication within the graft finally lead from viruria to viremia. Therefore our findings show that the most reliable indicator to predict and especially to monitor BKVAN is QPCR-urine detection which may also be considered an early indicator of viremia onset or progression. In fact BK viremia should not be automatically interpreted as 'of kidney origin' as reported by authors who correlated BKV viremia in bone marrow transplant recipients with clinical signs of hemorrhagic cystitis (Erard *et al.*, 2004.; Erard *et al.*, 2005). Thus, viremia can only serve as a general marker of 'BK-virus activation'. In addition our results support data in literature that report the persistence of BK viruria after viral clearance from the kidney, although at much lower numbers of copies than seen at the time of initial diagnosis (Randhawa *et al.*, 2004, Tong *et al.*, 2004). Finally, since BKV loads fluctuate during BKVAN, measurements of viruria and viremia can provide crucial information on a patient's therapeutic response to anti-viral treatment and can guide the duration of therapy. However these observations should be confirmed by further clinically studies and should be precisely defined in multicenter trials.

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