Development of a BK Virus Real-Time Quantitative Assay Using the bioMérieux Analyte-Specific Reagents in Plasma Specimens

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Key Words: BK virus quantitation; Viral loads; Real-time PCR test; Renal nephropathy

ABSTRACT

Objectives: Viral load testing for BK virus (BKV) has become the standard of care for diagnosing BKV infection and monitoring therapy in kidney transplant patients. However, there are currently no US Food and Drug Administration–approved assays and no standardization among available tests.

Methods: This study evaluated the performance of the analyte-specific reagent (ASR) BKV primers r-gene and probe r-gene reagents (bioMérieux, Marcy l’Étoile, France) soon to become available on the US market for accuracy, linearity, precision, analytical sensitivity, specificity, and correlation with the Qiagen (Germantown, MD) BKV ASR test using commercial material and patient plasma samples.

Results: The assay was linear from 204 to 3.92 million (2.31-6.6 log_{10} DNA copies/mL (coefficient of determination; R^2 = 0.999). A dilution series demonstrated limits of detection and quantitation of 2.14 log_{10} and 2.30 log_{10} copies/mL (95% hit rate detection), respectively. Interrun precision was highly reproducible, with coefficients of variance ranging from 2.2% to 6.0%. A comparison of 34 matched samples showed a good agreement (R^2 = 0.87) between the bioMérieux BKV laboratory test and the Qiagen BKV ASR assay results, with an average negative bias (~0.28 log_{10} copies/mL).

Conclusions: The laboratory-developed test with bioMérieux BKV reagents is a reliable and sensitive assay for BKV DNA quantitation compared with the Qiagen ASR test.

BK virus (BKV) is a common polyomavirus that establishes subclinical and persistent infection predominantly in the kidney in up to 90% of adults, typically without adverse effects. Renal transplant recipients are at risk of developing BKV-associated nephropathy (BKVAN), which frequently leads to graft failure in 15% to 80% of patients, while BKV reactivation in bone marrow transplant patients may result in hemorrhagic cystitis. Disseminated disease due to BKV has also been reported in patients with AIDS, chronic lymphocytic leukemia, and congenital immunodeficiency, in whom immunosuppression could not be reduced. The pathology associated with BKV is thought to result from destruction of infected urothelial cells undergoing active viral replication. BKVAN results in a decline in urinary tract function, and 30% to 60% of the patients experience irreversible graft failure. In some instances, antiviral therapy with cidofovir and leflunomide may be used, and the patient should be monitored closely for the onset of rejection.

Polymerase chain reaction (PCR) to detect and quantify BKV DNA in plasma has been promoted as a noninvasive way to identify patients at risk of BK nephropathy and to monitor response to therapy. Detection of BKV in plasma has a stronger correlation with disease than BKV in urine, since urine specimens may have very high viral loads that may overlap between symptomatic and asymptomatic patients.

Although the viral load cutoff associated with nephropathy is not well established, a small number of studies have shown that plasma BKV loads more than 10^4 copies/mL have a predictive value greater than 80%, while the absence of viremia and/or viruria rules out the diagnosis.
of BKVAN.\textsuperscript{10,12} Viral loads of more than $10^7$ copies/mL in urine are more likely associated with disease.\textsuperscript{11,13,14} Quantitative measurements of BKV DNA have also shown that decreasing virus levels over time correlate with response to treatment and improved outcome.\textsuperscript{11,14} Moreover, screening guidelines from the Infectious Diseases Society of America (2005) currently recommend that renal transplant patients be screened for BK viral load (BKVL) in the urine every 3 months and in plasma every 1 to 3 months up to 2 years posttransplant or when allograft dysfunction occurs, or when allograft biopsy is performed.\textsuperscript{2}

Virus levels are commonly quantitated by direct measurement of BKV DNA in plasma using real-time PCR amplification technologies.\textsuperscript{1,15-17} However, most assays are laboratory-developed tests (LDTs), and there are no international standards to compare among the different tests. In this study, we evaluated the performance of an LDT using the BKV primers r-gene and BK virus probe r-gene and an unassayed DNA Internal Control r-gene (bioMérieux, Marcy l’Étoile, France). This combination is coupled with an automated extraction and PCR setup. We show that this assay is comparable to the Qiagen (Germantown, MD) BKV analyte-specific reagent (ASR) assay, providing additional testing options for clinical laboratories.

