Toward Standardization of BK Virus Monitoring: Evaluation of the BK Virus R-gene Kit for Quantification of BK Viral Load in Urine, Whole-Blood, and Plasma Specimens

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Screening of BK virus (BKV) replication is recommended to identify patients at increased risk of BKV-associated diseases. However, the heterogeneity of molecular techniques hinders the establishment of universal guidelines for BKV monitoring. Here we aimed to compare the performance of the CE-marked BK virus R-gene kit (R-gene) to the performance of our in-house assay for quantification of BKV DNA loads (BKVL). A 12-specimen panel from the Quality Control for Molecular Diagnostics (QCMD) organization, 163 urine samples, and 88 paired specimens of plasma and whole blood (WB) from transplant recipients were tested. Both the R-gene and in-house assays showed a good correlation within the QCMD panel (r = 0.995 and r = 0.989, respectively). BKVL were highly correlated between assays, although positive biases were observed with the in-house assay in analysis of urine (0.72 ± 0.83 log_{10} copies/ml), plasma (1.17 ± 0.63 log_{10} copies/ml), and WB (1.28 ± 0.37 log_{10} copies/ml). Recalibration with a common calibrator significantly reduced the bias in comparisons between assays. In contrast, BKVL was underestimated with the in-house PCR in eight samples containing BKV genotype II, presenting point mutations at primer-annealing sites. Using the R-gene assay, plasma and WB specimens were found to be equally suitable for quantification of BKVL, as indicated by the high correlation coefficient (r = 0.965, P < 0.0001). In conclusion, the R-gene assay demonstrated reliable performance and higher accuracy than the in-house assay for quantification of BKVL in urine and blood specimens. Screening of BKV replication by a well-validated commercial kit may enable clinical laboratories to assess viral loads with greater reproducibility and precision.

The BK virus (BKV)-associated disease most frequently seen after renal transplantation is BKV-associated nephropathy (BKVN). BKV reactivation occurs in up to 50% of kidney transplant recipients (KTR) within the first year of transplantation, and 7% to 10% of patients progress to BKVN, resulting in graft dysfunction or loss (1). BKVN has also been reported in other immunocompromised patients such as lung transplant recipients (2, 3) and cardiac transplant patients (4, 5). This is a growing medical problem as the population of transplant recipients continues to increase. The underlying pathogenic mechanism of BKVN is not well defined (6). Disruption of the balance between BKV replication and host immune control is generally viewed as a key element of viral pathogenesis (7). Thus, to date, the mainstay therapeutic option for BKVN is a reduction of immunosuppression, which allows reconstitution of the immune response to clear the virus (8) but potentially increases the risk of graft rejection.

According to Kidney Disease Improving Global Outcomes (KDIGO) (9) and the 2014 European guidelines (10), all KTR should be regularly screened for BKV replication in plasma or urine to identify patients at increased risk of BKVN. A sustained BKV viremia level above the threshold of 4 log_{10} copies/ml has been defined as indicating "presumptive" BKVN (11), and high-level BKV viruria usually precedes viremia and potential nephropathy by 4 to 12 weeks. Thus, it is recommended to screen the BKV DNA load (BKVL) in plasma monthly in the first 6 months posttransplant, followed by 3-monthly screening until 2 years posttransplant, in order to guide therapeutic intervention for KTR patients with probable or proven BKVN. However, most BKV quantitative PCR methods are in-house techniques, and marked variability among assays has been described (12). A recent study demonstrated that, depending on the PCR assay, the currently recommended BKV viremia cutoff of ≥4 log_{10} copies/ml may underestimate the prevalence of BKVN (13). The variability of the assays may be attributable to various criteria: features of primers and probe design, including the size of the amplicon and the choice of reference material and/or types of matrices used for the blood compartment (plasma or whole blood [WB]) (12, 14). Hence, the wide heterogeneity of molecular techniques limits interlaboratory comparison and hinders the establishment of universal cutoff points of BKVL with high predictive positive values for the identification of patients at risk for BKVN. Therefore, there is a need to standardize BKVL determination in order to improve its reliability and allow unequivocal comparison of different patient populations.

