

PCR real time assays for the early detection of BKV-DNA in immunocompromised patients

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SUMMARY

Testing for viral BKV-DNA in urine is a non-invasive early detection and monitoring tool in the diagnostic of BKV-related pathologies: quantitative analysis by Real-Time PCR can provide useful information in addition to cytologic analysis, although our study suggests that high BKV viruria is not necessarily associated with kidney or bladder damage.

KEY WORDS: Polyomavirus, Renal transplant, Nephropathy

BK virus (BKV) is a polyomavirus, ubiquitous in human populations: over 90% of adults worldwide are infected during early childhood (Hirsch 2002), mostly transmitted by the respiratory route (Goudsmit 1982, Sundsfjord 1994).

After primary infection, asymptomatic in the majority of cases, BKV maintains low-level replication in different sites in the body, most frequently in the kidney (Heritage 1981); replication can occasionally surge to detectable levels in urine.

In immunocompromised patients, viral reactivation is often followed by clinical disease.

In particular, in patients who undergo hematopoietic stem cell transplantation (HSCT), reactivation of BKV infection is associated with hemorrhagic cystitis (incidence 15%) (Sencer 1993), while in kidney transplant recipients acute

tubulointerstitial nephritis is more frequent (incidence 5-8%) (Randhawa 1999, Hirsch 2002B). Acute tubulointerstitial nephritis is accompanied by a reduced renal function and allograft failure occurs in over 10% of patients affected (Hirsch 2002B). The increasing incidence of disease associated with BKV in immunocompromised patients requires further studies for the diagnosis and the management of infection. In fact, BKV viruria is not *per se* predictive of disease, given the frequency of asymptomatic reactivation in immunocompromised patients, 30-40% (Mylonakis 2001). Some authors claim that viruria beyond the threshold of 10⁷ copies/ml in urine can be a predictive marker of BKV pathology (Hirsch 2005). The aim of this study is to define the prevalence of BKV reactivations in different categories of immunocompromised patients, and to investigate the relationship between viral load in urine, immunosuppression and disease.

We studied 120 consecutive immunocompromised patients followed at Ospedali Riuniti, Ancona between May 2006 and January 2007 by evaluating renal function (creatinine and nitrogen in blood, cytology of urine sediment, when available) and CD4⁺ T cell number.

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Viral load was evaluated in urine by a Real-Time PCR technique. A qualitative PCR was also performed on all samples to exclude false negative results due to potential probe mismatches.

The patients were divided into three groups:

Group I: 70 HIV-positive (46 males, 24 females, aged 18-77 years, mean 43.4) all free from BKV-associated disease. They were classified on the basis of CD4⁺ T cell count into two subgroups; 21 patients with CD4⁺ T cell count <200/mm³ and 49 patients with CD4⁺ T cell count >200/mm³.

Group II: 12 kidney transplant recipients (9 males and 3 females, aged 1-74 years, mean 36) all free from BKV-associated disease.

Group III: 38 bone marrow transplant recipients (23 males, 15 females, aged 5-73 years, mean 43.8), five of whom had hemorrhagic cystitis.

Urine samples were collected from each patient and were centrifuged at 18000 rcf for 30 min. The sediment was re-suspended in 200 µl of lysis buffer containing EDTA 1mM, Tris-HCl 10mM pH 8.0, Tween 20 and NP40 (0.5% each) and 25 µg of proteinase K.

After incubation for 4 hours at 56°C and denaturation for 10 min at 94°C to inactivate proteinase K, each sample was stored at -20°C until amplification.

Quantitative-PCR (Q-PCR) was carried out by real-time amplification using the MX3000p (Stratagene) amplifier. PCR primers for the BKV VP1 gene were designed after thorough alignment of BKV sequences available in the GenBank database to identify a highly conserved region (including strains from different geographical areas).

The primer sequences are as follows (as synthesized): VP1f (forward primer: 5' ACT GTA ACA CCT GCT CTT GAA GCA T 3'), VP1r (reverse primer: 5'AGC TGC CCC TGG ACA CTC T3'), and the TaqMan probe (5' TGA AGA TGG CCC CAA CCA AAA GAA AAG 3') dual-labelled at the 5' end with 6-carboxyfluorescein (FAM) and the 3' end with 6-carboxytetramethylrhodamine (TAMRA). Primers and probe were designed using the Primer Express® software (PE Biosystems).

Q-PCR amplifications were set up in a 25 µl reaction volume containing 12.5 µl of TaqMan Universal PCR Master Mix (PE Biosystems), 5 µl of sample, 2.5 µl of VP1f and VP1r 500 nM each and 2.5 µl of TaqMan probe 100 nM.