**Materials and Methods**

**Samples and Performance Evaluation Panels**

A total of 68 consecutive plasma samples from kidney transplant patients submitted to our clinical laboratory for BKV testing were prospectively collected during 2014. Thirty-four samples were negative and 34 positive, allowing a broad dynamic range of BKV quantitation. Samples were deidentified and stored at −70°C, until further analysis. No clinical information was available on these patients. All samples had undergone BKVL testing by New York Presbyterian Hospital Clinical Laboratories (New York, NY) before validation. The study was approved by the Institutional Review Board Committee at Weill Cornell Medical College. Assay linear range, precision, and reproducibility acceptability criteria were based on New York State Department of Health Clinical Lab Evaluation Program Guidance in the Microbiology Molecular Checklist (February 2011; www.wadsworth.org/labcert/TestApproval).

The OptiQuant BK Linearity Panel (Acrometrix, Benicia, CA) was used to evaluate accuracy, reproducibility, linearity, and sensitivity. The six-member panel, formulated with intact BKV (subtype Ia) particles in a defibrinated, delipidized human plasma matrix, spanned the clinically significant range ($5 \times 10^2$ to $5 \times 10^6$ BKV copies/mL) for BKV-infected individuals, allowing characterization of the linear range. For determining the limit of detection (LOD) and the limit of quantitation (LOQ), the $5 \times 10^2$ copies/mL OptiQuant BKV panel member was further diluted to six concentrations ranging from 200 copies/mL to 6.25 copies/mL in BKV-negative plasma (Acrometrix) and analyzed in 12 replicates. Panel members were quantitated by the manufacturer’s real-time PCR assay (Luminex, Austin, TX), targeting a 62–base pair (bp) region in the VP3 gene and calibrated against a commercial purified BKV DNA. The between-run precision was determined with replicates using OptiQuant BKV panel samples (range, 275 copies/mL to 5.9 million copies/mL) measured on 3 different days. Cross-reactivity (specificity) was determined using high viral load samples titered in-house (cytomegalovirus, Epstein-Barr virus, human immunodeficiency virus, hepatitis C virus, and hepatitis B virus) or by Viracor (IBT Laboratories, Lee’s Summit, MO) (human herpesvirus [HHV]–7). Herpes simplex virus (HSV)–1, HSV-2, HHV-6, HHV-8, and JC viruses (JC MADI strain, quantitated viral DNA catalog 08-943-250) were obtained from Advanced Biotechnologies (Columbia, MD) and varicella zoster virus from the Centers for Disease Control and Prevention (Atlanta, GA). Adenovirus was from Acrometrix.

**bioMérieux BKV r-gene Primers/Probe LDT**

The LDT assay used in this study comprised two ASRs—a BKV primers r-gene (20-000-01) and BKV probe r-gene (20-001-01) (bioMérieux)—and an unassayed DNA Internal Control r-gene (71-120-01) (bioMérieux, Ivry sur Seine, France). BKV DNA was extracted from 200 μL plasma in the presence of 10 μL BKV internal control (IC) r-gene, using the QIAcube/QIAamp DNA extraction reagents (Qiagen), and eluted in a final volume of 50 μL. The BKV viral load was then measured by a quantitative TaqMan real-time PCR on the Rotor-Gen Q thermal cycler (Qiagen). These primers and probes amplify a 158-bp specific sequence located in the BKV small T antigen (StAg), covering all available BKV sequences, including the rare strains of types II, III, and IV (bioMérieux, Marcy l’Étoile, France, unpublished communications, 2014). A plasmid DNA containing the whole BKV genome plasmid, diluted 10-fold to produce a 4-point standard curve ranging from 5,000 to 5 copies/μL, was used for BKV quantitation (bioMérieux).

