

Polyomavirus-associated nephropathy: critical issues in virological monitoring

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SUMMARY

Polyomavirus-associated nephropathy is one of the most common viral complications in renal transplant recipients. Viral replication is the single common feature of all patients at risk of nephropathy.

Therefore, screening for virus replication allows for the identification of patients at risk of developing nephropathy and permits earlier intervention, in particular a pre-emptive reduction of immunosuppression, with improvement of outcome.

This paper describes the modes of virological monitoring and its role in the clinical management of renal transplant patients.

KEY WORDS: BKV, JCV, SV40, Polyomavirus-associated nephropathy, Renal transplantation

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Polyomavirus-associated nephropathy (PVAN) is an emerging disease affecting renal transplant patients and is currently one of the most common viral complications in this context. Since its first description in 1995, PVAN has increased in prevalence from 1% to 10% (Hirsch *et al.*, 2006): this could be due to the introduction of new deeply immunosuppressive drugs and/or the relative decline in acute rejection rates. PVAN may lead to kidney graft loss in 10% up to 100% of the cases, with return to hemodialysis within 6-60 months, thus markedly and significantly decreasing the graft survival rate (Hariharan 2006). As most of the problems associated with these findings are due to the limited information regarding pathogenesis, uncertainty about diagnostic procedures, and paucity of effective interventions, management strategies have to focus on close monitoring

and early diagnosis, thereby prompting adjustment of the immunosuppressive regimen and use of effective antiviral agents, thus requiring a multidisciplinary approach.

Polyomaviruses BK, JC and SV40

Polyomaviruses BK, JC and SV40 are members of the *Polyomaviridae* Family that is constituted by 40-45 nm non-enveloped virions with a supercoiled circular dsDNA genome of about 5,000 base pairs. The genome homology between the three viruses ranges from 68% to 72% and the genome is divided into three regions (early, late, and non-coding-control region - NCCR). BKV and JCV are specific for the human host, with which they have coevolved, and are independently transmitted from one another. BKV and JCV are highly seroprevalent (rate ranging from 70% to 90% and approximately 30% in adults, respectively). Primary infection usually occurs early in childhood, at a median age of five years, and is characterized by low upper respiratory tract morbidity or is asymptomatic. Following primary infection, BKV and JCV remain latent in the renourinary tract, as the epidemiologically most important site, B cells, brain, spleen, and probably oth-

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er organs. Asymptomatic reactivation with shedding into the urine may occur in both immunocompetent subjects (in 0% up to 62% [Hirsch and Steiger, 2003]) and immunocompromised patients. Hirsch (2005) has proposed the use of the following definitions to describe the different types of interactions between BKV and the human host:

- BKV infection: case with serological or virological evidence of virus exposure, including replicative and nonreplicative states;
- BKV replication: case with evidence of virus multiplication (active or lytic infection) obtained by detection of infectious virus, virions, structural proteins or their mRNA, or cell-free DNA in nonlatency sites (e.g., plasma or cerebrospinal fluid); by cytological analysis (of decoy cells in urine); or by histological analysis;
- BKV disease: case with histological evidence of BKV-mediated organ pathology.

In renal transplant recipients, viral replication may cause PVAN consisting in interstitial nephritis and/or ureteral stenosis. The polyomavirus SV40 naturally infects some species of macaques and may cause nephropathy in these primates in immunocompromised conditions, such as infection with simian immunodeficiency virus. SV40 was inadvertently introduced as a pathogen into the human population as a contaminant of early polio vaccines, both Salk inactivated and Sabin oral. It has been estimated that up to 150,000,000 persons worldwide (both children and adults) were exposed to infectious SV40 between 1955 and 1963 (Butel and Lednicky, 1999). However, Cutrone *et al.* (2005) evidenced that some polio vaccines prepared by an Eastern European manufacturer were contaminated with SV40 and distributed in the former Union of Soviet Socialist Republic as late as 1978. Based on serological studies, the prevalence of SV40 infection in humans ranges from 3% to 10% (Martini *et al.*, 1996; Engels *et al.*, 2004; Lopez-Rios *et al.*, 2004; Poulin and DeCaprio, 2006).