This study aimed to evaluate the performance of the BK Virus
R-gene kit (R-gene) (bioMérieux, France) for quantification of BKV in urine and blood samples from transplant recipients compared to the performance of our referral in-house assay, with both techniques targeting the stable genomic region encoding the small t antigen (StAg). Additionally, we compared the quantitative results of analyses of paired plasma and WB samples using the R-gene assay to determine if both matrices could provide appropriate biological material for BKV quantification. Finally, we evaluated the relative diagnostic values of BKV in plasma and WB samples longitudinally collected from 3 KTR.

MATERIALS AND METHODS

Specimen collection. A total of 163 urine specimens from 134 patients and 88 paired aliquots of plasma and WB samples from 39 patients submitted to our clinical laboratory for BKV testing were prospectively collected and stored at −80°C. Urine and blood samples were collected simultaneously for 15 patients. The study population comprised 146 KTR, 6 lung transplant recipients, and 6 hematopoietic stem cell transplant patients. Among the KTR, 7 patients had developed BKVN, 6 of the 7 within 4 months after kidney transplant. BKVN was confirmed histologically on kidney biopsy specimens. For 3 KTR patients, plasma and WB samples were longitudinally collected in order to compare BKV dynamics in the two blood compartments. Twelve samples of the Quality Control Molecular Diagnostics (QCMD) 2012 BKV proficiency panel were tested by the two assays. This study was approved by the Institution Review Board of Strasbourg University Hospital.

BKV DNA load quantification. Viral DNA was extracted from 200 μl of urine, plasma, or WB samples. An internal inhibition control was added to the lysis buffer and simultaneously purified with viral DNA using specific protocol B on the NucliSENS easyMAG system (bioMérieux, France). Urine and blood samples were eluted in 100 μl and 50 μl of elution buffer, respectively. Ten microliters of purified DNA was processed by the in-house quantitative real-time PCR assay using a LightCycler 2.0 instrument (Roche Diagnostics, Basel, Switzerland) as described previously (15). The R-gene PCR was performed in a 25-μl volume containing 15 μl of the amplification premix and 10 μl of standard or sample DNA using a LightCycler 480 II instrument (Roche Diagnostics, Basel, Switzerland). The two methods amplify a conserved region in the small t antigen (StAg) gene but target different sites. The dynamic quantification ranges are 2.40 to 11.40 log_{10} copies/ml for the in-house PCR assay and 2.70 to 11.00 log_{10} copies/ml for the R-gene assay.

Sequencing of VP1 and small t antigen regions. Amplification of the StAg gene was performed by nested PCR on 5 μl of eluted DNA, with outer primers BK2Reverse (5'-CCACGAGGGTCTAATAAAGGTAATCTGGA-3' [positions 1075 to 1094, Dunlop sequence]) and BK9Forward (5'-TACATAGCTTCAAAACACATCCACACTGACT-3' [3929 to 3962]) and inner primers BK1Reverse (5'-GAACCATGGGCCTTTGTCCAG-3' [375 to 394]) and BK5Forward (5'-TACACAGGAAAGGCAAGGGAAGG-3' [4319 to 4338]). Amplification products were sequenced with the BK1Reverse and BK5Forward primers, and sequencing results were aligned using Gentle software (University of Cologne, Cologne, Germany). Amplification of the BK capsid viral protein 1 (VP1) gene was performed by PCR on 5 μl of eluted DNA, using a primer set consisting of upstream sequence BK3F (5'-CCCCAGAGGGTCTAATAAAGGTAATCTGGA-3' [positions 1465 to 1484]) and downstream sequence BK3R (5'-CCCTGCAATTCTCCAAGGGTTTC-3' [2035 to 2054]). The BK gene “typing region” was sequenced using primer set F1n (5'-CTTGGAAGCATGAGATGGGCGC-3') and R1n (5'G ACCCTGCTGAAGGTTACGG-3'), as described previously (16). BKV genotypes were determined using BLAST alignment software.

