Quantitation of DNA of Polyomaviruses BK and JC in Human Kidneys

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Background. Renal allograft recipients can be monitored for polyomavirus-associated nephropathy (PVAN) using urine samples. Because virus in urine can be derived from the kidneys, ureter, or urinary bladder, we evaluated whether measurement of intrarenal concentrations of viral DNA might serve as a more reliable monitoring tool.

Methods. Real-time quantitative polymerase chain reaction was used to quantitate DNA of polyomaviruses BK (BKV) and JC (JCV) in renal tissue obtained from various clinical settings.

Results. Renal biopsy samples from 28 nonimmunosuppressed patients contained very low viral copy numbers. Minimally higher BKV loads (mean ± SE, 3.4 ± 1.7 copies/cell) were observed in 74 renal biopsy samples from renal allograft recipients with BKV viruria. The BKV DNA concentration was ∼10-fold higher in renal allograft recipients with BKV viruria, but 58 (50.4%) of 115 renal biopsy samples tested negative for BKV DNA, reflecting the focal nature of infection. JCV DNA was found in only 2 renal biopsy samples.

Conclusions. The BKV load is better measured in urine than in tissue, because a urine sample represents material from the entire kidney. An increase in the BKV load is usually not accompanied by a proportional increase in the JCV load, which indicates that these 2 related polyomaviruses are subject to different mechanisms regulating viral replication.

Polyomavirus (PV) belongs to the family Polyomaviridae. Polyomaviruses BK (BKV) and JC (JCV) are the 2 species that primarily affect humans. BKV has a worldwide seroprevalence of 60%–90% [1–4]. Initial infection occurs in childhood probably via the respiratory tract, and this leads to viral latency in the urogenital tract. Viral reactivation characterized by active viruria occurs in 10%–60% of renal allograft recipients. BKV nephropathy with renal dysfunction was initially described in the setting of congenital immunodeficiency and AIDS [5, 6]. More recent studies have shown that BKV is responsible for PV-associated nephropathy (PVAN) in up to 8% of renal allograft recipients [7–13]. Typically, a diagnosis of PVAN is made when viral inclusions are found in renal tubular epithelial cells.

JCV was initially isolated from a patient with progressive multifocal leukoencephalopathy (PML). As is true of BKV infection, there is widespread serologic evidence of JCV infection in humans [14]. After primary infection, JCV becomes latent in the kidneys and B lymphocytes [15–17]. By use of Southern blotting, JCV DNA was found in 10% of kidneys obtained at autopsy [18]. JCV is excreted with significant frequency in the urine of elderly individuals [19–24]. Unlike the case with BKV viruria, some studies have failed to find a correlation between the frequency of JCV viruria and the degree of immunosuppression [25–27]. Nonetheless, PML occurs primarily in the setting of immune deficiency, particularly in patients with AIDS [14, 28, 29].

Although latency of both BKV and JCV in the renal parenchyma is well documented, limited information is available on how tissue viral load is affected by different disease processes affecting the kidneys. Reliable methods to quantitate viral DNA in human tissues have only recently become available, and published studies of PV infection have mostly focused on blood and urine...
PATIENTS, MATERIALS, AND METHODS

Protocols for sample collection and clinical data review were approved by the University of Pittsburgh Institutional Review Board. Patients were investigated for PV infection by polymerase chain reaction (PCR) assays performed on randomly collected urine samples, as described elsewhere [36]. Histopathologic examination of renal tissue samples was performed using standard techniques. The diagnosis of PVAN was based on the presence of viral inclusions in tubular epithelial cells and was confirmed by in situ hybridization using a commercially available probe (Enzo Diagnostics). The following set of renal tissue samples was collated for the purposes of this study:

(1) Biopsy samples of native kidneys were obtained from 28 patients with histologic diagnoses that included glomerulonephritis, diabetic nephropathy, hypertensive nephropathy, and interstitial nephritis.

(2) Renal tissue samples that had been removed at autopsy and frozen were obtained from 5 patients with HIV-associated nephropathy. These samples were procured through the AIDS and Cancer Specimen Resource at the National Cancer Institute.

(3) Renal biopsy samples were obtained from renal allograft recipients with no evidence of PV, BKV, or JCV infection \((n = 74\) samples from 60 patients), as indicated by results of PCR assays performed on urine samples. Histologic examination of the samples showed acute cellular rejection with varying degrees of underlying chronic allograft nephropathy.

