UNIVERSITÀ DEGLI STUDI DI TORINO

POLYOMAVIRUSES BK- AND JC-DNA QUANTITATION
IN KIDNEY ALLOGRAFT BIOPSIES

Abbreviations: Ct, threshold cycle; GAPDH**, glyceraldehyde-3-phosphate-dehydrogenase;** Geq, genome equivalents; IHC, immunohistochemistry; PVAN, polyomavirus-associated nephropathy.

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Abstract

Background. Polyomavirus-associated nephropathy (PVAN) is one of the most common viral disease affecting renal allograft, with BK being the most frequent causal agent and JCV being considered responsible in <3% of the cases.

Objectives. To quantify polyomaviruses BK and JC load by real-time TaqMan PCR in tissue specimens (renal and ureteral) from kidney transplant recipients.

Study design and Methods. One-hundred-thirty-eight specimens (125 kidneys, 13 ureters) obtained from 109 patients were evaluated by quantitative real-time PCR for the detection of BKV- and JCV-DNA. Demographic, virological, and histopathological data were collected.

Results. BKV-DNA was positive in 32 of 109 patients (29.6%) and JCV-DNA in 20 of 109 patients (18.3%). The highest BK viral loads ($>10^4$ genome equivalents/cell) were found in two renal samples with histopathologically confirmed PVAN; while JC viral load was $> 10^4$ genome equivalents/cell in one ureteral sample.

Conclusions. Although quantitation of viral DNA on renal allograft biopsies could be complementary to histopathological evaluation and the highest viral load are detectable in renal specimens with PVAN, the identification of a diagnostic cut-off should require further studies.

Keywords: kidney transplantation, BKV, JCV, renal biopsy, polyomavirus-associated nephropathy; ureteral stenosis

Introduction

BKV and JCV are members of the *Polyomaviridae* family with a genome homology of approximately 72%. Human polyomaviruses BK and JC are ubiquitous and following primary infection remain latent in the renourinary tract and B cells. Asymptomatic viruria may occur in both immunocompetent subjects and immunocompromised patients, while in transplanted kidney viral replication may determine polyomavirus-associated nephropathy (PVAN) in 1-10% of the patients, leading to graft failure in 30 up to 80% of the cases (Hirsch et al., 2005; Hirsch et al., 2006). Today, PVAN is one of the most common viral disease affecting renal allografts, with BKV being the most frequent causal agent and JCV being responsible in less than 3% of the cases (Kazory et al., 2003; Wen et al., 2004; Cavallo et al., 2007). However, 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%. The definitive diagnosis of PVAN is made on the basis of histopathology, although this presents some drawbacks, including being invasive and limited sensitivity due to (multi)focal involvement. We present here results of quantitation of polyomaviruses BK and JC by Real-time TaqMan PCR in renal and ureteral specimens from renal transplant recipients.

Material and Methods

All 109 renal transplant recipients who underwent at least one graft biopsy for clinical and/or laboratory suspicion of rejection, PVAN, or other causes, in a 18-month period were evaluated. In 13 patients ureteral specimens, obtained during pyelostomy performed for ureteral stenosis, were studied (for three of these patients a renal sample was also available). Overall, 138 clinical samples were evaluated. Relevant clinical and laboratory data were abstracted from clinical charts and kidney transplant database. Informed consent was obtained from all the patients. BK viral load on concomitant serum and urine samples were available for 55 patients. Extraction from serum and urine samples was performed as previously described (Bergallo et al., 2006). Tissue specimens were formalin-fixed and paraffin-embedded; for DNA extraction, two-to-four sections (thickness, 4 μ) were incubated with 400 μL of lysis buffer (400 mM Tris-HCl 400 pH 7.5; 500 mM NaCl; 50 mM EDTA; 1% SDS) by vortexing for 30 sec, boiled for 10 min, vortexed for 30 sec, boiled for 10 min and vortexed again for 30 sec, then centrifuged for 1 min at 13.000 rpm at room temperature. Three hundred μL of the supernatant were mixed with 750 μL of absolute ethanol and 30 μL of 3 M sodium acetate pH 5. After centrifugation for 5 min at 13.000 rpm at room temperature, the pellet was washed with 70% ethanol, air-dried for at least 30 min, resuspended in 50 μL of double-distilled H_2O, and stored at -20°C prior to use.