Apart for polio vaccines, strong serological and molecular evidence suggests that new SV40 infections may be occurring in the human population although the route of transmission remains unknown. The pathogenic role of SV40 in humans is controversial. A recent study reported the risk of false positive results because of contamination by common laboratory plasmids contain-

ing SV40 sequences (Butel *et al.*, 1999a). Like other polyomaviruses, SV40 displays renotropism and is believed to persist latently in the kidney after primary infection.

Etiopathogenesis of PVAN

BKV is the most common etiologic agent of PVAN, while JCV has been recognized as responsible in less than 3% of all reported cases, alone (Kazory *et al.*, 2003; Wen *et al.*, 2004) or in association with BKV (Cavallo *et al.*, 2007). In a recent study (Drachenberg *et al.*, 2007), a biopsy-proven PVAN was diagnosed in six renal transplant recipients with exclusive JCV viruria out of 75 patients (8%) with BKV and/or JCV viruria, with an overall incidence during the study period of 0.9%. A role for SV40 in the etiology of PVAN has been recently suggested. Butel *et al.* (1999b) evidenced that SV40 seropositivity in children increased with age and was significantly associated with kidney transplantation. Moreover, co-infection with BKV and SV40 has been described in two out of six renal transplant patients with PVAN (Li *et al.*, 2002).

Based on the clinical setting, Hirsch has considered the pathogenic role of BKV as divided into four patterns: cytopathic, cytopathic-inflammatory, immune reconstitution and autoimmune pattern. This spectrum of pathogenic patterns has been suggested to depend on the reciprocal interaction between virus replication and immune response (Hirsch 2005). In particular, the cytopathic pattern is characterized by predominant polyomavirus replication and connotes PVAN histological pattern type A, while the cytopathic-inflammatory pattern is characterized by polyomavirus replication plus immune response and connotes PVAN histological pattern type B or ureteral stenosis. The immune reconstitution pattern has been proposed in the pathogenesis of hemorrhagic cystitis in bone marrow transplant recipients, while the autoimmune pattern has been hypothesized in relation to the development of systemic lupus erythematosus, although this requires further investigations (Sundsford *et al.*, 1999; Hirsch *et al.*, 2006; Colla *et al.*, 2007; Costa *et al.*, 2008).

History of PVAN

BKV was first isolated in 1971 from the urine of a renal transplant patient with ureteral stenosis (Gardner *et al.*, 1971). However, at that time, the

pathogenic role of BKV remained elusive and BKV was considered an orphan virus for many years afterwards.

In 1978, Mackenzie *et al.* (1978) first described four features of nephropathy in renal transplant: the detection of urine decoy cells, the presence of viral inclusions in uroepithelial cells in graft biopsies, the difficulties in differential diagnosis with acute rejection, and the role of immunosuppression in the development of these findings. In 1995, Purighalla *et al.* first described a case of PVAN and recognized it as a definite disease entity (Purighalla *et al.*, 1995). Subsequently, several reports followed with increasing prevalence rates from many transplant centres worldwide.

Risk factors for PVAN

It is now recognized that the development of PVAN requires the interaction of multiple risk factors. In all cases *condicio sine qua non* is the presence of intense immunosuppression. Particularly, but not exclusively, PVAN has been related to triple immunosuppressive therapy, including mycophenolate mofetil (MMF), tacrolimus (Tac), and steroids (Hirsch *et al.*, 2005). Although the level of immunosuppression rather than a specific drug seems involved in the onset of PVAN, this does not exclude a drug-specific mechanism promoting viral replication (Hirsch *et al.*, 2006). The prominent role of immunosuppression is also evidenced by the fact that reducing, switching or discontinuing immunosuppression represent the primary modes of intervention.

