Real-Time Polymerase Chain Reaction and Laser Capture Microdissection for the Diagnosis of BK Virus Infection in Renal Allografts

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ABSTRACT

We used real-time polymerase chain reaction (PCR) technology to detect BK virus (BKV) in H&E-stained kidney biopsy sections, using laser capture microdissection. Renal allograft biopsy specimens from 4 patients with the histopathologic diagnosis of BKV-associated nephropathy (BKVAN; group 1) and 3 patients suspected to have BKVAN but without diagnostic histologic features (group 2) were retrieved. Diagnostic inclusion-bearing cells were microdissected by laser capture microscopy from group 1. Renal tubular epithelial cells were microdissected randomly in group 2. DNA was extracted and real-time amplification performed using primers targeting the large “T” and small “t” regions of the BKV and JC virus genomes. Tubular epithelial cells from a case without evidence of BKV infection were used as negative controls in a similar reaction. BKV presence was demonstrated only in epithelial cells containing typical viral inclusions. Group 2 and negative control samples were confirmed as negative for BKVAN. Real-time PCR technology can be used to detect BKV in H&E-stained, paraffin-embedded tissue sections. This technique detected BKV in tubular epithelial cells of renal allografts. To our knowledge, this is the first report of detecting BKV in laser capture microdissected renal biopsy specimens using real-time PCR.

Laser capture microdissection (LCM) has been used in many useful ways for diagnostic and other investigative purposes, especially in the field of oncology, often to isolate material of interest for molecular evaluation.1 In the field of virology, LCM, together with the polymerase chain reaction (PCR), has found applicability in detecting HIV, Epstein-Barr virus, herpes simplex virus, and varicella viruses in a variety of clinical scenarios.2-4 To the best of our knowledge, this technology has not been applied to the detection of the BK virus (BKV), an agent associated with poor renal allograft function, and in only a few cases has LCM been used to detect related viruses at extrarenal sites.5

The standard methods of diagnosis of BKV-associated nephropathy (BKVAN) rest on histopathologic identification of viral inclusions in renal biopsy specimens. Unfortunately, these inclusions sometimes are not identified as a result of inadequate sampling or variable distribution within tissues, and such biopsy specimens can be interpreted mistakenly as showing acute cellular rejection.6 Immunohistochemical and electron microscopic methods have contributed to improved detection, but these techniques lack the specificity to differentiate between the BKV and the other major human polyomavirus, the JC virus (JCV).6 Yet, by an average of 40 weeks after transplantation, up to 5% of renal allograft recipients can be affected by the BKV, and progression to irreversible failure of the allograft has been observed in up to 45% of these cases.6

To improve the ability to detect the BKV, others have applied molecular methods to urine and/or serum samples, but the presence of viral particles in these specimens has not always translated into active renal infection or BKVAN.7,8 There is, therefore, a need to develop more sensitive and specific methods of demonstrating infection when other methods
prove inadequate and when clinical findings indicate a critical need to know.

We demonstrate the usefulness of LCM in combination with real-time PCR techniques for the detection of the BKV in a series of renal allograft transplant biopsy specimens. To the best of our knowledge, this report represents the first study combining the use of real-time PCR and LCM in this setting.

**Materials and Methods**

**Cases**

A total of 8 renal biopsy specimens were retrieved from the files of the Department of Pathology, Dartmouth-Hitchcock Medical Center, Lebanon, NH, that were accessioned between January 1999 and August 2004. Four were consecutive cases with histopathologic features diagnostic of BKVAN Image 1A and Image 1B. Two were clinically suggestive of BKV infection but were negative or had equivocal features for BKVAN by histopathologic criteria Image 1C. One case had classic diagnostic inclusions in earlier levels of histopathologic material but not on the deeper sections obtained for microdissection Image 2. A native kidney with no significant pathologic abnormalities was used for negative control samples Image 1D.

