Quantitative viral load measurement for BKV infection in renal transplant recipients as a predictive tool for BKVAN

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SUMMARY

Infection by polyomavirus BK (BKV) is an emerging problem in the clinical management of renal transplant patients because it is responsible for nephropathy and consequently can cause loss of the transplanted organ (BKV associated nephropathy, BKVAN).
Aim of this study was to evaluate the use of blood viral load measurement as a screening tool for diagnosis of BKV infection and to identify a threshold value for the management of patients.
A total of 75 kidney transplant patients, corresponding to 338 consecutive plasma samples, were analyzed by an automatic system for nucleic acid extraction and quantitative real-time polymerase chain reaction (PCR) for detection of BKV. BKV was detected in 170 samples (26 patients) with a median viral load of 4.1 $\log_{10}$ copies/mL; among these 26 patients, seven (34.7%) were found to have BKVAN on allograft biopsy together with a median viral load of 5 $\log_{10}$ copies/mL. The ROC curve analysis identified a viral load equal to 4.1 $\log_{10}$ copies/mL as the best discriminant cut-off value to predict the disease and to identify patients at risk of developing BKVAN.

KEY WORDS: BKV, Renal Transplantation, Real time PCR

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INTRODUCTION

Human polyomavirus BK is a double-stranded DNA virus, which together with JC virus causes primary infection in early childhood with seroprevalence ranging from 60-100% in adults (Major, 2001).
Following primary infection, BKV establishes subclinical and persistent infection predominantly in kidney where replication is controlled by the immune system (Reploeg *et al.*, 2001). Reactivation in infected urothelial cells with viral shedding in urine (viruria) appears to be related to the impaired immune functions. In pregnancy and older age groups, physiological disfunctions of the immune system can be associated with BKV replication and viruria, but with no clinical significance (Hirsch *et al.*, 2003). On the contrary, in immuno-compromised hosts, BKV reactivation causes disease of the urinary tract (cystitis, ureteral stenosis, nephritis) (Nickeleit *et al.*, 2003; Hariharan, 2006). Especially in renal transplant recipients BKV infection can induce a tubular interstitial nephropathy (BKVAN), whose frequency can reach 5% and can be the cause of premature loss of 30-60% of transplanted organs and subsequent return to haemodialysis (Hirsch *et al.*, 2001; Ramos *et al.*, 2002).
The risk factors for BKV infection associated with nephropathy are not known, but introduction of the systematic use of potent immunosuppressive drugs has played an important role (Binet *et al.*, 1999; Nickeleit *et al.*, 1999). Otherwise, the main-

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stay of therapy is a reduction of immunosuppression and /or a change of immunosuppressive drugs because the use of antiviral drugs, such as cidofovir, is nephrotoxic (Hirsch *et al.*, 2005). Patients with BKVAN do not have any clinical sign or specific symptom, except a decline in renal function; definitive diagnosis is made by demonstration of viral cytopathic changes in renal biopsy (Hirsch *et al.*, 2002).
Recently, the use of molecular tests to detect and measure viral load of BKV in plasma (viremia) became an important non–invasive tool to identify patients at risk of BKVAN during post-transplantation follow-up (Hirsch *et al.*, 2000). Indeed, several studies have demonstrated a correlation between higher viral loads in plasma and disease, with positive predictive value in the range of 60-85% (Limaye *et al.*, 2001; Randhawa *et al.*, 2004).
The purpose of this study was to evaluate the incidence of BKV infection and its effects on renal function in kidney transplant recipients by the activation of a monitoring protocol of BK viral loads in plasma during the first year after transplantation, and to assess the advantages of early diagnosis of BKVAN by systematic follow-up.

PATIENTS AND SAMPLES

Renal transplant patients at Spedali Civili of Brescia (Italy) from January 2007 to September 2010, were retrospectively reviewed.
A total of 75 kidney transplant recipients (59 male and 16 female, median age 51.8 years ±11.6, range 37-67) were included in this study and detection of BKV replication was performed on 338 plasma samples. Also, urine samples were collected monthly after transplantation for a period of 1 to 33 months for urinary cytology (Decoy cells/CU). BKV replication monitoring was performed twice monthly during the first year post transplantation in patients with viremia or renal function abnormalities, otherwise every 3 months.
Monitoring intervals were reduced to one/month during follow-up (33 months).
Patients with positive viremia had a renal biopsy to detect BKVAN. Patients with BKV-associated nephropathy were administered antiviral therapy with cidofovir (0.5 mg/kg for 4 administrations) and/or leflonumide (100 mg/die for 3 days and then 40-60 mg/die) and the follow-up of viremia was continued for six months after negative BKV DNA detection. Immunosuppressive protocols were as follows: induction therapy was obtained in 57 patients by alemtuzumab and in 18 by basilixumab; the 7 subjects who resulted positive to BKVAN were treated with tacrolimus (FK) in association with rapamycin (Sirolimus-Sir) (6 patients) and 1 patient was treated with FK and mycophenolate mofetile (MMF) in association with steroid (St). During follow-up 5 patients continued immunosuppressive therapy with Sir, one with MMF and 1 patient with FK and St.
In the presence of BK viremia, the immunosuppression level was reduced. Urine specimens were collected for decoy cell detection as midstream samples; plasma samples were collected in sodium citrate blood tubes. Both sets of samples were frozen at -80°C until used.