Statistical analysis. Statistical analysis was performed using GraphPad Prism6 software and GraphPad StatMate 2.0 software (GraphPad; San Diego, CA). Agreement of results and correlations between R-gene and in-house assays were assessed with Bland-Altman analyses and Deming regression analyses. Correlations between BKVL levels measured in plasma and WB samples were determined by a Spearman correlation test.

Power analysis was performed to detect a difference of 0.45 log_{10} between means with a significance level (alpha) of 0.05 (two-tailed test).

RESULTS

Analytical precision and sensitivity and specificity of the R-gene quantification kit. Two BKV-positive samples of the QCMD 2009 proficiency panel were chosen to perform intra- and interassay precision analyses through two serial dilutions in negative plasma 10 times. The coefficient of variation ranged from 0.83% to 3.99% for intra-assay precision and from 1.76% to 3.61% for interassay precision (Table 1). For determinations of limits of detection (LOD), the contents of an AcroMetrix BKV panel (Life Technologies, France) were diluted in plasma, urine, or WB. Each dilution was extracted and then amplified 15 times. The 95% LOD were 2.44 log_{10} copies/ml, 2.30 log_{10} copies/ml, and 2.48 log_{10} copies/ml in urine, plasma, and WB samples, respectively. No cross-reaction was observed with JC virus (ATCC reference VR-21583), adenoviruses (VR-3), or the following human herpesviruses: herpes simplex virus 1 (HSV1; VR-260), HSV2 (VR540), varicella zoster virus (VZV; VR-916), human herpesvirus 6 (HHV-6; VR1480), cytomegalovirus (CMV; VR-977), and Epstein-Barr virus (EBV; Raji cells).

Results from the QCMD 2012 BKV proficiency panel. Twelve samples from the QCMD 2012 BKV proficiency panel were tested with the in-house and the R-gene assays. Results are depicted in Table 2. The two assays were highly concordant, as the 7 samples for which a negative result was expected were found to be negative and 5 samples were found to be positive. A perfect correlation for the assays in comparison to the QCMD expected values (in-house r = 0.989; R-gene r = 0.995) was obtained. For quantitative analysis, our results were compared to the consensus mean and standard deviation calculated from all the data returned by the participants to the QCMD 2012 BKV proficiency panel. The values of the positive results obtained with the in-house assay were slightly higher than those of the QCMD expected results, with a mean difference of 0.71 ± 0.31 log_{10} copies/ml. Meanwhile, the R-gene PCR results were close to the QCMD expected values, with a mean difference of 0.17 ± 0.15 log_{10} copies/ml.

High concordance of qualitative results between R-gene and in-house PCR assays. Qualitative analysis revealed a 98.8% concordance level between assays in urine, a 98.9% concordance level in plasma, and a 95.5% concordance level in WB specimens. Totaals of 155 urine, 87 plasma, and 84 WB samples were positively detected by both PCR techniques. One urine specimen and one WB specimen were weakly positive with the in-house PCR assay and negative with the R-gene kit, while 1 urine sample and 4 blood samples were identified as positive with the R-gene assay only.
Correlation of quantitative results between R-gene and in-house PCR assays. Overall, the samples tested were representative of the entire dynamic range. As shown in Fig. 1A, B, and C, BKVL levels were highly correlated between assays for urine and blood specimens. The slopes of the Deming regression lines were 1.05 (95% confidence interval [CI], 0.99 to 1.11), 0.84 (95% CI, 0.68 to 0.99), and 1.12 (95% CI, 1.01 to 1.23) for the urine, plasma, and WB specimens, respectively. Bland-Altman analysis showed a bias of 0.72 ± 0.83 log_{10} copies/ml (mean difference ± standard deviation) for urine samples (Fig. 1D). Values of mean differences between the two assays were slightly higher for plasma and WB samples, with biases of 1.17 ± 0.63 log_{10} copies/ml and 1.28 ± 0.37 log_{10} copies/ml, respectively (Fig. 1E and F). These data suggest that BKVL are overestimated by the in-house PCR compared to the R-gene assay. This could result from the use of different standard materials in the two techniques to generate the calibra-