PCR amplification was performed in a final volume of 25 μL reaction comprising 15 μL PCR master mix and 10 μL IC-containing BKV DNA. The two primers sets (BKV genome and IC) were amplified by quantitative PCR (qPCR) using the Rotor-Gen Q instrument (Qiagen) with the following amplification conditions: 95°C for 15 minutes, followed by 45 cycles of 95°C for 10 seconds and 60°C for 40 seconds. The cycle threshold (Ct) for BKV and the IC were calculated by the system software, and the resulting Ct was
used to quantitate the BKV copy number by interpolation against standard curve values generated using quantitation standards.

QIagen BKV ASR Assay

The laboratory-developed QIagen BKV ASR test was performed using the BKV primer and probe ASRs as previously described. Briefly, nucleic acid was extracted from 200 μL plasma in the presence of a BKV positive control, using the QIAcube automated isolation system and the Total Nucleic Acid Isolation kit (QIagen). The DNA was eluted into a final volume of 50 μL, of which 15 μL was added to the master mix containing primers, probes, Taq polymerase, magnesium chloride, and buffers. The BKV viral load was then measured by qPCR using primers that amplify the conserved VP2 and VP3 region of the BKV genome and the Rotor-Gene Q system (Qiagen). Amplification conditions were 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds, 65°C for 30 seconds, and 72°C for 20 seconds with 10 cycles of touchdown during the annealing step (1°C).

Statistical Methods

The correlation of log_{10} BKV loads in plasma and QIagen validation specimens was determined by the least squares regression model using Excel 2003 (Microsoft, Redmond, WA). A log_{10} difference of 1.0 was used as the cutoff for the determination of concordance between the two assays as previously described. The between-run precision of the qPCR method for BKV quantitation was expressed by coefficient of variation (CV). Agreement between the bioMérieux BKV r-gene LDT and the QIagen BKV Rotor Gene PCR was determined using a Bland-Altman plot of all samples (34 samples). The LOD (95% hit rate detection) was determined by probit analysis (MiniTab 16 Statistical Software; MiniTab, College State, PA). The LOQ was defined as the lowest dilution with an SD within approximately 0.2 log_{10}.

Results

Testing of a commercial linearity panel across the range of detection from 204 to 392 million (2.31-6.60 log_{10} DNA copies/mL) demonstrated an excellent agreement between the expected and observed viral load with a coefficient of determination (R^2) of 0.999. The assay was linear with a slope approaching 1 (1.1) across six logs of detection. A high-titer specimen to measure the linear range beyond 6.60 log_{10} was not available for inclusion in the analyses.

When a larger number of interassay dilutions were tested to assess the LOD, the concentration at which BKV DNA was detected in at least 95% of the replicates was 2.144 log_{10} copies/mL (95% confidence interval [CI], 1.94-2.55 log_{10} copies/mL), corresponding to 140 copies/mL (95% CI, 85-355 copies/mL) DNA. The LOQ was 2.30 log_{10} copies/mL (200 copies/mL) based on an SD less than or equal to 0.2 log_{10} copies/mL (Table 1).

Interassay precision analysis using the QIagen OptiQuant BKV panel samples (2.70-6.70 BKV log_{10} copies/mL) measured on 3 different days exhibited excellent reproducibility throughout the linear range of detection with low interassay variation. The commercial validation specimens yielded BKV viral load means (SD, CV) ranging from 2.44 log_{10} copies/mL (0.15, 6.0%) and 6.77 log_{10} copies/mL (0.15, 2.2%) for the low and high panel members, respectively. A high degree of intra-assay precision was also observed.

Table 1.

<table>
<thead>
<tr>
<th>Dilution Series for Limit of Detection Determination</th>
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Table 2.

