Evaluation of a BK virus real-time PCR assay designed using novel bioinformatics technology

Joel A. Lefferts a, Nathan D. Hicks b, Gregory J. Tsongalis a,⁎

a Department of Pathology, Dartmouth Medical School and Dartmouth-Hitchcock Medical Center, Lebanon, NH 03756, USA
b Department of Biology, University of Vermont, Burlington, VT, USA

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ABSTRACT

Monitoring of active polyomavirus BK (BKV) infections by quantitative real-time PCR is becoming a progressively more routine practice in the care of renal transplant patients due to the potential for these infections to injure transplanted kidneys. Quantitative BKV results from a previously validated, laboratory-developed real-time PCR assay based on commercially available MGB Alert® reagents were compared to results obtained from the same urine and plasma specimens using a commercially designed real-time PCR assay by IntelligentMDx. When compared qualitatively, the two assays performed identically with the exception of one urine specimen in which BKV DNA was detected near the lower limit of quantification by the MGB Alert® assay. A quantitative comparison of the results showed an average 0.55 log10 copies/mL difference between the two assays. These findings suggest that despite small differences, the IntelligentMDx assay could be adopted for clinical BKV monitoring of renal transplant patients.

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Introduction

Primary infection of the polyomavirus BK (BKV) occurs in the majority of the human population as an upper respiratory tract infection during childhood and is followed by viral latency, mainly in the urogenital tract (Hirsch and Steiger, 2003; Knowles et al., 2003). The widespread exposure to BKV can be demonstrated by seropositivity rates in the human population being reported at approximately 80% (Kean et al., 2009; Knowles et al., 2003; Rollison et al., 2003). Reactivation of latent BKV may occur in immunocompromised patients and is of particular concern in immunosuppressed renal transplant patients where it has been implicated in allograft dysfunction and rejection. This type of damage to the renal allograft has been termed polyomavirus-associated nephropathy (PVAN) and can be visualized histologically by the presence of tubulointerstitial nephritis (Randhawa et al., 1999, 2002). As many as 60% of patients experiencing PVAN will advance to allograft loss. Increased monitoring, however, has been shown to reduce this risk. Detection rates by PCR of BKV DNA in the urine of renal transplant recipients vary from study to study but are approximately 20–40% while PVAN rates among renal transplant patients is between 2% and 10%. A purely qualitative urine PCR test for BKV DNA, therefore, would not be ideal for identifying renal transplant patients with PVAN (Babel et al., 2009; Munoz et al., 2005; Randhawa et al., 2005a,b; Viscount et al., 2007). BKV DNA can also be detected at lower levels in the plasma of a smaller percentage of renal allograft recipients. A sequential progression of PVAN can be observed in which BKV DNA is first detected in the urine (vireuria) and then in the blood (viremia). In some cases persistent BKV viruria and viremia can progress to PVAN, which can be diagnosed by tissue biopsy of the transplanted kidney (Babel et al., 2009; Viscount et al., 2007).

Although BKV viruria can be an earlier marker of potential PVAN it is not as specific for PVAN as viremia. With the use of quantitative real-time PCR assays for detection of BKV DNA and specific viral load thresholds, the clinical utility of BKV DNA testing can be improved. One study found that BKV DNA at concentrations above 1.6 × 10⁷ copies/mL demonstrated 100% sensitivity, 96% specificity, 50% positive predictive value and 100% negative predictive value when plasma was tested and 100% sensitivity, 92% specificity, 31% positive predictive value and 100% negative predictive value when urine was evaluated using a threshold of 2.5 × 10⁷ copies/mL (Viscount et al., 2007).

Despite a lack of standardization in both BKV PCR assays and standards or calibrator material, quantitative detection of BKV by real-time PCR has been used with increasing frequency during the past few years to monitor plasma and urine BKV concentrations in immunosuppressed transplant patients as a marker for PVAN (Babel et al., 2009; Brennan et al., 2005; Wadei et al., 2006). Currently, an FDA-approved IVD is not available for quantitative clinical testing of BK viral levels and individual laboratories must rely on their own laboratory-developed tests (LDTs). Due to the inter-laboratory variability and variability in assay design and format, quantitative results reported from different laboratories may not always be in agreement. The region of the BKV genome chosen as the target for
real-time PCR amplification has been demonstrated to be a source of inter-assay variability but other possible reasons for differences in assay performance between laboratories include differences in specimen storage and processing, DNA isolation method, real-time PCR primer and probe design, detection chemistry, real-time PCR instrumentation and cycling conditions (Deback et al., 2009; Hoffman et al., 2008).