### TABLE 2 Results from the QCMD 2012 BKV proficiency program

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>QCMD 2012 result or consensus log_{10} virus concn</th>
<th>R-gene assay result</th>
<th>In-house PCR result</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>BKV load (log_{10} copies/ml)</td>
</tr>
<tr>
<td>QCMD 2012-01</td>
<td>Negative</td>
<td>ND</td>
<td>Negative</td>
</tr>
<tr>
<td>QCMD 2012-02</td>
<td>3.62</td>
<td>0.56</td>
<td>3.62</td>
</tr>
<tr>
<td>QCMD 2012-03</td>
<td>2.67</td>
<td>0.47</td>
<td>2.93</td>
</tr>
<tr>
<td>QCMD 2012-04</td>
<td>Negative</td>
<td>ND</td>
<td>Negative</td>
</tr>
<tr>
<td>QCMD 2012-05</td>
<td>Negative</td>
<td>ND</td>
<td>Negative</td>
</tr>
<tr>
<td>QCMD 2012-06</td>
<td>Negative</td>
<td>ND</td>
<td>Negative</td>
</tr>
<tr>
<td>QCMD 2012-07</td>
<td>1.95</td>
<td>0.57</td>
<td>(2.13)</td>
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<tr>
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<td>4.71</td>
<td>0.56</td>
<td>4.77</td>
</tr>
<tr>
<td>QCMD 2012-09</td>
<td>Negative</td>
<td>ND</td>
<td>Negative</td>
</tr>
<tr>
<td>QCMD 2012-10</td>
<td>Negative</td>
<td>ND</td>
<td>Negative</td>
</tr>
<tr>
<td>QCMD 2012-11</td>
<td>Negative</td>
<td>ND</td>
<td>Negative</td>
</tr>
<tr>
<td>QCMD 2012-12</td>
<td>5.28</td>
<td>0.49</td>
<td>5.66</td>
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</table>

Consensus log_{10} virus concentrations were calculated as the mean values reported from 181 datasets submitted by clinical laboratories. Abbreviations: SD, standard deviation; ND, not determined; delta log_{10}, log_{10} copies/ml difference between the R-gene and the in-house PCR assays in the QCMD panel. Values in parentheses are below the limit of quantification for both tests.

**FIG 1** Deming regression and Bland-Altman analysis for the BK virus R-gene and the in-house assays. (A to C) The Deming regression curve equation and 95% confidence interval (CI) of the slope are indicated for urine (A), plasma (B), and whole-blood (C) specimens. (D to F) Bland-Altman plots of urine (D), plasma (E), and whole-blood (F) specimens are shown. Solid lines represent the means of the differences between the results of the two assays; dashed lines indicate the limits of agreement between the two methods (1.96 × the standard deviation). BKV, BK virus.
tion curves. To test this hypothesis, the two assays were recalibrated to a common calibrator, the AcroMetrix BKV panel, and 25 randomly selected blood samples were retested. As shown in Fig. 2, the use of a common calibrator significantly reduced the bias between assays from 1.09 to 0.69 log_{10} \text{ copies/ml} (P < 0.0001), demonstrating that using different standard materials impacts the agreement between assays.

**Analysis of discrepant samples.** While BKVL values were on average higher with the in-house assay, 5/163 urine and 3/88 blood samples showed lower viral loads with the in-house PCR assay than with the R-gene assay (mean difference, −2.95 log_{10} \text{ copies/ml}). Because of the low DNA viral loads, the StAg gene could be amplified by PCR in only 4 samples. Sequence analysis of the StAg gene revealed one and two mismatches at the 3’ position of the forward and reverse primers, respectively, and an additional mutation at the 5’ position of the probe of the in-house PCR. These mismatches are likely to hinder annealing and amplification of the primers. Further sequencing of the VP1 gene typing region showed the presence of BKV genotype II in all discrepant samples. The same mutations observed in the discrepant samples can be found in several genotype II reference strains (GenBank accession no. AB263916 [ETH3] and AB263920 [GBR-12]). As a result of excluding these discordant samples, Deming regression slopes were 1.06 (95% CI, 1.04 to 1.08), 1.09 (95% CI, 0.98 to 1.19), and 1.12 (95% CI, 1.01 to 1.23) and biases were 0.85 ± 0.32 log_{10} \text{ copies/ml}, 1.25 ± 0.33 log_{10} \text{ copies/ml}, and 1.28 ± 0.37 log_{10} \text{ copies/ml} for urine, plasma, and WB specimens, respectively.