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</table>

Figure 1. Linear range of the bioMérieux (Marcy l’Étoile, France) BK virus (BKV) test using reference samples. The linearity and dynamic range were measured using the Acrometrix (Benicia, CA) OptiQuant BKV panel, ranging from 2.3 to 6.6 log_{10} copies/mL. The linearity was determined by plotting the log of the mean measured titers against the log of the mean input titers. The results demonstrate a linear response over 4 logs of detection with an R^2 value of 0.99, y = 1.0626x – 0.5654; R^2 = 0.9994.
and 34 were negative in both assays. There was good agreement in the performance of the bioMérieux BKV LDT compared with the in-house Qia gen BKV assay viral load test, demonstrating an excellent correlation with a coefficient of determination ($R^2$) of 0.874 Figure 3. A Bland-Altman plot demonstrated that the average difference (bias) of –0.28 log was within approximately twofold, indicating that on average, the bioMérieux BKV LDT yielded viral load values that were twofold higher than the values obtained with the Qiagen BKV ASR assay. Fifty percent of the positive samples were within 0.3 log of the Qia gen assay, while 68% of the positive samples were within 0.5 log. All results were within 1 log of the Qia gen method Figure 4, and agreement between the assays is demonstrated by the 95% CI ($\pm$1.96 SD) for the mean difference, including zero. The greatest difference from the Qia gen results was a single result of 0.8 log copies/mL observed at the low value of 1.73 log copies/mL.

There were no biological false-positive results with any other pathogens potentially found in human plasma due to shared targets with the viruses tested as described under Materials and Methods.

Discussion

Quantitative testing for BKV plays an important clinical role in the monitoring of immunocompromised patients, especially in the setting of posttransplantation BKV nephropathy. However, the marked variability among laboratory-developed PCR tests and the lack of standardized reference material make the interpretation of clinical management guidelines and BKV testing results difficult. In this study, we used a validation panel comprising commercial and patient samples to evaluate the analytical characteristics of a new LDT using the bioMérieux BKV ASR reagents. To our knowledge, this is the first study validating the use of these reagents. A study validating the European Conformity–marked BKVs r-gene kit was recently performed by Sueur et al in Europe.

Performance characteristics results indicate that the new BKV assay has a dynamic range of at least six logs ($R^2 = 0.999$),

![Figure 2](#) Probit analysis. The limit of detection (LOD) was determined using serial dilutions of the 500-copy/mL OptiQuant BKV panel member in EDTA plasma (Acrometrix, Benicia, CA) to six concentrations from 200 to 6.25 copies/mL (0.8-2.3 log_{10} copies/mL) assayed in replicates of 12. LOD 95% = 2.144 log copies/mL; LOD 95% = 140 copies/mL.

![Table 2](#) Intra-Assay Reproducibility of the BK Virus ASR Test Using Reference Material

<table>
<thead>
<tr>
<th>Nominal, Log_{10} Copies/mL</th>
<th>No. of Samples Tested</th>
<th>Mean Viral Load, Log_{10} Copies/mL</th>
<th>Log_{10} Difference Expected-Observed</th>
<th>SD, Log_{10} Copies/mL</th>
<th>% CV: Intra-assay</th>
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<td>6.59</td>
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<td>3.70</td>
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<td>3.36</td>
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<tr>
<td>2.70</td>
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<td>2.31</td>
<td>0.39</td>
<td>0.135</td>
<td>5.86</td>
</tr>
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</table>

ASR, analyte-specific reagent; CV, coefficient of variation.

![Table 3](#) Inter-Assay Precision of the BK Virus ASR Test Using Reference Material

<table>
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<tr>
<th>Nominal, Log_{10} Copies/mL</th>
<th>No. of Samples Tested</th>
<th>Mean Viral Load, Log_{10} Copies/mL</th>
<th>SD, Log_{10} Copies/mL</th>
<th>% CV: Inter-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.70</td>
<td>3</td>
<td>6.77</td>
<td>0.15</td>
<td>2.2</td>
</tr>
<tr>
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<td>5.72</td>
<td>0.20</td>
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<td>4.70</td>
<td>3</td>
<td>4.58</td>
<td>0.12</td>
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<td>3.70</td>
<td>3</td>
<td>3.51</td>
<td>0.10</td>
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<tr>
<td>2.70</td>
<td>3</td>
<td>2.44</td>
<td>0.15</td>
<td>6.0</td>
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</table>