DNA extraction

DNA was extracted by an automatic nucleic acid platform (Nuclisens EasyMag - Biomerieux). Aliquots of 200 µl of each plasma sample were added to 800 µl of lysis buffer and the mix was loaded in the instrument; after lysis step (10 minutes at room temperature) 65 µl of magnetic silica, 10 µl of internal control (CPE, Nanogen Advanced Diagnostics S.r.l.), represented by purified beta globin sequence, and 55 µl of wash reagent were added to each specimen. DNA was automatically extracted, eluted in 55 µl of specific buffer and immediately used in real time PCR. The remaining aliquot was stored at -80°C.

Real-time quantitative BK viral load

BKV DNA quantification was carried out by BKV Q.Alert Kit (Nanogen Advanced Diagnostics, Italy), using ABI Prism 7300 Fast Real Time PCR System (Applied Biosystems, Italy). This test is based on simultaneous amplification of the BKV Large T Antigen gene and of internal control human beta globin gene.
Beta globin DNA was added to each sample to monitor the efficiency of extraction and amplification procedures.
Specific TaqMan probes labelled with different reporter molecules were used to detect amplification of the targets. Quantification of BKV samples was obtained by interpolation of fluorescent signal intensity with a standard curve derived

from amplification of serially diluted BKV DNA standards. The assay was linear in the interval between 316 and 12.5x10^6 BKV DNA copies/mL of plasma.

Statistical analysis

Data were expressed as median value and ranges. Receiver-operating characteristic (ROC) curve analysis was used to determine the BKDNA cut-off for diagnosis of BKVAN; ROC curve was obtained using commercially available software (MedCalc, version 9.5.2.0).

RESULTS

A total of 338 plasma samples, corresponding to 75 kidney recipients, were analysed by Real-time PCR. Quantitative BKV DNA detection was positive in 170/338 (50.3%) plasma samples, obtained from 26/75 (34.7%) patients (median consecutive samples/patient: 5).

A histologically confirmed diagnosis of BKVAN was made in seven of the 26 patients giving a 9.3 % prevalence of disease in the 75 kidney transplant patients and even up to 26.9% for patients

TABLE 1 - *BKV DNA in plasma of 75 kidney transplant recipients.*

Plasma samples	*Positive*	*Negative*	*Patients*	*Negative viremia*	*Positive viremia*	*Patients with BKVAN*
338	170 50.3%	168 49.7%	75	49 65.3%	26 34.7%	7/26 26.9%

TABLE 2 - *Correlation between viral load and clinical status in 75 renal transplant patients.*

Viral load (copies/mL)	*Group 1 <1000*	*Group 2 1000-10000*	*Group 3 >10000*	*Total*
Median (range)	632 (336-962)	3783 (1012-9680)	41.4X10^4 (10.1X10^3-3.4X10^6)	
Plasma samples (n)	20	53	97	170/338 50.3%
Patients (n)	4	5	17	26/75 34.7%
BKVAN (n)	-	1	6	7/75 9.3%

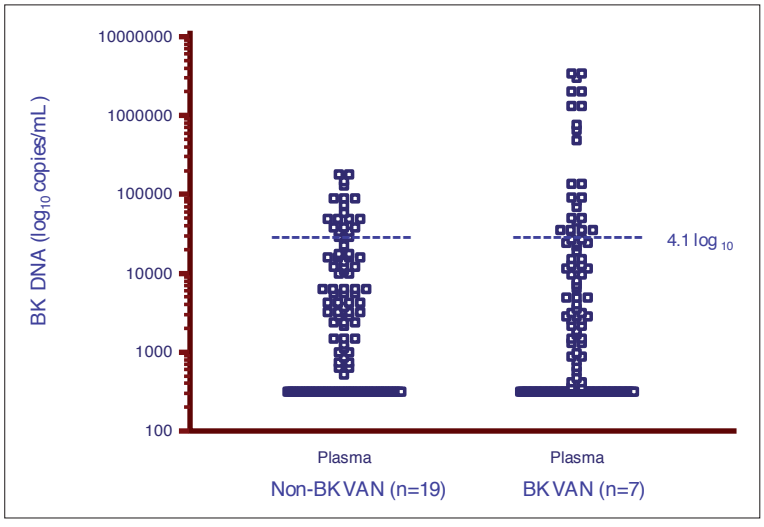

FIGURE 1 - *Distribution of BKV DNA load in BK virus-associated nephropathy (BKVAN) and non-BKVAN patients. Dashed line indicates the suggested viral load cut-off for diagnosis of BKVAN.*