**Both plasma and WB matrices are appropriate for BKVL measurement.** Although European guidelines recommend monitoring BKVL in plasma, numerous diagnostic laboratories quantify BKVL in WB (personal communication). In order to determine if plasma and WB would provide equivalent materials, BKVL levels were compared in 88 paired aliquots of plasma and WB. Eighty-seven of 88 samples were positive in both plasma and WB specimens with the R-gene quantification assay. One sample was positive in plasma only, with a very low viral load of 2.59 log_{10} \text{ copies/ml}. BKVL levels were highly correlated between plasma and whole blood, as shown by the correlation coefficient r = 0.965 (P < 0.0001; 95% CI, 0.947 to 0.977) (Fig. 3A) and a low bias of 0.40 ± 0.23 (Fig. 3B). Given that technical variability of <0.5 log_{10} \text{ copies/ml} is considered acceptable (17, 18) and of low clinical relevance (19), our data suggest that both plasma and WB specimens are appropriate for BKVL determination. The statistical power of this analysis reached 90%.

Furthermore, to compare the BKVL dynamics in the two blood compartments, BKVL was measured in plasma and WB samples that were longitudinally collected from 3 KTR. As shown in Fig. 4A and B, patients A and B showed a first positive BK viremia result at day 119 and day 163 posttransplant, respectively, previous blood samples having been negative. Of note, their viruria had been tested positive in our laboratory at day 92 and day 59 posttransplant, respectively. The first BKVL measurement in urine for patient C occurred 36 days posttransplant and was highly positive (>7.6 log_{10} \text{ copies/ml}). BKVL were consequently investigated in blood samples, and a positive viremia result was found as soon as day 54 posttransplant (Fig. 4C). This patient developed a BKVN 72 days after transplant, which first prompted a reduction of immunosuppression and then further required a switch from mycophenolate mofetil treatment to leflunomide treatment. For these 3
patients, BKVL values were highly correlated between plasma and WB specimens. Taken together, these data suggest that plasma and WB are equally suitable for BKVL monitoring in the blood compartment. The StAg gene was sequenced at days 163 and 198 for patient A, days 133 and 161 for patient B, and days 54, 79, 90, 114, 135, and 177 for patient C. No variability between the StAg gene sequences could be observed over time for the three patients.

DISCUSSION

The wide variety of in-house quantitative real-time PCR assays and the lack of international standards result in an important interlaboratory disagreement with respect to BKVL measurements (12) and thus far have hindered the establishment of a universal BKVL cutoff value with a high positive predictive value for presumptive BKVN (13, 14). Commercially available quantitative PCR kits are believed to provide more repeatable and reproducible results, consequently contributing to the efforts of clinical laboratories regarding the currently ongoing accreditation process. This study aimed to compare the performance of a commercially CE-marked assay, the R-gene kit, to the performance of our referral in-house method, with both techniques targeting the stable genomic region encoding the StAg.

Regarding the qualitative results, a high level of concordance between the two PCR methods was obtained for urine and plasma as well as for WB specimens. As for the quantitative results, a high correlation was observed between assays, although BKVL values appeared to be systematically overestimated by the in-house technique for each matrix, with blood results displaying the highest bias. This finding was further corroborated by the results obtained with QCMD samples, as the R-gene kit provided values much closer to the reference data (delta log, 0.17 ± 0.15 log10 copies/ml) than our in-house technique. This overestimation arises partly from the different reference materials used for quantification. Indeed, recalibration of the two assays to a common material resulted in a statistically significant reduction of the bias. The overestimation by our in-house technique may result in viral loads readily overstepping the threshold of 4 log10 copies/ml for presumptive BKVN and possibly leading to therapeutic intervention (20, 21). These data highlight the significance of the choice of reference material and the need for the development of an international standard.