However, the preferential manifestation in renal allograft as compared to other allografts and to the autologous kidneys of other organ graft recipients suggests the role of other risk factors, including patient, allograft, and viral determinants. To summarize, the following factors are believed to contribute possibly to PVAN:

- immunosuppression: triple therapy (the antimetabolite MMF, the calcineurin inhibitor Tac, steroid), antirejection treatment (anti-lymphocyte preparations, i.v. steroid boluses);
- patient determinants: age >50 years, male sex, white ethnicity, pre-transplantation BKV seronegativity in children, interferon-gamma production by specific T cells, presence of comorbidities (diabetes mellitus, cytomegalovirus coinfection);

- allograft determinants: HLA mismatching, previous episode of acute rejection, latent infection load in the renourinary tract, presence of renal injury (including calcineurin-inhibitor toxicity);
- viral determinants: new BKV serotypes (VP1, mutations in domain), rearrangements in NC-CR with presumably increased viral fitness (Gosert *et al.*, 2008).

Clinical features of PVAN

PVAN is typically diagnosed within the first year posttransplantation, but about 25% of the cases are diagnosed later (range 1.3-45.1 months). Clinical presentation may be inconspicuous, thus being not useful for diagnosis. Varying degrees of allograft dysfunction may be seen, although in the early stages even normal serum creatinine levels may be detected.

PVAN may consist in interstitial nephritis and/or ureteral stenosis with ureteric obstruction, hydronephrosis, and sometimes associated urinary tract infections. Progressive renal failure has been reported in approximately 30-60% of cases (Hariharan 2006). Rare fatal disseminated BKV infection after cadaveric transplantation has also been reported (Petrogiannis-Haliois *et al.*, 2001).

Screening for polyomavirus replication

Viral replication is the single common feature of all renal transplant patients at risk of PVAN. Therefore, screening for virus replication will identify patients at risk of developing PVAN, thus permitting earlier intervention, in particular a pre-emptive reduction of immunosuppression (Ginevri *et al.*, 2007), with improvement of outcome.

This approach presents a high negative predictive value (>99%), as in the absence of virus replication PVAN is excluded (Mischitelli *et al.*, 2007). Screening for viral replication is also the most important tool for monitoring the response to treatment in patients with diagnosed PVAN. Different screening assays are available:

- 1) urine cytology, i.e. detection of decoy cells that are present in 40-60% of transplant recipients, although it is a good screening test with a negative predictive value (NPV) of 100%, positive predictive value (PPV) is very low (about 20%)(Hariharan 2006);
- 2) quantification of urinary BKV-DNA, with a

load 100-fold higher than plasma values evidenced in 30-40% of transplant recipients, that has a PPV of approximately 40% (Hariharan 2006);

- 3) quantification of plasma BKV-DNA, with a viral load $>10^4$ copies/mL recommended for a presumed diagnosis of PVAN (Hirsch *et al.*, 2005; Marchetti *et al.*, 2007; Smith *et al.*, 2007);
- 4) quantification of urinary VP1 mRNA that is likely to mirror active viral replication.

As regards plasma BKV-DNA quantification, two recent studies (Viscount *et al.*, 2007; Costa *et al.*, 2008) reported that by adopting a cut-off of 1.6×10^4 copies/mL the following operating characteristics for the presumed diagnosis of PVAN were achieved: sensitivity 100%, specificity 99.6%, PPV 75%, and NPV 100%.

As regards VP1 mRNA quantification, Ding *et al.* (2002) first proposed its use as a tool for noninvasive diagnosis of PVAN adopting a cut-off of 6.5×10^5 copy number/nanogram of total RNA. It is noteworthy that the relevance of these data, as pointed out by Hirsch (Hirsch 2003), is dependent on the purity of the RNA preparation before reverse-transcription to cDNA. In fact, contamination by the encoding BKV genomic DNA could yield falsely high results, and to estimate the degree of viral genomic VP1 DNA contaminating the VP1 cDNA preparation, VP1 should be quantified after prior DNase digestion. More recently, the same authors considered BKV replication positive subjects those renal transplant recipients with urinary cell VP1 mRNA copy number greater than 1 copy/1 pg of total RNA (Dadhania