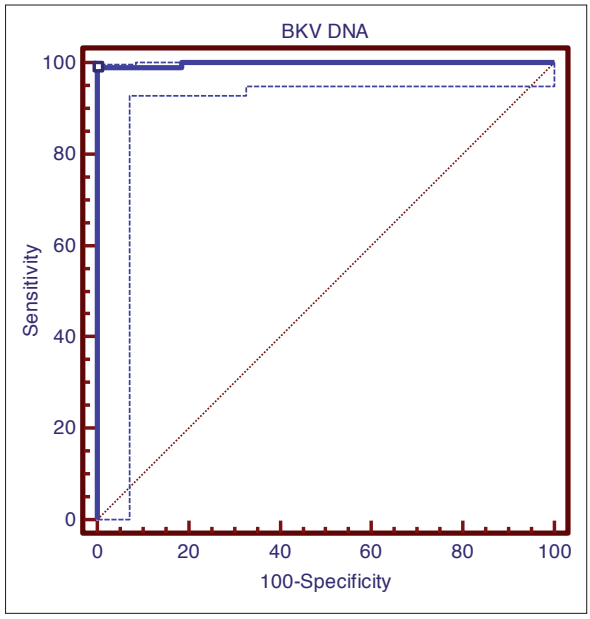

FIGURE 2 - *ROC curve for diagnosis of BKVAN and plasma BKV levels. A cut-off of 4.1 log_{10} copies/mL was used.*

with BKV viremia (26). In addition 6 out of 7 (86%) patients had a BKV viral load $>10^4$ copies/mL. Thirteen out of the 49 (26.5%) patients negative for viremia were positive for urinary cytology (CU). All 26 patients with positive viremia were also positive for CU.

In these patients, we also investigated viruria: a persistent and constant viruria was present during the period of follow-up (range 10^4->10^6 copies/mL); in BKVAN patients viruria resulted higher than $10x10^6$ copies/mL (Table 1).

Quantification of viral load in the 170 sample specimens showed a variability in viremia from 10^3 to 10^6 copies/mL. We divided the samples into 3 groups on the basis of BKV viral load in blood. Group 1 included 20 plasma samples with viral load <1000 copies/mL (median 632, range 336-962 copies/mL) derived from 4 renal transplants; in group 2 there were 53 samples with viral load between 1000-10000 copies/mL (median 3783, range 1012-9680 copies/mL) obtained from 5 patients and the last group included 97 samples with viral load >10000 copies/mL (median $41.4x10^4$, range $10.1x10^3$-$3.4x10^6$copies/mL) from 17 kidney-recipients (Table 2).

BKVAN was diagnosed by kidney biopsy in 7 (26.9 %) of the 26 viremic patients; 6 (87.5%) patients had a viral load $>3.5x10^4$ copies/mL and one patient had a viral load between 1000 and 10000 copies/mL (Figure 1). The area under ROC curve was 0.95 (95% CI: 0.975-1.00) and using a threshold value of 4.1 log_{10} copies/mL yielded a sensitivity of 94.9% (95% CI: 94.5-99.8%) and a specificity of 100% (95% CI: 94.8-100%) for BKVAN screening (Figure 2).

Median time for onset of viremia was 6 months (range: 2-25) post transplantation. All 7 BKVAN patients exhibited some degree of renal dysfunction and mean creatinine level at diagnosis was 2.1 mg/dL (range: 1.6-2.7), remaining stable during follow-up. They showed a reduction in viral