A recently designed quantitative real-time PCR assay developed by IntelligentMDx, Inc., (Cambridge, MA) using a proprietary bioinformatics approach was evaluated in a molecular diagnostic laboratory to determine its clinical utility. This assay, currently designated for research use only (RUO) was compared to a previously validated quantitative BKV assay using the MGB Alert® BK Virus ASR reagents to evaluate its performance with plasma and urine specimens.

Materials and methods

Patient specimens

Residual clinical plasma \((n = 32)\) and urine \((n = 38)\) specimens submitted to the Molecular Pathology Laboratory at Dartmouth-Hitchcock Medical Center for quantitative BK virus analysis were collected for use in this study. Automated total nucleic acid isolations were performed using a QIAGEN EZ1 BioRobot. Briefly, two 0.4 mL volumes of each plasma sample were subjected to the EZ1 Virus Mini Kit 2.0 (QIAGEN) protocol in which predetermined amounts of either the MGB Alert internal control plasmid or the IMDx internal control plasmid (containing Caenorhabditis elegans and Arabidopsis thaliana chloroplast genomic sequences, respectively) were spiked into the carrier RNA used during the automated isolation process. Two 1.0 mL urine samples were processed in a similar fashion for each urine specimen, but to reduce carry-over of PCR inhibitors found in urine, each 1.0 mL urine sample was first centrifuged for 2 min at 16,000 × g. The supernatant was removed from each resulting pellet which was then re-suspended in 0.4 mL of TE buffer and then subjected to isolation with the EZ1 Virus Mini Kit.

BKV DNA detection assays

Samples extracted for the IntelligentMDx assay (IntelligentMDx, Cambridge, MA) were then subjected to 40 cycles of a proprietary real-time quantitative TaqMan PCR assay (25 \(\mu\)L reactions containing 1.0 \(\mu\)L extracted nucleic acid sample) on a 7500 Fast Real-Time PCR System (Applied Biosystems) using reagents and protocol supplied by IntelligentMDx (IMDx). The primer and probe (FAM-labeled) sequences used in this assay were designed with a proprietary bioinformatics process to optimize for the highest predicted analytic specificity and sensitivity for BKV genomic DNA. In addition to the primers and probe specific for the BKV target, a CAL FLUOR® Orange 560-labeled probe was created to competitively detect the internal control plasmid added to each specimen during the automated isolation.

The MGB Alert real-time PCR assay was performed on the SmartCycler® II (Cepheid, Sunnyvale, CA) using 50 cycles of PCR followed by melt curve analysis. Each reaction consists of a 25 \(\mu\)L volume containing: 12.5 \(\mu\)L TaqMan FAST Universal PCR Master Mix (Applied Biosystems, Foster City, CA); 1.25 \(\mu\)L of a BK detection reagent made by mixing equal volumes of MGB Alert BK Virus Primers, BK Virus Probe, Internal Control 2B Primers and Internal Control 2B Probe (ELITech Molecular Diagnostics, formerly Nanogen, San Diego, CA); 6.25 \(\mu\)L \(\mathrm{H}_2\mathrm{O}\); and 5 \(\mu\)L of total nucleic acid isolations. The fluorescent data for the real-time amplification were collected on the FAM channel and the internal control data on the Cy3 channel.

For real-time PCR quantification using the IMDx assay, calibration curves were created for each assay by plotting cycle threshold \((C_T)\) values against the known concentrations of a dilution series \((1.5 \times 10^1\) copies/\(\mu\)L–\(1.5 \times 10^9\) copies/\(\mu\)L) of a of a plasmid containing the entire BK virus genome (Advanced Biotechnologies Inc, Columbia, MD). Quantification of BKV with the MGB assay was similarly achieved by creating a calibration curve using dilutions of a plasmid containing the segment of the BKV genome amplified by the MGB Alert BK primer set \((2 \times 10^1\) copies/\(\mu\)L–\(10^8\) copies/\(\mu\)L). All results were converted from \(\log_{10}\) copies/\(\mu\)L of isolated nucleic acid to \(\log_{10}\) copies/mL of plasma or urine using dilution factors of 60 and 150 for urine and plasma, respectively, based on the specimen input and nucleic acid elution volumes for each specimen type.

