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Sequence Variation in Amplification Target Genes and Standards Influences Interlaboratory Comparison of BK Virus DNA Load Measurement

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International guidelines define a BK virus (BKV) load of ≥4 $\log_{10}$ copies/ml as presumptive of BKV-associated nephropathy (BKVN) and a cutoff for therapeutic intervention. To investigate whether BKV DNA loads (BKVL) are comparable between laboratories, 2 panels of 15 and 8 clinical specimens (urine, whole blood, and plasma) harboring different BKV genotypes were distributed to 20 and 27 French hospital centers in 2013 and 2014, respectively. Although 68% of the reported results fell within the acceptable range of the expected result ±0.5 $\log_{10}$, the interlaboratory variation ranged from 1.32 to 5.55 $\log_{10}$. Polymorphisms specific to BKV genotypes II and IV, namely, the number and position of mutations in amplification target genes and/or deletion in standards, arose as major sources of interlaboratory disagreements. The diversity of DNA purification methods also contributed to the interlaboratory variability, in particular for urine samples. Our data strongly suggest that (i) commercial external quality controls for BKVL assessment should include all major BKV genotypes to allow a correct evaluation of BKV assays, and (ii) the BKV sequence of commercial standards should be provided to users to verify the absence of mismatches with the primers and probes of their BKV assays. Finally, the optimization of primer and probe design and standardization of DNA extraction methods may substantially decrease interlaboratory variability and allow interinstitutional studies to define a universal cutoff for presumptive BKVN and, ultimately, ensure adequate patient care.

The emergence of BK virus-associated nephropathy (BKVN) as a major cause of graft dysfunction and loss in kidney transplant recipients (KTR) arises from the use of highly potent immunosuppressive drugs (1–3). This is a growing medical problem as the population of transplant recipients continues to increase. In Europe and the United States, the number of kidney transplantations has increased up to 50% in the last 20 years (www.kidney.niddk.nih.gov and http://www.era-edta.org). BKV reactivation or reinfection occurs in 40 to 50% of KTR, followed by BKVN in 6.6% of KTR at 5 years posttransplant, ultimately leading to graft dysfunction and loss in up to 50% of cases (4). The diagnosis of BKVN is based on the documentation of viral cytopathic effects observed in tubular epithelial cells accompanied by inflammatory cell infiltration after renal biopsy (5, 6). Immunohistochemistry with SV40 staining is the gold standard for diagnosing definitive BKVN (7). Nevertheless, in the early stages of BKVN, kidney allograft biopsy results may be falsely negative at an estimated rate of 10 to 30% (8). Prospective studies showed that high BKV viruria usually precedes viremia by 4 to 12 weeks, with a sustained BKV viremia above the threshold of 4 $\log_{10}$ copies/ml defined as presumptive of BKVN, with a positive predictive value of >80% (9, 10). These studies showed that BKVN can be effectively and safely prevented using a preemptive reduction in immunosuppression (11, 12). Therefore, European and Kidney Disease Improving Global Outcomes (KDIGO) guidelines recommend monthly KTR screening for BKV replication in urine and plasma specimens in the first 6 months posttransplant and then every 3 months until 2 years posttransplant (13, 14) to guide therapeutic intervention for KTR with presumptive BKVN.

Monitoring of BKV replication has been improved by the development of real-time quantitative PCR (qPCR) assays showing high sensitivity and specificity (15). However, the wide variety of available qPCR assays and the lack of international standards limit interlaboratory comparison (16, 17).

The distribution of proficiency panels constitutes a relevant approach to assess the variability of BK virus DNA load (BKVL) and to compare interlaboratory results, as shown for other opportunistic viruses, such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) (18, 19). High interlaboratory variability prompted international collaboration groups to establish WHO reference standards for these viruses (20, 21).

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TABLE 1 Quantitative results of the 2013 and 2014 BKV panels[a]