et al., 2008). In a study we are conducting on VP1 mRNA quantification in urine specimens from kidney transplant recipients, we have developed a method comprising a first extraction step followed by DNase digestion and subsequently a second extraction step, and normalization of results based on the number of urinary cells (by evaluating the housekeeping gene GAPDH copy number). Considering preliminary data we have found by using this method and adopting an arbitrary cut-off level of 10^3 copies/ 10^3 cells, VP1 mRNA quantification does not exhibit operating characteristics superior to those obtained by monitoring viremia, in particular while negative predictive value was 100%, positive predictive value resulted $<20\%$. However these findings should be more properly addressed on a large population of kidney transplant recipients. Given a limited experience of monitoring response to treatment (both reduction/switching of immunosuppressive treatment and antiviral treatment) based on VP1 mRNA, we found that this appeared to be more closely and temporarily related to viral replication than viremia, thus allowing for a prompt adjustment of therapy (Sessa *et al.*, 2008). Screening assays present some drawbacks: no study has compared the different screening methods; urine cytology and urinary VP1 mRNA assays are susceptible to preanalytic hazards due to the type and duration of processing; viruria may differ depending on the type of specimen (supernatant, cell pellet, resuspended urine), micturition intervals and fluctuations of urine content may contribute to intercurrent variations; inhibition of

TABLE 1 - Virological monitoring of BK virus replication in renal transplant recipients.

Test	Notes
Urine BKV-DNA	100% negative predictive value, low positive predictive value
Plasma BKV-DNA	100% negative predictive value, 75% positive predictive value by using a cut-off level of 1.6×10^4 copies/ml
VP1-mRNA	100% negative predictive value, very low positive predictive value; potentially useful for monitoring response to treatment; further studies needed
Tissue BKV-DNA	$>10^3$ copies/cell in renal specimens with PVAN

Timing:

Twice monthly in the first 3 months posttransplantation. Three-month intervals in the first two years posttransplantation. Confirm positivity of replication screening within 2-4 weeks; consider renal biopsy in the presence of renal dysfunction (increased serum creatinine level).

PCR in urine (e.g. urea concentration) for viruria (amplification controls). According to recommendations of an international panel of experts (Hirsch *et al.*, 2005), other studies (Giraldi *et al.*, 2007), and our Renal Transplant Unit practice (Table 1), to balance cost and efficiency, renal transplant patients should be screened twice monthly in the first three months posttransplantation, thereafter every three months during the first two years and then yearly until the 5th year. Due to the possibility of self-limiting (transient) replication, positive screening assays should be confirmed within 2-4 weeks. The persistence of an elevated plasma viral load for >3 weeks is highly suggestive of PVAN (presumed diagnosis of PVAN) and should prompt an evaluation with renal biopsy. A definitive diagnosis of PVAN is made by the demonstration of typical viral cytopathic findings at histopathology. However, given the focal involvement of early PVAN and the possibility of tissue sampling errors, clinical management often is based on the surrogate markers of viral replication. On the other hand, the requirement of tissue evaluation in patients who are suspected to have PVAN remains, also taking into account that a renal biopsy is necessary to exclude other pathologic processes, such as acute rejection that may coexist (Drachenberg *et al.*, 2006). In this context, considering that viral inclusions may be absent in the early stages of PVAN, inflammation may be scarce (Schmid *et al.*, 2005) and the focal nature of renal involvement, the quantification of polyomavirus DNA on renal graft biopsies and/or ureteral specimens could also be taken into account besides histopathological evaluation. Polyomavirus-DNA quantitation could be useful in the presence of little evidence of viral cytopathy (Schmid *et al.*, 2005). A study by Randhawa *et al.* (2005) found a mean BKV load of 7738.9 DNA copies/cell in renal allograft biopsies with active PVAN, while it was 185.8 and 28.8 in pre-PVAN renal biopsies and in specimens from patients with asymptomatic BKV viruria, respectively. In a study we performed on kidney and ureter specimens from renal transplant recipients by quantitative Real Time TaqMan PCR, BKV load in allograft biopsies with histologically confirmed PVAN was $>10^4$ Geq/cell, while it ranged between 0.3 and 13.7 in patients with asymptomatic BK viruria (data not shown) and was <1 Geq/cell in the patient with pre-PVAN