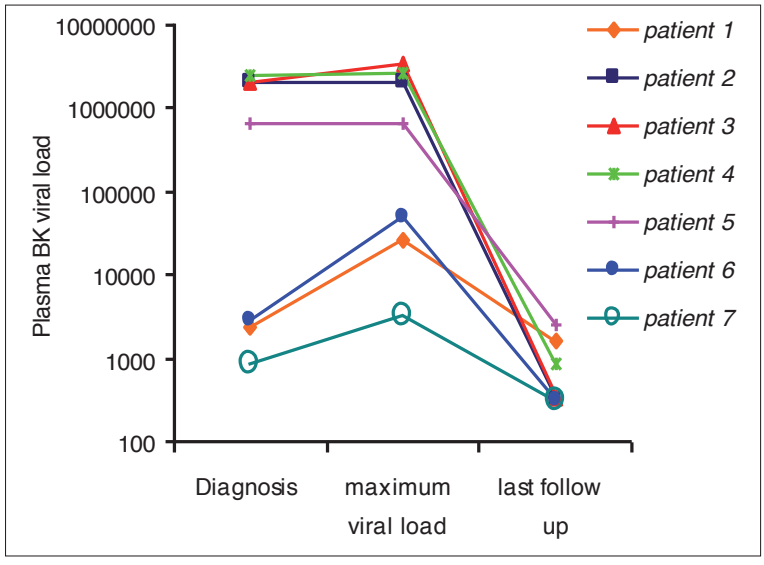

FIGURE 3 - *Monitoring of BK viral load in 7 patients with BKVAN.*

load during the follow-up period, ranging from 1 log to more than 3.8 log (Figure 3).
Induction therapy in these patients consisted of alemtuzumab (4 patients) and basiliximab (3 patients); at diagnosis all were treated with antiviral therapy (cidofovir and leflunomide) and reduction of immunosuppression therapy. Five patients were treated with FK and Sir and continued with Sir during follow-up, 1 patient received FK and Sir and then MMF. Lastly, 1 patient was treated with steroids (St) and MMF/FK and in follow-up with FK and St. All patients were receiving leflunomide; cidofovir was added in 3 patients. Two patients were treated with leflunomide for 6 months after diagnosis and both had stable creatinine levels at the end of the study. None of them suffered a graft loss.

DISCUSSION

The efficient early diagnosis of BKVAN, with subsequent decrease in immunosuppressive therapy leads to stabilization of nephropathy, improvement in renal function and resolution of the infection with a successful outcome (Drachenberg *et al.*, 2004).
Therefore, measurement of BK viral load in plasma of kidney transplant recipients proved a valid tool to identify patients at risk of disease and to monitor the efficacy of pharmacological therapy (Koukoulaki *et al.*, 2009).
The relationship between BK viremia, its cut-off and predictive value for progression of nephropathy, is still widely studied (Hirsch *et al.*, 2000; Limaye *et al.,* 2001; Nickeleit *et al.*, 2003; Cavallo *et al.*, 2009). The present study describes the results of BKV replication monitoring in renal transplant patients during 33 months post-transplantation to underline the effectiveness of laboratory markers for prediction of the development of BKVAN.
During the follow-up period, we monitored viremia; BKV was detected in 170/338 (50.3%) of plasma samples of 26 patients (34.7%). BKVAN was diagnosed by histopathological examination in 7 (7/26, 26.9%) viremic patients. We found that 4/7 BKVAN cases were diagnosed within 6 months after transplantation, whereas another 3 were diagnosed after 10 months. BKVAN has been reported to occur from 2 to 60 months post transplantation (Trofe *et al.*, 2004). The mean creatinine level at time of diagnosis is similar to what others have reported in literature (median value: 2.1 mg/dL).
Serial samples from these patients demonstrated a high viral load in plasma >10000 copies/mL which decreased from 1 to 3.8 log during follow up, as found in many papers (Costa *et al.*, 2008; Bressollette-Bodin *et al.*, 2005); so high viremia should be considered a predictive marker for BKV associated nephropathy. In our study, only one patient had a viral load <10000 copies/mL and developed BKVAN.
As other authors suggest, BK viral load in blood correlates with the immunosuppression status of a patient more than with the kind of drug used (Brennan *et al.*, 2005).
None of the patients with BKV-associated nephropathy showed any sign of graft failure and renal function was kept stable during the follow-up period by reducing the level of immunosuppression and by use of leflunomide.
The recipients with BK positive viremia during the first 6 months post transplantation showed good renal graft function and did not present progressive deterioration or damage of renal tissue; while those who had positive viremia detected after 10 months post transplantation, showed early renal dysfunction, which stabilized after reduction of immunosuppressive therapy during follow-up.
A recent study substains that BK viremia is not infrequent after kidney transplantation, but often associated with a nephropathy that improves with reduction of immunosuppressive therapy (Basse *et al.*, 2007).
Therefore, serial determination of BK viremia is the best tool to predict the evolution of disease during follow-up and to allow a reduction of immunosuppressve regimens when necessary (Koukoulaki *et al.*, 2009; Costa *et al.*, 2008). Correct setting of cut-off levels of BK viremia to predict a BKVAN becomes an essential requirement for clinical management of renal transplant patients, as suggested by many authors (Hirsch *et al.*, 2005; Costa *et al.*, 2008; Viscount *et al.*, 2007; Pang *et al.*, 2007) who consider BK viremia $>10^4$ copies/mL the most reliable value. We found that use of cut-off of 4.1 $\log_{10}$ copies/mL of plasma improved the specificity around to 100%. Since renal biopsy is highly invasive, accurate quan-

tification of BKV viral loads in plasma by routine monitoring to identify recipients at risk, could be the best strategy for early clinical intervention and efficient treatment, with benefits regarding damage progression and prevention of deterioration of renal function.

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