(4) Renal biopsy samples were obtained from renal allograft recipients with JCV viruria \((n = 22\) samples from 11 patients) or BKV viruria \((n = 115\) samples from 43 patients), as indicated by results of PCR assays performed on urine samples. The male:female ratio of viruric patients was 2:1. The renal biopsy samples were negative for PV DNA by in situ hybridization. The histologic diagnoses included acute cellular rejection, chronic allograft nephropathy, calcineurin inhibitor toxicity, and donor disease.

(5) Renal biopsy samples were obtained from renal allograft recipients with active PVAN \((n = 68\) samples from 39 patients).

Renal biopsy samples obtained from the same patients either before the diagnosis of PVAN (pre-PVAN, \(n = 106\) samples from 36 patients) or after the resolution of PVAN (post-PVAN, \(n = 38\) samples from 26 patients) were also studied.

Renal tissue samples were subjected to routine formalin fixation and paraffin embedding. Quantitation of viral DNA in the tissue was performed using biopsy lysates prepared as described elsewhere [37]. DNA extraction was performed by use of the QIAamp Blood Mini Kit (Qiagen). The technical details of the BKV quantitative PCR assay used in this study have now been published elsewhere [37]. An essentially similar assay has now been developed to simultaneously quantitate JCV DNA in renal tissue.

The assay is based on the TaqMan platform and uses the Prism 7700 Sequence Detector (ABI). The following oligonucleotide sequences derived from the JCV VP1 gene were used: 5'-TCA ATG CAT GTC GCC TTT ACT TT-3' (JCFB forward primer), 5'-ACG GGG TCC TGC TTC CTT C-3' (JCRB reverse primer), and FAM-5'-AGG GTT GTA CGG GAC TGT AAC ACC TGC TC 3'-TAMRA (probe).

All real-time quantitative PCR analyses were conducted at the TaqMan Core Facility of the Genomics and Proteomics Core Laboratories of the University of Pittsburgh. PCR amplifications were set up in a reaction volume of 50 \(\mu\)L that contained the TaqMan Universal PCR Master Mix (PE Biosystems), 5 \(\mu\)L of tissue lysate, 300 nmol/L each forward and reverse primer, 200 nmol/L probe, 300 \(\mu\)mol/L dNTPs, 5 mmol/L magnesium, and 1.25 U of Taq Gold polymerase. Thermal cycling was begun with an initial denaturation step at 95°C for 12 min that was followed by 45 cycles at 95°C for 15 s (denaturation) and 60°C for 1 min (reannealing and extension).

Standard curves for the quantitation of JCV DNA were constructed by plotting the threshold cycle \((Ct)\) against the logarithm of serial 10-fold dilutions of a plasmid containing full-length JCV DNA (ATCC 45027). 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 \((6.023 \times 10^{23})\) and the number of nucleotide pairs in the plasmid, and the average molecular weight of a nucleotide pair was assumed to be 660 \(\mu\)g.

To correct for the variable amount of DNA in different renal tissue samples, each sample was subjected to simultaneous TaqMan PCR for a control gene for the enzyme aspartoacylase (ACY), as described elsewhere [37]. The PCR conditions for the gene for ACY were the same as those used for JCV DNA, and both reactions were run simultaneously on the same plate. Standard curves for the quantitation of the gene for ACY were constructed by plotting the Ct against the logarithm of doubling dilutions of DNA extracted from peripheral blood mononuclear cells (PBMCs) from a healthy human volunteer \((0.08–50\ ng of...
DNA/PCR equivalent to 12–7500 diploid sets of the gene for ACY). JCV DNA concentrations were normalized to ACY DNA content, only 1 diploid set of the gene for ACY was assumed to be present per cell, and a value of 6.6 pg was assigned to the DNA content of a human cell.

All renal tissue samples were tested in duplicate, their respective Ct values were determined, and the number of BKV DNA copies, JCV DNA copies, and diploid sets of the gene for ACY were calculated from the standard curve. To control for plate-to-plate variation in PCR efficiencies, standard curves for BKV DNA, JCV DNA, and the gene for ACY were constructed with serial dilutions of the same stock solutions, which were frozen at −70°C in small aliquots.