**Histologic Materials and LCM**

Original slides stained with H&E were reviewed, and additional 5-µm-thick sections were prepared and stained

**Image 1** (Case 3) A and B, Allograft tubules with typical nuclear inclusions of the BK virus. C, Renal allograft tubules with artifactual nuclear enlargement, equivocal for BK virus–associated nephropathy. D, Normal renal tubules from the control specimen (A-D, H&E, original magnification ×400).
with H&E. Noncharged glass slides were used, and the baking steps were omitted during processing. Otherwise, standard methods of slide preparation for histopathologic examination with the H&E method were followed, using an automated stainer (Varistain Gemini; Shandon Scientific, Cheshire, England). The sections were not coverslipped to permit subsequent microdissection.

LCM was performed using the Pixcell II LCM System (Arcturus, Mountain View, CA) following a National Institutes of Health–recommended procedure and using the CapSure Macro LCM caps or the CapSure HS caps (Arcturus). In the positive cases, identified intranuclear inclusions within renal tubular epithelial cells were targeted and dissected together with the adjacent tubular epithelium. In each of these dissections, an average of 750 pulses (range, 555-1,021) were delivered per case. The glomeruli, all of which revealed no pathologic abnormalities, also were microdissected, with an average of 344 pulses each (range, 245-500). The 2 cases with negative or equivocal histologic features and the native kidney biopsy specimen were treated in an identical manner, except that renal tubular epithelia were dissected randomly in the absence of clearly identifiable inclusion-like structures and glomeruli were not targeted for dissection. Before and after microdissection, images were compared to ensure that target tissues actually were captured.

**Image 2** Typical inclusions in the original set of slides (A; inset) but not in the deeper sections prepared for microdissection (B; inset). In this case, the deeper level section was microdissected (A and B, H&E, original magnification x100; insets, H&E, original magnification x400).

**Image 3** Typical intranuclear inclusion before (A) and after (B) microdissection (A and B, H&E, original magnification x400).
Real-Time PCR

A crude cell lysate was made from the material obtained by LCM using the Pico Pure DNA Extraction Kit (Arcturus). For samples on the CapSure Macro LCM caps, 30 µL of Proteinase K solution was added. For samples on the CapSure HS, 10 µL was added. Caps were incubated overnight in a 55°C to 58°C oven using the alignment tray and heating block (available from Arcturus). The caps were briefly and gently centrifuged to collect the crude lysate in the microtube. This was heated to 95°C for 10 minutes to inactivate the Proteinase K.

BKV and JCV real-time PCR was performed using the primers described by Beck et al.¹¹ and using SYBR Green (ABI, Foster City, CA) detection rather than fluorescence resonance energy transfer. Post-PCR melting was done to differentiate the BKV from JCV amplicons. An internal control gene (factor V) also was amplified to determine whether the LCM sample was adequate for amplification. Briefly, 3 µL (Macro caps) or 1 µL (HS caps) of lysate was added to a master mix containing 1× SYBR Green Master Mix (ABI) and 1 µmol/L of forward and reverse primers. The primer sequences were as follows: BKJC forward, 5’-TTTTGGGGGACCTAGTTGC; BKJC reverse, 5’-CTCTACAGTAGCAAGGGATGC; factor V forward, 5’-GCTGCCCATGAATAGCACTG; and factor V reverse, 5’-CTACTTCTAATCTGTAAGAGCAG. Cycling was performed in the Smart Cycler II (Cepheid, Sunnyvale, CA) using the following conditions: 95°C, 10 minutes; and 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds (optics on), and 72°C for 30 seconds.

Melting Curve Analysis

Melting curve analysis has become a routine method for the analysis of sequence variants using common, real-time PCR platforms. Based on the melting temperature, or Tₘ, of a specific amplicon, the melting curve identifies a characteristic Tₘ that can distinguish between amplicons that differ by only a single base. For our study, melting curve analysis was performed following PCR amplification. By using the SYBR Green channel, melting data were obtained from 40°C to 95°C at a ramping rate of 0.2°C/s with the optics on.

Results

Sampled tissues from all 4 cases with diagnostic nuclear inclusions were positive by real-time PCR amplification for BKV. Melting curve analysis revealed sequences with Tₘs equivalent to those for the corresponding BKV+ control sample. Similarly, melting curve analysis was able to differentiate between the BKV+ and JCV+ control amplicons Figure 1. The 3 cases with no diagnostic nuclear inclusions were negative for the presence of BKV or JCV by real-time PCR (Figure 1). Negative, blank, and positive control samples for these cases all showed the expected results.