Sample no. by source	BKV genotype(s)	Quantitative results (log_{10} copies/ml) for: All data sets (maximum $n = 28$) GM ± SD	All data sets (maximum $n = 28$) Spread (range)	Commercial assays (maximum $n = 23$) GM ± SD	Commercial assays (maximum $n = 23$) Spread (range)	In-house assays (maximum $n = 5$) GM ± SD	In-house assays (maximum $n = 5$) Spread (range)
Urine							
BKV13-01	I	4.79 ± 0.64	2.23 (3.92–6.15)	4.68 ± 0.53	2.12 (4.03–6.15)	5.11 ± 0.84	1.99 (3.92–5.91)
BKV13-02	I	6.14 ± 0.45	1.89 (5.34–7.23)	5.97 ± 0.28	1.05 (5.34–6.39)	6.65 ± 0.49	1.12 (6.11–7.23)
BKV13-03	I, IV	6.98 ± 0.71	3.20 (5.28–8.48)	6.80 ± 0.65	2.20 (5.28–7.48)	7.52 ± 0.63	1.62 (6.86–8.48)
BKV13-04	II, IV	8.81 ± 0.67	2.60 (7.72–10.32)	8.80 ± 0.41	1.23 (8.18–9.41)	8.85 ± 1.18	2.60 (7.72–10.32)
BKV13-05	I	9.32 ± 0.65	2.32 (8.12–10.44)	9.23 ± 0.48	1.50 (8.45–9.95)	9.55 ± 0.96	2.32 (8.12–10.44)
BKV14-01	IV	7.37 ± 0.66	3.30 (5.32–8.62)	7.47 ± 0.53	2.00 (6.62–8.62)	6.90 ± 1.03	2.63 (5.32–7.95)
BKV14-02	I	9.85 ± 0.47	2.10 (8.54–10.64)	9.87 ± 0.49	2.10 (8.54–10.64)	9.75 ± 0.39	0.97 (9.33–10.30)
BKV14-03	IV	7.28 ± 0.64	2.98 (5.36–8.34)	7.37 ± 0.54	2.06 (6.28–8.34)	6.89 ± 0.99	2.63 (5.36–7.99)
BKV14-04	II	8.58 ± 1.07	5.55 (4.58–10.13)	8.91 ± 0.31	1.38 (8.12–9.50)	7.25 ± 2.29	5.55 (4.58–10.13)
WB[b]							
BKV13-06	I, II, IV	3.69 ± 0.84	3.43 (1.85–5.28)	3.52 ± 0.78	3.18 (1.85–5.03)	4.30 ± 0.89	2.00 (3.28–5.28)
BKV13-07	I, IV	4.91 ± 0.50	1.98 (4.21–6.19)	4.75 ± 0.35	1.10 (4.21–5.31)	5.39 ± 0.57	1.61 (4.58–6.19)
BKV13-09	I, II	4.96 ± 0.61	4.91 (3.72–6.61)	4.74 ± 0.44	1.72 (3.72–5.44)	5.63 ± 0.57	1.39 (5.22–6.61)
BKV13-10	I, IV	3.72 ± 0.72	2.67 (2.51–5.18)	3.54 ± 0.65	2.44 (2.51–4.95)	4.24 ± 0.71	1.84 (3.34–5.18)
BKV14-05	I	3.99 ± 0.52	2.60 (2.23–4.83)	3.88 ± 0.52	2.60 (2.23–4.83)	4.50 ± 0.14	0.36 (4.25–4.61)
Plasma							
BKV13-12	I	4.99 ± 0.63	2.81 (4.35–7.16)	4.75 ± 0.26	1.03 (4.35–5.38)	5.71 ± 0.83	2.03 (5.13–7.16)
BKV13-13	I	4.26 ± 0.48	1.88 (3.52–5.40)	4.07 ± 0.32	1.32 (3.52–4.84)	4.85 ± 0.38	1.03 (4.37–5.40)
BKV13-14	I, IV	6.34 ± 0.45	1.73 (5.79–7.52)	6.14 ± 0.24	0.97 (5.79–6.76)	6.93 ± 0.39	0.95 (6.57–7.52)
BKV13-15	I, IV	6.07 ± 0.52	2.12 (5.23–7.35)	5.91 ± 0.41	1.67 (5.23–6.90)	6.55 ± 0.55	1.47 (5.88–7.35)
BKV14-08	I	6.27 ± 0.33	1.32 (5.47–6.79)	6.20 ± 0.31	1.30 (5.47–6.77)	6.61 ± 0.17	0.37 (6.42–6.79)

[a] Geometric mean (GM), SD, and spread were calculated with quantitative positive results reported from all participants. Results reported as negative or under or over the limit of quantification were excluded. BKV genotypes constituting each panel sample are reported.
[b] WB, whole blood.

In this study, we assessed BKVL variability in several French hospital centers that conduct nearly 90% of the kidney transplantation activity in France. Two panels of clinical samples, including BKV genotype II and IV for the first time, were distributed to compare the performances of individual laboratories and analyze factors that may influence interlaboratory comparison (22).