No template control lanes were included in PCR runs, randomly dispersed negative blanks were used in each PCR run, and all standard precautions designed to prevent contamination during PCR were followed. Reaction setup, TaqMan PCR analyses, plasmid preparation, and DNA extraction were performed in separate laboratories.

Real-time PCR amplification data were analyzed with software provided by the manufacturer of the sequence detector. Quantitative PCR data were imported into Microsoft Excel and sorted into different diagnostic categories. Comparisons between groups were performed using the Mann-Whitney rank sum test or 1-way analysis of variance (Dunn’s test). All statistical calculations were performed using commercially available software (SigmaStat 2.03 for Windows; Systat).

## RESULTS

The BKV assay used in this study has been described elsewhere [37]. Briefly, the assay has a detection limit of 1 BKV genome/cell and is linear in the range $1 \times 10^3$–$1 \times 10^5$ BKV DNA and 12–7575 diploid sets of the gene for ACY. The JCV assay had similar performance characteristics. The specificity of both assays was confirmed when no amplification was shown when up to $1 \times 10^7$ copies of BKV, JCV, or simian virus (SV) 40 DNA were added to the reaction mix. BKV, JCV, and SV40 DNA standards used in the specificity assays were derived from plasmids containing full-length BKV, JCV, or SV40 genomes obtained from the American Type Culture Collection (ATCC 45025, 45027, and 45019, respectively). A “nucleotide-nucleotide blast” search for short nucleotide sequences performed at a Web site maintained by the National Center for Biotechnology Information and the National Library of Medicine (available at: http://www.ncbi.nlm.nih.gov) confirmed that the primer pair used should not amplify BKV, JCV, or other viruses pathogenic to humans.

Native renal biopsy samples from 5 (17.9%) of 28 nonimmunosuppressed patients contained detectable BKV DNA. The maximum BKV load was 0.07 DNA copies/cell (mean ± SE, 0.007 ± 0.003 DNA copies/cell; median, 0 DNA copies/cell). Marginally higher BKV loads (mean ± SE, 3.4 ± 1.7 DNA copies/cell; median, 0 DNA copies/cell) were observed in 12 (16.2%) of 74 renal biopsy samples obtained from patients with detectable BKV or JCV viruria, but the difference was not statistically significant. The BKV load was 10-fold higher in renal biopsy samples from patients with asymptomatic BK viruria (mean ± SE, 28.8 ± 12.8 DNA copies/cell; median, 0.003 DNA copies/cell) or JCV viruria (mean ± SE, 26.2 ± 25.7 DNA copies/cell; median, 0 DNA copies/cell) ($P = .001$, renal biopsy samples from patients with BKV viruria vs. native renal biopsy samples). However, 16 (72.7%) of 22 renal biopsy samples from patients with JCV viruria and 58 (50.4%) of 115 renal biopsy samples from patients with BKV viruria showed no detectable BKV DNA. The highest BKV load (mean ± SE, 7738.9 ± 1580.4 DNA copies/cell; median, 1770 DNA copies/cell) were found in renal biopsy samples from patients with active PVAN. Elevated BKV loads were found even in pre-PVAN renal biopsy samples. This latter group of samples had a mean ± SE BKV load of 185.8 ± 110.4 DNA copies/cell (median, 0.0045 DNA copies/cell) (table 1). The reduction of immunosuppression, prompted by PVAN, led to the disappearance of histologic inclusions and a reduction of the BKV load in post-PVAN re-

### Table 1. BK polyomavirus (BKV) load in different clinical settings.

<table>
<thead>
<tr>
<th>Clinical setting</th>
<th>Sample, no. (%)</th>
<th>BKV load, DNA copies/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Positive</td>
</tr>
<tr>
<td>Native kidney</td>
<td>28</td>
<td>5 (17.8)</td>
</tr>
<tr>
<td>Allograft kidney</td>
<td>74</td>
<td>12 (16.2)</td>
</tr>
<tr>
<td>JCV viruria</td>
<td>22</td>
<td>6 (27.3)</td>
</tr>
<tr>
<td>BKV viruria</td>
<td>115</td>
<td>57 (49.6)</td>
</tr>
<tr>
<td>PVAN</td>
<td>68</td>
<td>67 (99.5)</td>
</tr>
<tr>
<td>Post-PVAN</td>
<td>38</td>
<td>29 (76.3)</td>
</tr>
<tr>
<td>Pre-PVAN</td>
<td>106</td>
<td>65 (61.3)</td>
</tr>
</tbody>
</table>