Thermal cycling conditions were: 2 min incubation at 50°C, followed by a 10 min at 95°C and then 45 cycles each of 95°C for 15 s and 60°C for 1 min. The standard curve for the quantification of BKV-DNA in sample was obtained by a 10-fold serial dilutions of the plasmid pBKV-VP1, obtained by inserting the 72 bp fragment amplified by the Q-PCR primers in the pGEM T-EasyA TA vector (Promega). The procedure was performed following the manufacturer's instructions and, after transfection of JM109 competent cells, plasmid DNA from positive bacterial clones was purified by Wizard® Plus SV Minipreps, DNA purification system (Promega) and the DNA concentration was assessed by spectrophotometric analysis at 260 nm.

Primers for qualitative PCR targeted the BKV LT-Ag gene, and they were also designed after thorough alignment of BKV sequences for the identification of a conserved region; these sequences are (as synthesized): LT-Agf (forward primer: 5' TAC AGC ATT TCC ATG AGC TC 3'), LT-Agr (reverse primer: 5' GTA GAT ACC CTT CAT ATG AC 3').

Qualitative amplification was carried out as follows: 5µl of sample extract was added to 45 µl of reaction mixture containing 2.5 U of Taq DNA polymerase (AmpliTaq, Roche Diagnostic), 0.5 µM of each primer, 200 µM of each deoxynucleotide triphosphate, 50 mM of KCl, 4mM of MgCl₂ and 10 mM of Tris-HCl.

Thermal cycling conditions were 45 cycles of 30 seconds each at 45°C, 30 seconds at 72°C and 30 seconds at 94°C, to allow the amplification of a 102 bp fragment.

The overall prevalence of BKV reactivation in the population studied was 22.5%, the results are summarized in table I. In particular, the prevalence of BKV viruria in group I (HIV-positive) was 22.8% (16/70), however, reactivation occurred in 57% (12/21) of patients with CD4⁺ T cell number <200/mm³, in contrast to 8.2% (4/49) in those with >200 cells. In these subjects BKV viruria inversely correlated with CD4⁺ T cell count (p=0.03 Spearman Correlation) and values exceeding 10⁷ copies/ml were found in 50% (6/12) of patients with <200 CD4⁺ T cells, in contrast to 25% (1/4) of patients with >200.

Nevertheless all subjects within this group were free from BKV-related disease and had normal renal function.

TABLE 1 - Prevalence of BKV in urine samples from different immunocompromised patients.

Group I: HIV-positive					
CD4	n. (%)	viruria	Viruria >10 ⁷	Media viral load	Altered renal function
<200	21 (30%)	12 (57%)	6 (50%)	6x10 ⁸	0
>200	49 (70%)	4 (8,2%)	1 (25%)	2x10 ⁵	0
Group II: kidney transplant					
nd	12	3 (25%)	1 (33,3%)	4x10 ⁸	4
Group III: bone marrow transplant					
<200	38	9 (23,7%)	6 (66,6%)	4x10 ⁹	3

In group II, kidney transplant recipients, BKV DNA in urine was detected in only 3 patients (25%), but none displayed any sign of disease. One patient, despite a BKV DNA copy number exceeding 10⁷ copies/ml, was asymptomatic and had no altered marker of renal function.

Finally, in group III, bone marrow transplant recipients, the prevalence of BKV viruria was 23.7% (9/38). Interestingly, in contrast to the other groups, 5 out of the 6 patients with BKV viruria exceeding 10⁷ copies/ml, suffered from hemorrhagic cystitis (severe hematuria and dysuria). During the period of surveillance a female patient entered the study at the onset of the cystitis. This 25-year-old, was transplanted in November 2006 and was treated with cyclosporine and micophenolate mofetil. Hemorrhagic cystitis

occurred 40 days after transplantation. At the worsening of symptoms, 57 days after transplantation, cidofovir (a single 250mg dose) was administered to the patient, first by intravesical route, subsequently, at day 59, cidofovir was administered again, both intravesical (250mg) and intravenous (250 mg) achieving a two logarithm reduction in viruria (Figure 1). At the same time the dosage of the immunosuppressive therapy was increased to prevent a possible rejection.

Despite the improvement of symptoms and the reduction of hematuria, viruria remained stable (3 subsequent determinations, mean value 2x10⁸ copies/ml) up to 80 days after transplantation. Plasma samples were also evaluated in this case and BKV load in plasma was also stable (day 77:

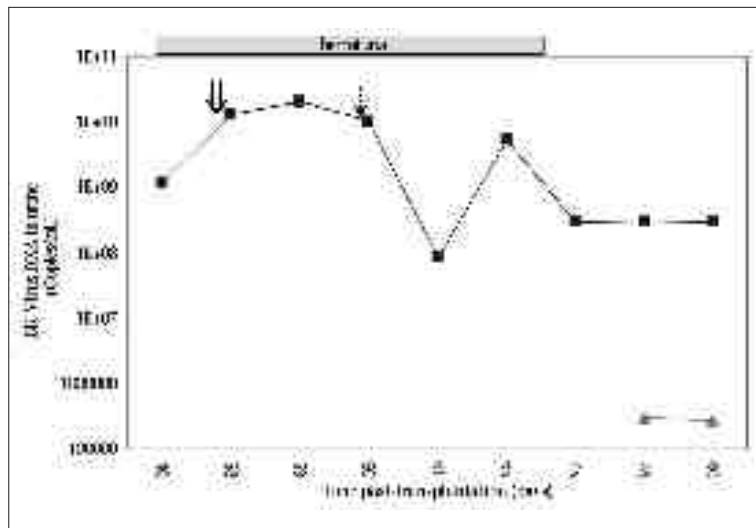


Figure 1 - Clinical course and blood/urine viral load in patient with hemorrhagic cystitis. -■- BKV DNA in urine (copies/ml), -▲- BKV DNA in plasma (copies/ml), ↓ 1° cidofovir dose (intravesical route 250 mg), ↓ 2° cidofovir dose (intravenous route 250 mg and intravesical route 250 mg).

3×10^5 copies/ml, day 79: 2.7×10^5 copies/ml) (Figure 1).

In conclusion, the following evidence emerged from this study: BKV reactivation occurs at comparable frequency in the different groups of immunocompromised patients, mean value 22.5%.

However, in HIV positive patients with CD4⁺ T cell number <200, reactivation is more frequent (mean value 57%) than in transplant recipients who are generally more immunosuppressed and have CD4⁺ T cell mostly <200. Interestingly the level of viruria is inversely correlated with CD4⁺ T cell counts (p 0.03 Spearman Correlation). Reactivations in HIV positive patients, despite being very frequent, do not seem to progress to clinical disease. In contrast, in bone marrow transplant recipients, the high viral load (>10⁷ copies/ml in urine) is relatively rare but correlates with the occurrence of hemorrhagic cystitis. The same threshold did not appear to be a reliable predictive marker of BKV related nephropathy, either in HIV+ or in hematologic patients. Further studies are needed to clarify these aspects and to understand the role of viruria in the pathogenesis of BKV-related disease in different categories of immunocompromised patients.

REFERENCES

- GOUDSMIT, J., DILLEN, P.W., VAN STRIEN, A., VAN DER NOORDAA, J. (1982). The role of BK virus in acute respiratory tract disease and the presence of BKV DNA in tonsils. *J Med Virol* **10**, 91-99.
- HERITAGE, J., CHESTERS, P.M., McCANCE, D.J. (1981). The persistence of papovavirus BK DNA sequences in normal human renal tissue. *J Med Virol* **8**, 143-150.
- HIRSCH, H.H. (2002). Polyomavirus BK nephropathy. A re-emerging complication in renal transplantation. *Am J Transplant* **2**, 25-30.
- HIRSCH, H.H., KNOWLES, W., DICKENMANN, M., PASSWEG, J., KLIMKAIT, T., MIHATSCH, M.J., STEIGER, J. (2002). Prospective study of polyomavirus type BK replication and nephropathy in renal transplant patients. *N Engl J Med* **347**, 488-496.
- HIRSCH, H.H., BRENNAN, D.C., DRACHENBERG, C.B., GINEVRI, F., GORDON, J., LIMAYE, A.P., MIHATSCH, M.J., NICKELEIT, V., RAMOS, E., RANDHAWA, P., SHAPIRO, R., STEIGER, J., SUTHANTHIRAN, M., TROFE, J. (2005). Polyomavirus-associated nephropathy in renal transplantation: interdisciplinary analyses and recommendations. *Transplantation* **79**, 1277-1286.
- MYLONAKIS, E., GOES, N., RUBIN, R.H., COSIMI, A.B., COLVIN, R.B., FISHMAN, J.A. (2001). BK virus in solid organ transplant recipients: an emerging syndrome. *Transplantation* **72**, 1587-1592.
- RANDHAWA, P.S., FINKELSTEIN, S., SCANTLEBURY, V., SHAPIRO, R., VIVAS, C., JORDAN, M., PICKEN, M.M., DEMETRIS, A.J. (1999). Human polyomavirus-associated interstitial nephritis in the allograft kidney. *Transplantation* **67**, 103-109.
- SENCER, S.F., HAAKE, R.J., WEISDORF, D.J. (1993). Hemorrhagic cystitis after bone marrow transplantation. *Transplantation* **56**, 875-879.
- SUNDSFJORD, A., SPEIN, A., LUCHT, E., FLAEGSTAD, T., SETERNES, O.M., TRAAVIK, T. (1994). Detection of BK virus DNA in nasopharyngeal aspirates from children with respiratory infections but not in saliva from immunodeficient and immunocompetent adult patients. *J Clin Microbiol* **32** (5), 1390-1394.