MATERIALS AND METHODS

Panel constitution and preparation. The 2013 panel consisted of 15 clinical samples, including 5 urine (BKV13-01 to BKV13-05), 5 whole blood (WB) (BKV13-06 to BKV13-10), and 5 plasma (BKV13-11 to BKV13-15) specimens. Positive samples were collected from 20 patients, including 14 kidney, 4 lung, and 2 hematopoietic stem cell transplant recipients. The 2014 panel included 4 urine (BKV14-01, BKV14-03 [a replicate of BKV14-01], BKV14-02, and BKV14-04), 2 WB (BKV14-05 and BKV14-06), and 2 plasma (BKV14-07 and BKV14-08) specimens. Positive samples in the 2014 panel were collected from 4 kidney transplant recipients and 1 hematopoietic stem cell transplant patient. Clinical samples of the same matrix and with equivalent DNA viral loads were pooled to prepare adequate volumes of panel samples. The samples were homogenized before and after pooling by gentle mixing using a tube rotator for 20 min. Samples with mixed genotypes were obtained by mixing 2 to 3 single-genotype samples. As shown in Table 1, 11 BKV-positive samples harboring a single BKV genotype and 8 samples harboring multiple BKV genotypes were chosen to represent a wide range of BKV DNA concentrations. The negative samples (BKV13-08, BKV13-11, BKV14-06, and BKV14-07) were prepared from plasma and WB specimens collected from KTR who tested negative for BKV DNA ≥3 months before and 3 months after the collection date.

The panel samples were aliquoted, coded, and frozen at −80°C until shipment to the participating laboratories. Aliquots from the same panel were prepared the same day by a single technician using 1 calibrated pipette to minimize any variation and possible errors in preparations. Urine and blood samples were collected at the Strasbourg University Hospital (Strasbourg, France) and previously tested for BKV by our routine diagnostic testing with the CE-marked BK virus R-gene kit (bioMérieux, Marcy l'Etoile, France). The performance of our routine test is evaluated annually by the Quality Control for Molecular Diagnostics (QCMD) (23). This study was approved by the institutional review board of Strasbourg University Hospital.

Study participants. Twenty and 27 French hospital laboratories performing diagnostic testing of BKVL were invited and agreed to participate in this study in 2013 and 2014, respectively. Each participant completed a questionnaire detailing the procedure employed for BKV testing. Panel samples were shipped by a laboratory-specialized courier. The participants were asked to test the samples as if they were routine diagnostic specimens and to return the results within 10 weeks.

BKV genome sequencing. Amplification of the small T-antigen gene (*StAg*) and the BKV capsid viral protein 1 gene (*VP1*) was performed, as previously described (23). The large T-antigen gene (*LTAg*) was sequenced using primers BK6F (5′-GCCATTCCTTGCAGTACAGG-3′ [positions 4859 to 4878]) and BK6R (5′-GAGCTCATGGACCTTTTAGGC-3′ [positions 5120 to 5100]). BKV genotyping was performed as previously described (24).

Results analysis and statistics. For qualitative analysis of the data sets, results reported as negative, undetectable, or below the limit of detection were considered negative, while positive values reported with an absolute quantification or above the limit of detection were considered positive. False-positive and false-negative results and re-