Tissue samples were tested for BKV- and JCV-DNA by Real-time quantitative TaqMan PCRs targeting viral genes encoding for the large T-antigens of each virus with the 7300 Real Time PCR System (Applied Biosystems, Monza, Italy). For BKV-DNA a commercial kit was used (Q-BKV, Nanogen Advanced Diagnostics, Milano, Italy), following the manufacturer's instruction, while JCV-DNA was tested as previously described (McNees et al., 2005). Details are reported in Table 1. Amplifications were set up in a reaction volume of 25 μL, including 5 μL of extracted sample or plasmid dilutions. No template control (sterile double-distilled H_2O) was included in each PCR run. Standard curves for BKV- and JCV-DNA quantification were constructed by plotting the threshold cycle (Ct) against the logarithm of serial 10-fold dilutions (ranging from 10^2 to 10^5) of pBKV provided with the Real-Time kit and a plasmid containing full-lenght JCV-DNA (courtesy of T. Musso), respectively. The plasmid DNA concentration was determined by measuring the optical density at 260 nm. The DNA content in micrograms was converted to genomic copies using Avogadro's number (6.023×10^{23}) and the number of nucleotide pairs in the plasmid, and the average molecular weight of a nucleotide pair was assumed to be 660 μg. All measurements were performed in duplicate. To correct for the

variable amount of DNA in different tissue samples, each sample was subjected to simultaneous TaqMan PCR for the housekeeping gene Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, Accession No. J04038), targeting the region between exon 6 and 8; primers and probe were designed using the Primer Express software (version 3.0, Applied Biosystems;Table 1). Results were considered acceptable only in the presence of GAPDH-positivity. Standard curves for the GAPDH gene quantitation were constructed by plotting the Ct against the logarithm of serial dilutions of DNA extracted from peripheral blood leukocytes. The Ct values and number of BKV-DNA copies, JCV-DNA copies, and diploid sets of GAPDH-gene were calculated from the standard curve. The assay was linear in the range 10^2-10^5 copies per reaction. In case of results above the linear range serial 10-fold dilutions of extracted samples were made and quantification was obtained considering the mean of the results within the linear range. A "nucleotide-nucleotide blast" search for short nucleotide sequences performed at the National Center for Biotechnology Information and the National Library of Medicine web site confirmed that the primer pairs used for JCV amplify only 92 clinical isolates of JCV and should not amplify other viruses pathogenic to humans. Amplification data were analyzed by the Sequence Detection System software (Applied Biosystems).

For histopathologic evaluation periodic acid-Schiff, Masson's trichrome, phosphotungstic acid hemotoxylin, and acid fucsin-orange G stains were performed. In case of suspected PVAN, immunohistochemistry (IHC) with immunoperoxidase staining was performed on fixed material using polyclonal anti-SV40 antibody (dilution 1:20,000; Lee Biomolecular Research Labs. San Diego CA), cross-reacting with BKV and JCV.

Statistical analysis was performed using the chi square test with a commercially available software (MedCalc; version 9.2.1.0). A p value <0.05 was considered significant.

Results

Results are summarized in Table 2. Real-time PCR for BKV-DNA was positive in 37/138 transplant biopsies (26.8%) from 32/109 patients (29.6%): 30/124 (24.2%) renal and 7/14 (50%) ureteral samples, from 26/96 (27.9%) and 6/13 (46.2%) patients, respectively (for two patients BKV-positivity was concordant on renal and ureteral specimens). The positivity rate did not significantly differ between renal and ureteral samples. Concordant negative and positive results were found in all the cases, thus the highest load was considered in each patient. Median BKV viral load was 125 Geq/10^4 cells (range 40-1023067263; 25th percentile 40, 75th percentile 361); tissue viral load was $>75^{th}$ percentile in ten samples and $>90^{th}$ percentile (i.e. 66668.8) in five: 137126 in a renal specimen with post-transplantation glomerulopathy, 36448 and 601057 in two ureteral samples from a patient in whom renal specimen resulted negative to BKV and JCV, 493047821 and 1023067263 in two patients with PVAN. Viral load was 2058 Geq/10^4 cells in a renal specimen from a patient in which PVAN was subsequently diagnosed (pre-PVAN). JCV-DNA was positive in 24/138 biopsies (17.4%) from 20/109 patients (18.3%): 21/124 (16.9%) renal and 3/14 (21.4%) ureteral specimens, from 17/96 (17.7%) and 3/13 (23.1%) patients, respectively, without differences between renal and ureteral specimens. Median JCV viral load was 40 Geq/10^4 cells (range 36-23060245; 25th percentile 40, 75th percentile 265); tissue viral load was $>75^{th}$ percentile in 7 specimens and $>90^{th}$ percentile (i.e. 1336) in three specimens: 1470 in a renal specimen with post-transplantation glomerulopathy, 2002 and 23060245 in two ureteral samples.

BKV and JCV were detected alone in 23 and 11 patients, respectively; both BKV-DNA and JCV-DNA were detectable in nine patients.

Demographic, virological, and histopathological data are summarized in Table 3.

Discussion

The presence of latent BKV in renal tissue from kidney allograft recipients is well documented, ranging from 2.6% (Schmid et al., 2005) up to 16.2% (Randhawa et al., 2005). In our study BKV was detected in 26.8% of tissue specimens: 24.2% of renal biopsies and 50.0% of ureteral samples. Although our data are higher than those previously reported, the possibility of plasmid contamination was ruled out as the control plasmids were not produced in our laboratory; moreover, plasmid contamination during sample processing would affect a large number of samples. Polyomaviruses are normally latent in the urothelium, thus DNA-positivity could be simply indicating latency, as well as we cannot exclude contamination from urinary shedding or that virus might be derived from a reservoir present in infiltrating B-cells or other inflammatory cell population. The detection of BK viral load >90th percentile on two ureteral specimens in the absence of positivity on renal biopsy of the same patient obtained two months later suggest a role for BK in the development of ureteral stenosis and a localized ureteral reactivation, this also taking into account the negativity of viremia. JCV-DNA has been demonstrated in up to 10% of autoptic kidneys (Randhawa et al., 2005) and in 17.4% of both renal and ureteral specimens from our patients; however, no case of JCV-PVAN was diagnosed, while a value of 2.3×10^7 Geq/10^4 cells was detected on a ureteral specimen from a case which had been already reported (Cavallo et al., 2007).