(Costa *et al.*, 2009). Although, like most studies investigating PVAN, our study was limited by the low number of affected patients, data obtained from our group and those by Randhawa *et al.* indicate that the highest BKV loads (in both the studies $>10^3$ copies/cell) are found in renal specimens from patients with active PVAN. The evidence that renal tubules accumulate thousands of viral particles before undergoing cell lysis underlines the role of cytopathic effect in renal allograft injury in PVAN (Randhawa *et al.*, 2002). Because of focal involvement of PVAN, especially during the early stage of infection, the sensitivity of histopathology and tissue PCR could be limited. Moreover, this could affect the degree of viral load; it has been suggested that BKV load is better measured in urine than in tissue, because urine represents material from the entire kidney (Randhawa *et al.*, 2005). However, as some glomeruli are physiologically shut off, it seems possible that the affected inflamed tubuli may be underrepresented in the urine flow, but overrepresented in the blood. Therefore, although quantitation of tissue viral DNA could be complementary to histopathological evaluation and the highest viral loads are detectable in renal specimens with PVAN, i.e. $>10^3$ copies/cell, the identification of a diagnostic cut-off should require further studies, also taking into account the potential sampling errors and the evaluation of surrogate markers of viral replication (Hirsch *et al.*, 2005).

To monitor the course of PVAN, quantitative evaluation of viral load in plasma and urine should be performed every 2-4 weeks. The relevance of early identification of the PVAN development emphasizes the importance of further validating threshold values, as well as standardisation of quantitative PCR methods. The definitive diagnosis of PVAN is made by demonstrating polyomavirus-induced cytopathic changes in tubular or glomerular epithelial cells. Typical histopathologic changes may be confirmed by immunohistochemistry with antibodies specific for the large T-antigen (anti-SV40, which is cross-reactive among BK, JC, and SV40).

Sensitivity and specificity of the histological diagnosis of PVAN is complicated by the focality of renal involvement, particularly early in the disease; associated changes, in particular inflammatory infiltrates, difficult to differentiate from

acute rejection; and pronounced tubular atrophy and fibrosis of late stages where only few viral cytopathic changes are seen (possible false negative result). The limited sensitivity of allograft biopsy is also critical for the definition of "resolved PVAN" as the goal of any intervention. Resolution of PVAN not only requires the disappearance of the histological signs of active disease (e.g., viral replication, inclusions, necrosis, inflammatory infiltrates) and negative immunohistochemistry, but should also include negative results of the surrogate replication markers such as BKV viremia and viruria.

Treatment of PVAN

The treatment of PVAN has three fundamental objectives: to eliminate the virus, to avoid the development of acute rejection, and to preserve the renal function. At moment, there is no approved treatment for PVAN. The primary mode of intervention consists of reducing immunosuppression. This can be accomplished by three different approaches: i.e. reducing, stopping or switching the immunosuppressive drug employed. No randomized controlled study has compared different protocols and an individualized approach to a single patient is usually considered, thus implying close monitoring of viral replication and renal function (Hirsch *et al.*, 2005; Hariharan 2006). Reduction of immunosuppression may prove insufficient to control viral replication or may not be appropriate in patients at high risk of rejection. There is no selective antiviral target and no randomized controlled study on antiviral agents has been performed.

To date, a switch to leflunomide and the antiviral agent cidofovir appears to be the most promising therapeutic approach (combined with reduced immunosuppression), although the mode and timing of administration remain to be defined (Hilton and Tong, 2008; Lamoth *et al.*, 2008; Wu and Harris, 2008; Kuypers *et al.*, 2009; Wu *et al.*, 2009).