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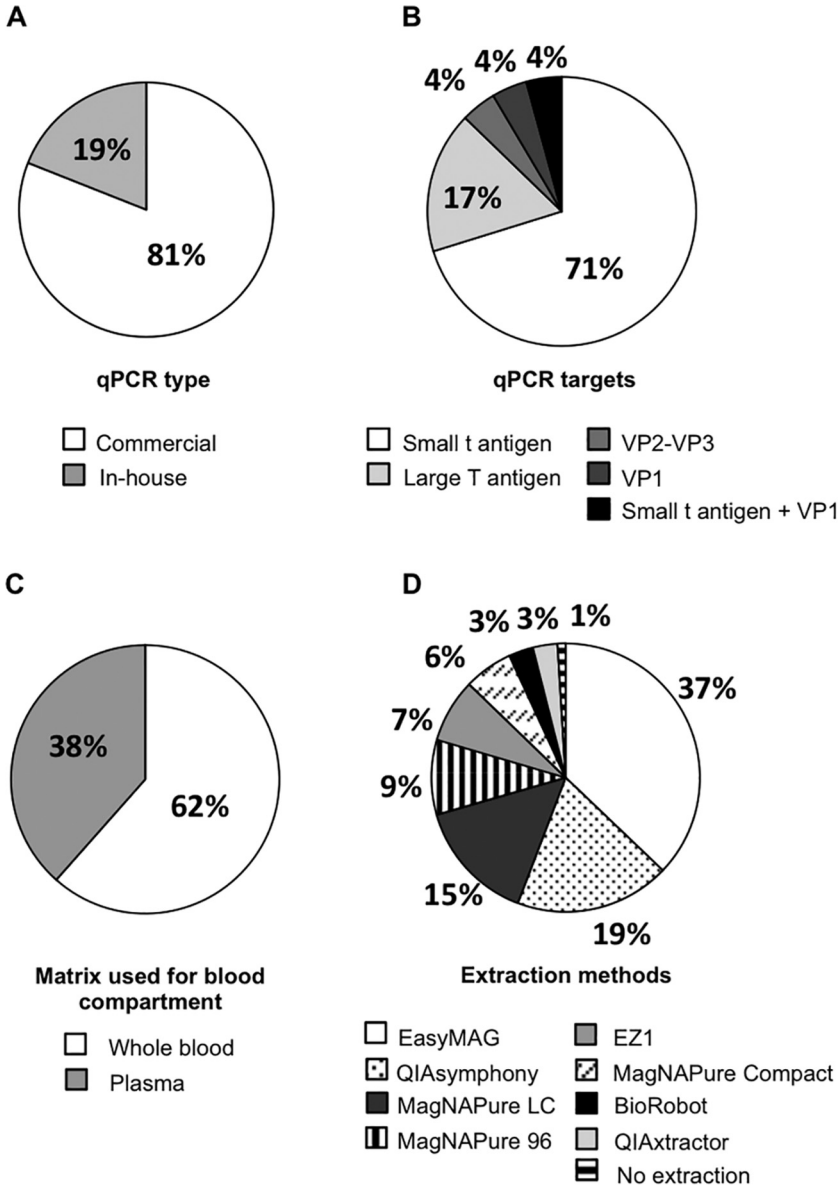


FIG 1 Diversity of diagnostic conditions among the participants. Participating laboratories were asked to describe the type (A) and target (B) of the qPCR assay routinely used for BK viral load quantification, the matrix routinely tested in the blood compartment (C), and the extraction methods used for DNA purification (D). For laboratories using different extraction methods for urine and blood samples, both techniques were considered in our analysis. The percentages of participants are indicated for each pie chart.

sults reported outside the limits of quantification were excluded from the quantitative analysis.

For each sample, the expected viral load to which all results were compared was defined as the geometric mean of the positive quantitative results reported by all participating laboratories. Variability according to viral load, matrix, and genotype was compared based on the standard deviations within each group, as previously described (25). Nonparametric tests (Mann-Whitney and Kruskal-Wallis tests) were used, and pairwise multiple comparisons between groups were analyzed using Dunn's multiple-comparison test. All statistical analyses were performed using the GraphPad Prism 6 software (San Diego, CA, USA).

RESULTS

Characteristics of participating laboratories and utilized assays. Twenty-seven French hospital laboratories were sent the 2014 8-sample study panel, including the 20 laboratories that participated in the 2013 15-sample study. Two laboratories performing different assays were sent >1 panel. Nineteen (95%) and 25 (93%) laboratories returned results in 2013 and 2014, respectively. A total of 47 data sets were used for the final analysis, including 19 from 19 laboratories in 2013 and 28 from 25 laboratories in 2014. Marked variability was observed in terms of techniques, target genes, matrix used for the blood compartment, and DNA extraction methods (Fig. 1). Most laboratories (81%) used commercial assays (Fig. 1A), including the BK virus R-gene kit ($n = 15$) (bioMérieux, Marcy l'Etoile, France), the RealStar BKV PCR kit ($n = 3$) (Altona Diagnostics/Eurobio, Courtaboeuf, France), the LightMix polyomaviruses JC and BK kit ($n = 2$) (TibMolBiol, Berlin,

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Germany), the artus BK virus RG PCR kit ($n = 2$) (Qiagen GmbH, Hilden, Germany), and the BKV ELITe MGB kit ($n = 1$) (ELITechGroup Molecular Diagnostics, Puteaux, France). Four in-house assays, including 2 previously published in the literature (26, 27) and one adapted from Hoffman et al. (17) were used by 5 (19%) laboratories. The unpublished assay (S. Rogez, S. Hantz, and S. Alain) targeted the *VP1* gene using the primer set forward (5′-TTTGGGACTTTCACAGGAGG-3′) (positions 2227 to 2246, Dunlop sequence]) and reverse (5′-GCCTCTCCACTGTTGTGT TCC-3′ [positions 2409 to 2389, Dunlop sequence]), and the probe (5′-TGTTTCAGCTGCTGATATTTGTGGCC-3′ [positions 2346 to 2371, Dunlop sequence]). Most of the assays targeted the *StAg* or *LTAg* gene (Fig. 1B). The 5 laboratories performing in-house assays used different standard materials: BKV strain MM quantitated DNA control (Advanced Biotechnologies, tebubio, Le Perray-en-Yvelines, France) (ABI control) ($n = 2$), which is a whole BKV genome linearized at the BamHI site in the *StAg* gene; the AmpliRun BK virus DNA control (Vircell S.L, Granada, Spain) (Vircell control) ($n = 2$), which is also a whole viral genome; or serial dilutions of qPCR amplification product cloned into a vector ($n = 1$). Each commercial kit contained its own standard material, the composition of which is proprietary.