ASR, analyte-specific reagent; CV, coefficient of variation.
a lower detection limit of 2.0 log$_{10}$ copies/mL, and an intra-assay CV of less than 6.0% for all concentrations, confirming a high degree of assay precision across the entire range of BKV concentrations tested. Moreover, there was complete agreement between this test and the laboratory BKVL ASR assay with an $R^2$ value of 0.874, indicating a high degree of accuracy throughout the assay range. A wide dynamic range is significant for identification and monitoring of patients with high levels of viremia at risk of developing BKVAN, while still effectively monitoring for prospective BKV reactivation at low viral loads. Several clinical studies reported median BKVL in plasma ranging from 2.84 to 4.1 log$_{10}$ copies/mL (range, 0-7.21) for patients with BKVAN. Moreover, serial determination of BK viremia is considered the best tool for predicting disease evolution during follow-up, allowing reduction of immunosuppression.

The LOQ is particularly important for the identification of patients who should be monitored for BKV reactivation. However, the relationship between cutoff levels of BKV viremia and prediction of BKVAN is still under investigation and varies widely. Recently, the American Society of Transplantation (AST) defined a BKVL of 4 log$_{10}$ copies/mL or more as presumptive BKVAN, recommending reduction in immunosuppression. Randhawa et al suggested predictive values of active BKVAN of 3.7 log$_{10}$ copies/mL of BKV in viremia, while in a separate study, a reduction in immunosuppression was done upon detection of more than 2.7 log$_{10}$ copies/mL. Although there is no accepted standard LOQ for BKV, we have determined the LOQ as the lowest concentration that could be reliably quantitated with an SD of 0.2 log$_{10}$ copies/mL or less to be 2.30 log$_{10}$ copies/mL, which is in agreement with the LOQ reported in the literature and our currently used Qiagen BKV ASR assay. The bioMérieux BKV LDT also showed excellent precision in BKVL quantitation with overall CV values (2.2%-6.0%) comparable to the values reported in plasma samples in other, similar studies. There was good agreement between the laboratory test and the bioMérieux LTD assay, with an overall negative bias of –0.28 log$_{10}$ (copies/mL) of the bioMérieux BKV test. The 95% (1.96 SD) limit of agreement is 0.80 log$_{10}$ (gray lines).
test misdiagnosed BKVAN compared with the LDT, resulting in renal graft loss.

BKV isolates are classified into four subtypes (I-IV), with subtype I exceedingly the most prevalent throughout the world among individual patients with BKV infection.\(^{1,3}\) Probe and primer design have been also described as significant sources of BKVVL discrepancies among different assays. Genomic regions encoding the VP1 capsid protein and the large T antigen have been shown to have sequence divergence,\(^{33,34}\) while the StAg gene used in this assay is described to be displaying only a few single-nucleotide polymorphisms.\(^{35}\) Here we demonstrate that the new LTD is highly comparable to the Qiagen ASR assay designed to amplify a consensus region of VP2/VP3 genes, indicating that the new test is highly accurate. Correct quantitation of BKVVL samples was also obtained by Sueur et al\(^ {22}\) using the r-gene kit.

Given BKV genetic heterogeneity and the lack of US Food and Drug Administration–approved assays, an important advantage of the study is that it provides information regarding standardized reagents for BKV testing produced under good manufacturing practices, offering additional testing options for clinical laboratories. Since PCR tests vary considerably among laboratories, the availability of such reagents is expected to greatly improve assay performance and generation of guidelines for clinical management. Finally, although correlation of results with the Qiagen ASR assay was excellent, possible limitations of the study include the relatively small number of patients and lack of a high-titer specimen to measure the linear range beyond \(7.0 \log_{10}\) copies/mL.

In summary, the LTD with bioMérieux BKV reagents is a highly sensitive, specific, and reliable assay for measuring BKV loads in plasma and identifying patients at risk of BKVAN.

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