The histological diagnosis of PVAN can be challenging. While in intermediate/advanced stages florid cytopathic changes and inflammatory infiltrates represent the most typical patterns, in early stages viral inclusions may be absent, as well as inflammation may be scarce (Schmid et al., 2005). Considering this and the focal nature of renal involvement, polyomavirus-DNA quantitation could be useful in the presence of little evidence of viral cytopathy (Schmid et al., 2005). In a study by Randhawa and coll., a mean BKV load of 7738.9 DNA copies/cell was found 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 (Randhawa et al., 2005). In our study BKV load in allograft biopsies with histologically confirmed PVAN was >10^4 Geq/cell, while ranged between 0.3 and 13.7 in patient with asymptomatic BK viruria (data not shown) and was <1 Geq/cell in the patient with pre-PVAN. Although, like most studies investigating BKVAN, our study was limited by the low number of affected patients, data obtained from our group and those by Randhawa and coll. indicate that the highest BKV load (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). Considering pre-PVAN, giving the evaluation of only one case, our study failed to evidence a higher viral load in comparison to patients with asymptomatic viruria; alternatively this could be due to to the sampling interval, as specimen was evaluated 2.1 months before the diagnosis of PVAN was made.

Because of focal involvement of PVAN, especially during the early stage of infection, sensistivity 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.

In conclusion, although quantitation of tissue viral DNA could be complementary to histopathological evaluation and the highest viral load 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).

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Table 1. Technical details on Real Time PCR assays employed in this study. GAPDH, glyceraldehydes-3-phopshate dehydrogenase.

	BKV	JCV (modified from McNees et al., 2005)	GAPDH
Primers Probe	Q-BKV Real Time PCR kit (Nanogen Adv. Diagnostics)	F 5'-TTCTTCATGGCAAAACAGGTCTT-3' R 5'- GAATGGGAATCCTGGTGGAA -3'	F 5'-GCCAAAAGGGTCATCATCTC-3' R 5'-GGGGCCATCCACAGTCTTCT-3'
		FAM-5'-CCACTTCTCATTAAATG-3'-MGB	VIC-5'-TGGTATCGTGGAAGGA-3'-MGB
Cycling conditions	50°C 2 min 94°C 10 min 95°C 15 sec 60°C 1 min } 45 cycles		

Table 2. Results of polyomaviruses BK- and JC-DNA detection in renal and ureteral specimens from renal transplant recipients. Pts, patients; R, renal samples; U, ureteral samples; SD, standard deviation. *Patients with histologically confirmed PVAN.

Total Samples N =138 Pts N = 109	Samples N (%)	Pts N (%)	Viral load (Geq/10^4 cells)
BKV-DNA	37 (26.8%)	32 (29.6%)	Median 125 (range 40-1023067263; 25th percentile 40; 75th percentile 361) *Two PVAN, 4.93 x 10^8 and 1.02 x 10^9
	- 30/124 (24.2%) R	- 26/96 (27.9%) R	Median 40, mean±SD 4907.0±25494.4
	- 7/14 (50.0%) U	- 6/13 (46.2%) U	Median 1059, mean±SD 106626±242646.4
JCV-DNA	24 (17.4%)	20 (18.3%)	Median 40 (range 36-23060245; 25th percentile 40; 75th percentile 265)
	- 21/124 (16.9%) R	- 17/96 (17.7%) R	Median 40, mean±SD 216.4±401.2
	- 3/14 (21.4%) U	- 3/13 (23.1%) U	Median 1020, mean±SD 1020.0±1385.9

Table 3. Main features of renal transplant recipients and relation to polyomaviruses positivity.

Patients Total N = 109	BKV-DNA		JCV-DNA	
	POS.	NEG.	POS.	NEG.
M/F 66/43	15/17	51/26	11/9	55/34
Age groups				
- <40 yrs (11)	3	8	-	11
- 41-60 yrs (64)	16	48	20	44
- >60 yrs (34)	10	24	-	34
PVAN (2)	2	-	-	-
Ureteral stenosis (13)	6*	7	3*	10
Acute rejection (4)	2**	2	1**	3
Post-transplantation glomerulopathy (6)	4***	2	3	3

*Viral load, JCV 2.3 x 10^7 Geq/10^4 cells in 1 patient and <10^4 in the remainings

**Viral load <10^4 Geq/10^4 cells

***Viral load, <10^5 Geq/104 cells