Features of primer and probe design have also been described as among the most significant sources of BKVL discrepancies in comparisons of individual specimens (12). The main BKV genomic regions depicted as showing sequence divergence are those encoding the VP1 capsid protein and the large T antigen (22, 23), while the StAg gene is described as displaying only a few single nucleotide polymorphisms (24). However, despite our assumption that choosing the StAg gene as a PCR target for our in-house PCR would provide a less variable technique for quantification, discrepant BKVL results were observed for some individuals due to mutations at positions G4830A, A4836T, G4876A, and G4877A. These mutations were located 2 bp from the 3′ end of the forward and reverse primers, thus explaining their impact on BKVL quantification (25). Sequence alignment using published BKV strains demonstrated that these mutations were specific to BKV genotype II (26). BKV genotyping performed by sequencing the VP1 gene typing region confirmed that all discrepant samples contained this rare BKV genotype. Nevertheless, the R-gene kit, although targeting the same gene, allowed correct quantification of these samples. It has to be noted that primers and probes of the R-gene kit were designed to allow the amplification of BKV strains from the four existing genotypes, while those of our in-house PCR target the Dunlop reference strain belonging to genotype I. These data indicate that PCR assays based on primers and probes designed solely for one BKV genotype may underestimate BKVL. Thus, if clinical assumption of the presence of BKVN is strong, albeit not corroborated by a high viral load, we would recommend performing BKVL quantification by another laboratory using an assay validated for all BKV genotypes. We would also suggest sequencing the VP1 gene typing region and the PCR target gene...
when possible in order to detect uncommon genotypes and point mutations potentially impeding PCR amplification.

The KDIGO and European guidelines recommend close screening of BKV in plasma and urine during the first months after kidney transplantation. Monitoring of BKV viremia is noninvasive and can be informative, as a high level of BK viremia usually precedes viremia and potential nephropathy by 4 to 12 weeks (1, 27). Even though a cutoff value or a positive predictive value is hard to define, a viremia level of $>7 \log_{10}$ should alert clinicians and indicate the need to increase surveillance of viremia (28). Regarding the assessment of BKVL in the blood compartment, validation of WB as an appropriate matrix was recently carried out (29), but no study thus far has endeavored to contrast plasma and WB samples for BKVL determination. Comparison between serum and plasma samples has been performed and revealed a significantly lower level of BKVL in serum than in plasma, demonstrating in that circumstance the importance of choosing one specific type of sample for monitoring viral load over time (30). In our study, BKVL values were highly correlated in plasma and WB and the follow-up of three patients showed similar kinetics of BKVL in the two types of samples. Both matrices could thus be used for BKVL determinations in the blood compartment. Nevertheless, WB is the preferred clinical sample for detection of other opportunistic viruses such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) (31, 32) in transplant recipients. Using the same type of sample for all viruses could be more convenient for the monitoring of these patients, as the extraction would need to be performed only once for the entire viral screening panel, with significant time and cost saving. Furthermore, WB samples do not necessitate centrifugation or other manipulations that would increase the risk of sample carry-over. Only a small volume of WB is required for DNA extraction, and long-term storage can be achieved by simple guanidine thiocyanate lysis. In this study, few KTR developed BKVN and only one was longitudinally followed up; hence, we were not able to define a clinical threshold of viremia for presumptive BKVN.

It has to be noted that our findings apply only to the R-gene assay and our in-house assay. Other laboratories that use different extraction platforms and/or different laboratory-developed tests (targeting a different gene) may show differing results. Whether the use of the R-gene assay factually reduces the interlaboratory variability is also to be investigated. A multisite study comparing the performances of different laboratories using various BKV quantitative PCRs to detect BKV in the same panel of samples is warranted.

In conclusion, the R-gene assay demonstrated reliable performance and higher accuracy than the in-house assay for quantification of BKVL in urine and blood specimens. Using a commercial kit may enable clinical laboratories to assess viral load with greater reproducibility and precision. Indeed, standards, reagents, and internal controls made according to good manufacturing practices are already provided and their use requires fewer manipulations and adjustments. Nevertheless, it is crucial to pursue efforts to develop international standards and reference material in order to improve the standardization of BKV quantitative PCR and eventually define consensus threshold values for therapeutic intervention.

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