Leflunomide is an antiinflammatory drug approved for the treatment of rheumatoid arthritis. Leflunomide has evidenced immunosuppressive potency in solid organ transplant recipients and antiviral activity against human cytomegalovirus, herpesvirus and BKV *in vitro* and in experimental animals, thus combining immunosuppressive and antiviral actions.

Cidofovir is a phosphonate purine analogue of cytosine that potently inhibits viral DNA polymerase, and displays a broad spectrum activity against herpesviruses, papillomaviruses and poxviruses. However, polyomaviruses do not encode a DNA polymerase, thus other mechanisms that are not fully understood should be involved in its effect. Doses, duration and timing of administration and selection of patients are not defined and further studies are needed to define specific therapeutic protocols.

The prevention of PVAN by regular monitoring of viremia/viruria remains the most appropriate approach and may prompt a pre-emptive reduction of immunosuppression.

As regards retransplantation in patients who have lost their kidney graft for PVAN, pre-emptive retransplantation experience is quite limited (Hirsch and Ramos, 2006; Womer *et al.*, 2006; Mindlova *et al.*, 2008) and many questions remain unanswered, such as whether or not adopt the same immunosuppressive drugs and combinations. Recommendations for screening and treatment of polyomavirus are the same as for patients with a first renal allograft.

Immunity to BKV

Another important factor to consider in the evaluation of renal transplant recipients at risk of PVAN is the immune response. As regards humoral immune response, seropositive recipients are not protected against BKV replication and PVAN (Hirsch *et al.*, 2002). In studies of pediatric patients, recipient seronegativity for BKV was associated with an increased risk of viral replication and nephropathy (Ginevri *et al.*, 2003; Smith *et al.*, 2004; Schaub *et al.*, 2007). Although IgG titer significantly increases in patients with decreasing viremia values and after resolution of PVAN (Comoli *et al.*, 2004; Chen *et al.*, 2006; Schaub *et al.*, 2007), subjects with elevated IgG levels can still develop PVAN, thus suggesting a role of defective cellular immunity in the onset of nephropathy. Polyomavirus BK-seropositive healthy subjects present CD4+ and CD8+ T-cells specific for BKV large T antigen and capsid protein VP1 (Zhou *et al.*, 2007). In renal transplant patients, a unfavourable balance between viral replication and cellular immunity is a common denominator in PVAN pathogenesis (Comoli *et al.*, 2004; Binggeli *et al.*, 2007). In transplant pa-

tients with PVAN a defective cellular immunity has been identified using Enzyme-Linked Immuno SPOT (ELISPOT) (Comoli *et al.*, 2004). Recent studies are focusing on the evaluation of the relation between specific cellular immunity and BKV replication for early prediction of possible PVAN development and to monitor its clinical course and response to therapy. This evaluation could be of great importance and further studies are needed to define protocols and guidelines for immunological monitoring of BKV-specific response.

CONCLUSION

PVAN is a major problem in renal transplantation and emphasizes the impact of immunosuppression in this context in which patient, organ and virus specific determinants closely interact. Screening of viral replication in renal transplant patients is warranted to enable early diagnosis of "possible", "presumptive" or "definitive" PVAN as well as "resolved" PVAN after appropriate intervention.

The detection of a plasma BKV DNA load above "alert" thresholds (such as 1.6×10^4 copies/ml) should trigger closer monitoring of viral replication and, in the presence of renal dysfunction, allograft biopsy to confirm PVAN and exclude acute rejection. Antivirals or immunosuppressants with antiviral activity are promising for the treatment of PVAN and their use should be evaluated in large controlled studies to define protocols and guidelines. Many open questions remain to be answered, such as the pathogenic mechanisms, the role of JCV and SV40, the most reliable tools and thresholds for early diagnosis (operating characteristics and predicting values), the strategies for a pre-emptive treatment, and management after graft loss.

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