**NOTE.** $P < .05$ for nontransplanted (native) renal biopsy samples vs. renal biopsy samples from patients with BKV viruria, native renal biopsy samples vs. renal biopsy samples obtained after the resolution of polyomavirus-associated nephropathy (post-PVAN), native renal biopsy samples vs. renal biopsy samples from patients with PVAN, and post-PVAN renal biopsy samples vs. renal biopsy samples obtained before the diagnosis of PVAN (pre-PVAN). JCV, polyomavirus JC.
nal biopsy samples. The post-PVAN renal biopsy samples had a mean ± SE BKV load of 1128.6 ± 767.4 DNA copies/cell (median, 1.3 DNA copies/cell). However, the BKV loads in post-PVAN renal biopsy samples remained higher than those in pre-PVAN renal biopsy samples \( (P = .02) \). The BKV loads in post-PVAN renal biopsy samples were also significantly higher than those in native renal biopsy samples and renal biopsy samples from patients with chronic allograft nephropathy \( (P = .001) \). When only the BKV DNA–positive renal biopsy samples were analyzed, the mean BKV load in the native renal biopsy samples was significantly lower than that in stable renal allograft biopsy samples, renal biopsy samples from patients with JCV viruria, and pre-PVAN renal biopsy samples. In an attempt to seek correlations between BKV loads in urine and renal biopsy samples, we analyzed 15 urine samples collected within 2 weeks of the biopsy. In this limited set of samples, BKV loads in urine and renal biopsy samples tended to increase together (Pearson correlation coefficient, 0.8).

Quantitative PCR for JCV was performed on 5 native renal biopsy samples, 5 samples of renal tissue obtained at autopsy from patients with HIV-associated nephropathy and later frozen, 48 renal biopsy samples from patients with no viruria, 32 renal biopsy samples from patients with BKV viruria, 8 renal biopsy samples from patients with JCV viruria, 1 renal biopsy sample from a patient with active BKV-associated nephropathy, and 4 renal biopsy samples from patients with resolved BKV-associated nephropathy. JCV DNA could be detected in only 2 samples, 1 from a patient with chronic rejection (43.1 DNA copies/cell) and 1 from a patient with HIV-associated nephropathy (1 DNA copy/3000 cells). The gene for ACY could be detected in all renal tissue samples, which indicates that the lack of detectable JCV DNA was not due to suboptimal integrity of the samples. Furthermore, in urine samples obtained from patients, BKV DNA and JCV DNA were found in approximately equal frequency (20%–30%). Thus, our relative inability to amplify JCV DNA from renal tissue samples is not due to our choice of primers or inadequately optimized PCR conditions.

**DISCUSSION**

The occurrence of latent BKV in human renal tissue is well documented \[18, 38\]. Heritage et al. found BKV DNA sequences in 50% of renal tissue samples tested, but, although the virus appeared to be present in a nonintegrated state in both the cortex and the medulla, not all fragments of tissue obtained from the same organ tested positive for its presence \[38\]. The present study has extended these observations by providing quantitative estimates of BKV load in native kidneys in nonimmunosuppressed patients. The low mean copy number (0.007 DNA copies/cell) is consistent with the presence of virus in a latent stage. The focal nature of infection explains why BKV DNA was found in only 17.8% of renal tissue samples analyzed, even though most adults show serologic evidence for exposure to BKV.

In renal biopsy samples from patients with no evidence of viruria, as indicated by results of PCR assays performed on urine samples, the proportion that tested positive for BKV DNA was similar to that observed in native kidneys. The mean BKV load (3.4 DNA copies/cell) was marginally higher, although this difference did not reach statistical significance. The detection of BKV DNA in this setting is of uncertain clinical significance. Long-term follow-up is needed to determine if the low levels of BKV DNA present contribute to the development of chronic allograft nephropathy, as has been suggested by some investigators \[39\].