Discussion

The human polyomaviruses, BKV and JCV, share a 70% homology with each other, as well as with the simian virus 40. Primary infection generally occurs in childhood, following which the virus remains latent in the urogenital tract. Reactivation of the virus can occur in patients who are immunocompromised, including renal allograft recipients, in whom persistent viral replication might cause progressive graft dysfunction. The BKV is the polyoma viral type that usually causes nephropathy, although the JCV, which is associated primarily with progressive multilocular leukoencephalopathy, has been implicated in renal disease less frequently.¹¹,¹²
The demonstration of BKV in renal allografts is the ultimate proof of infectivity, because no other surrogate methods of detection have successfully predicted allograft lesions. Histopathologic examination remains the “gold standard” for making these diagnoses, in which infected tubular epithelial cells are marked by large basophilic intranuclear inclusions and the tubulointerstitium shows a variable inflammatory infiltrate, which often includes plasma cells. Several factors, however, hamper the sensitivity and specificity of histopathologic diagnosis of BKVAN. Among these are the patchy distribution of the characteristic inclusions; the obscuring of details owing to inflammation or necrosis; inadequate sampling; and, potentially, confusion with inclusions from other viral diseases. Yet, the need to make a conclusive diagnosis usually is one of medical exigency because this information is critical to the administration of appropriate interventions and the prediction of long-term prognosis of the allograft.

The clinical importance of identifying BKVAN is related to the fact that appropriate treatment entails a reduction in immunosuppressive drug doses. This is opposite the treatment for rejection, which shares many histologic characteristics with BKVAN. Unfortunately, this reduction in immunosuppression increases the risk of rejection. It has been shown that graft failure can exceed 50% and even reach 100% in 2 years, especially if BKVAN is advanced at the time of diagnosis.13

Although it is clear that no single technique is likely to produce ideal specificity and sensitivity, a combination of tools could be applied efficiently to optimize diagnosis and treatment. To this end, immunohistochemical analysis and electron microscopy have found some success, except that these might not differentiate between BKV and the related JCV.6 Also, electron microscopy is complex, time-consuming, expensive, and lacks the desired sensitivity to justify the efforts it demands. In addition, successful differentiation between the different polyomaviruses might be clinically significant.14 The use of type-specific antibodies in tissue sections might partly overcome this problem,12 but no study has successfully demonstrated this phenomenon in tissue sections.

Given the rapid advances in the field of molecular pathology, it is hardly surprising that techniques such as real-time PCR, which have hitherto been recognized as research tools, are quickly becoming available as tools for clinical diagnosis. Real-time PCR has several advantages over the traditional PCR. Some of these include its more rapid turnaround time owing to the elimination of separate postamplification analysis steps and increased sensitivity and specificity owing to novel detection methods.15 Therefore, we were motivated to explore possible ways whereby real-time PCR could be of use in solving the diagnostic dilemma that BKV allograft nephropathy sometimes presents, especially because such a diagnosis represents vital information in overall patient management.

Our findings show that this technique is capable of detecting the presence of the BKV in renal allografts. In combination with the real-time PCR detection of the virus, melting curve analysis also is able to differentiate specifically between BKV and JCV, the 2 human polyomaviruses. The specificity of this assay is demonstrated by the following findings: (1) A sample known to be positive in initial sections for the BKV did not react when cells without inclusions in deeper sections were evaluated. (2) Two allografts with equivocal or negative findings by histopathologic examination were negative by real-time PCR. (3) Normal kidney did not show any detectable viral DNA. Careful re-review of the histopathologic findings and the subsequent clinical course and follow-up of these patients indicated that they more than likely did not have BKVAN. Therefore, even in cases with equivocal histopathologic findings, this real-time PCR assay has the potential to unequivocally exclude a BKV infection. It is not infrequent in our experience to observe artifactual nuclear enlargement and “smudging” of the nucleus in renal biopsy materials, even in patients with no risks for developing BKV nephropathy. When such artifacts occur in patients with a clinical suspicion of BKVAN as shown in Image 2, this real-time PCR assay followed by melting curve analysis might be indicated.

References


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