Calibration curves

A calibration curve for the IMDx assay \((R^2 = 0.996)\) was produced using dilutions of the Advanced Biotechnologies (Columbia, MD) BKV MM Strain Quantitated Viral DNA plasmid, containing the entire BKV genome while dilutions of a separate plasmid containing only the targeted region of the BKV genome were used for the MGB Alert calibration curve \((R^2 = 0.998)\). Since the control material for the MGB Alert assay was available in a more concentrated form (but has since been discontinued), the calibration curve for the MGB Alert assay spans several logs (copies/\(\mu\)L) beyond the highest concentration on the IMDx calibration curve. To allow for a quantitative comparison of all BKV-positive samples, the equation produced from the IMDx calibration curve was applied to all patient specimens including those with concentrations greater than the most concentrated calibrator.

Results

Qualitative comparisons

Of the 32 plasma specimens tested, BKV was detected in the same 18 plasma specimens by both the IMDx and MGB Alert assays. BKV was not detected in the remaining 14 plasma specimens by either assay (Table 1). Of the 38 urine specimens tested, the MGB Alert-based assay identified 15 specimens as positive and 23 as negative for BKV while the IMDx assay identified 19 urine specimens as positive and 25 as negative (Table 1). The two qualitatively discordant urine samples that were positive by the MGB Alert assay but negative by the IMDx assay were found to have a BKV concentrations lower than any of the other urine or plasma specimens tested, equal to and slightly below the lower limit of quantification for this assay (based on the calibrator sample with the lowest concentration). Additional clinical specimens from the same patients from which this discordant samples were obtained tested positive for BKV by the MGB Alert assay, suggesting that the MGB Alert results obtained for these samples were low-level, true-positive and not false-positive results.

Quantitative analysis

Quantitative comparisons of the results positive for BKV DNA by both assays showed linear relationships with correlation coefficients of 0.9245 and 0.8885 for plasma and urine specimens, respectively.

| Table 1 | Qualitative comparison of MGB Alert and IntelligentMDx (IMDx) real-time PCR assays for the detection of BKV DNA in plasma and urine specimens. |
| --- | --- | --- |
| Plasma specimens \((n = 32)\) | MGB Alert | IMDx |
| Assay | Positive | Negative |
| Positive | 18 | 0 |
| Negative | 0 | 14 |

| Urine specimens \((n = 38)\) | MGB Alert | IMDx |
| | Positive | Negative |
| Positive | 13 | 2* |
| Negative | 0 | 23 |

* Two urine specimens tested were found to be negative by the IMDx assay and positive, but near the lower limit of quantification, by the MGB Alert assay.
and of 0.9111 when results from the two specimen types were analyzed collectively (Figs. 1A–C). Quantitative results for the combined urine and plasma specimens as determined by the IMDx assay were found to be lower by a mean value of 0.55 log10 copies/mL than the results obtained from the MGB Alert assay. With the exception of a single outlier with an IMDx assay result 1.93 log10 copies/mL higher than the MGB Alert assay, all results fell within ±2 standard deviations of the mean value of the two assays (Fig. 1D).

Discussion

Both assays were found to be effective for detection and quantification of BKV DNA in plasma and urine specimens. The Intelligent MDx assay did not detect two samples found to be positive by the MGB assay, which may be attributed to multiple factors, including less DNA per PCR and fewer PCR cycles being used in this assay. Previous studies, however, have shown that low levels of BKV in plasma and urine specimens have limited clinical significance (Viscount et al., 2007). Established thresholds to define the border between low (clinically insignificant) and high (clinically significant) BKV levels in plasma and/or urine will need to be established. This will be difficult, however, without international standards or calibrators for BKV, like those for HCV or HIV.

Without such standardization, assay features including the required batch size and real-time PCR instrument needed may be more important factors for a laboratory deciding between different BKV testing strategies. As performed in this evaluation the MGB assay, run on a Cepheid SmartCycler, could be run in batches of 16 reactions (including controls) while the IMDx assay was performed using an Applied Biosystems 7500 instrument, capable of up to 96 reactions per run. Both assays, however, have the flexibility to be reconfigured to run on most real-time PCR platforms commonly used in clinical laboratories.

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References