Renal biopsy samples obtained from patients with BKV viruria demonstrated an \( \sim 1 \log \) increase in BKV load (mean, 28.8 DNA copies/cell), compared with that in renal biopsy samples obtained from patients without viruria. This is consistent with the hypothesis that the increased BKV load in the urine from these patients is secondary to interrenal BKV replication. The proportion of renal tissue samples positive for BKV DNA also increased from 17.9% to 49.6%. A likely explanation for the failure to detect BKV DNA in the remaining renal tissue samples is the focal nature of infection in the renal parenchyma. It is also worth emphasizing that the renal tissue samples studied by us were composed predominantly of renal cortex, but BKV might have greater predilection for replication in the renal medulla.

As was expected, the highest BKV loads were found in renal biopsy samples obtained from patients with active PVAN (mean, 7738.9 DNA copies/cell). Thus, in these patients, renal tubules can accumulate thousands of viral particles before they undergo cell lysis. These findings reinforce our present notion that viral cytopathic effect plays a prominent part in mediating renal allograft injury in PVAN \[37\]. In contrast, tissue quantitation of viruses that are thought to primarily remain in a latent phase yields substantially lower copy numbers. For example, using a competitive PCR technique, Serth et al. determined that benign and malignant prostatic tissue contains 100–200 copies of papilloma virus DNA/12,500 diploid cells (0.008–0.016 copies/cell) \[40\]. Similarly, Orii et al. reported that tissue from patients with posttransplant lymphoproliferative disease contains \( 1 \times 10^{12}–1 \times 10^{17} \) copies of Epstein-Barr virus DNA/1 \times 10^3 PBMCs (0.05–0.76 copies/cell) \[41\]. In our study, BKV DNA was detected more frequently and at a higher concentration in pre-PVAN renal biopsy samples than in native renal biopsy samples and renal biopsy samples obtained from patients without BKV viruria. This observation illustrates the potential use of serial quantitative PCR analyses to identify at-risk patients before they develop full-blown PVAN. However, because the renal biopsy is an invasive technique, regular monitoring of BKV load is better performed by testing urine or blood samples. Another reason for this is our observation that many patients with viruria tested negative for
viral DNA in renal tissue. Urine samples represent material from the entire kidney, and, therefore, are more informative about viral content than are small renal biopsy samples, which typically sample only a small fraction of the renal cortex. Once at-risk patients are identified as a result of screening, the likelihood of their developing PVAN can potentially be lowered by the more judicious use of immunosuppression.

JCV, like BKV, is latent in the kidneys [18, 38]. The incidence of JCV viruria in apparently healthy people is >70% in selected groups, such as elderly individuals, Native Americans, and Pacific Islanders [42]. In the present study, JCV DNA was found in only 1 patient with chronic rejection and 1 patient with HIV-associated nephropathy. Surprisingly, even patients with JCV viruria showed no detectable JCV DNA in renal biopsy samples, although these samples did have higher BKV loads. This may reflect the focal nature of JCV infection or that JCV DNA is present at a concentration that is much lower than that of BKV DNA. It should be recalled that most cases of PVAN are due to BKV, although, in rare cases, JCV may also be associated with this complication [43, 44]. An alternate explanation for our failure to detect JCV DNA in renal biopsy samples obtained from patients with active JCV viruria is that the excreted virus is derived from an extrarenal reservoir, such as the pelvicaliceal system, ureter, or urinary bladder. Successful amplification of the gene for ACY from >98% of the renal tissue samples argues against technical issues resulting in false-negative results.

In summary, we have quantitated BKV DNA and JCV DNA in renal tissue obtained from a variety of clinical settings. Low levels of BKV DNA were present in renal biopsy samples obtained from nonimmunosuppressed patients. Immunosuppression was associated with increased BKV load, and actual levels showed a broad correlation with the patient’s BKV replication status. However, ~50% of renal biopsy samples from patients with BKV viruria tested negative for BKV DNA. This low level is most likely the result of focal infection and provides a rationale for using urine rather than renal biopsy samples to monitor renal allograft recipients for PVAN. In the renal tissue samples, an increase in the BKV load was typically not accompanied by a proportional increase in the JCV load, which suggests that these 2 related PVs might be subject to different mechanisms that regulate viral replication.

Acknowledgments

Karen Weck graciously made available JC polyomavirus primer and probe sequences and helped design the TaqMan-based assay used in this study. Technical assistance in performing the assays was provided by Deborah Zygmunt and Bunnie Miller.

References