Amplicon sizes varied from 75 to 274 bp (see Table S1 in the supplemental material). All participating laboratories routinely performed BKVL assays with urine samples, whereas 62% of the laboratories used WB samples, and 38% used plasma samples for viral load determination in the peripheral blood compartment (Fig. 1C). Nine different automated DNA extraction platforms, 89% of which were based on a magnetic bead method, were used (Fig. 1D). Seventy-seven percent of the laboratories used the same extraction method for urine and peripheral blood matrices, while the remaining 23% used different extraction platforms depending on the matrix, hindering normalization of the results (see Table S2 in the supplemental material). Regarding the urine samples, most laboratories used resuspended or vortexed material, and no pellet concentrates were used.

Among the 25 participants that returned results, 22 (88%) participate annually in the QCMD program, including those using in-house assays.

High concordance of qualitative results. Data from both panels were pooled for qualitative analysis; only 4 out of 509 reported results were incorrect. All samples with an expected BKVL of >4.0 $\log_{10}$ copies/ml were detected as positive by all participants. One false-negative result was reported by 1 laboratory using an in-house qPCR assay targeting the *VP1* gene (positions 2227 to 2409) for a sample with an expected BKVL of 3.69 ± 0.84 $\log_{10}$ copies/ml. Three laboratories reported false-positive results for BKV13-08, BKV13-11, and BKV14-07, respectively; the 3 participants used different commercial qPCR assays targeting *StAg* or *LTAg*.

Important variability of quantitative results. The quantitative results are summarized in Table 1 and Fig. 2. The geometric mean BKVL ranged from 3.69 to 9.85 $\log_{10}$ copies/ml. The difference between the highest and the lowest viral loads reported by all participants varied from 1.32 to 5.55 $\log_{10}$, with a mean value of 2.61 $\log_{10}$. The BKVL standard deviation (SD) ranged from 0.45 to 1.07 $\log_{10}$ copies/ml. The greatest SD was observed for the BKV genotype II sample.

Technical variability of <0.5 $\log_{10}$ is considered acceptable (18, 28) and of low clinical relevance (29). Thus, we compared laboratory performance by calculating the percentage of results falling within the acceptable variation of ±0.5 $\log_{10}$ relative to the expected value. Sixty-eight percent of the reported results fell within the acceptable range (Fig. 3). Interestingly, for a plasma sample (BKV genotype I) approaching the cutoff for therapeutic intervention (BKV13-13, with an expected value of 4.26 $\log_{10}$ copies/ml), 74% of laboratories reported a BKVL of ≥4 $\log_{10}$ copies/ml (range, 4.00 to 5.40 $\log_{10}$ copies/ml), while only 26% reported BKVL of <4 $\log_{10}$ copies/ml (range, 3.52 to 3.98 $\log_{10}$ copies/ml).

The variability of BKVL in the 3 matrices (urine, WB, and plasma) was not statistically different ($P = 0.08$, Kruskal-Wallis test) (Fig. 4). Intralaboratory variability investigated by the sample replicated in the 2014 panel (BKV14-01/03) was significantly lower than the interlaboratory variability (SD, 0.10 versus 0.65; $P = 0.02$, Mann-Whitney test).

Number and position of BKV polymorphisms impact BKVL quantification. Several factors, including commercialized primers and probes, DNA extraction methods, amplification target genes, and standard materials, were previously associated with changes in viral load measurement (17, 30).

In our study, commercial assays demonstrated less variability and better performance according to the above-mentioned criterion, with 76% of the results falling within the acceptable range versus 44% for in-house assays ($P < 0.0001$, Mann-Whitney test). We further compared the results of the 2014 panel (which included 8 data sets of commercial assays other than the R-gene kit). The expected values obtained by laboratories using other commercial assays were not statistically different from those of laboratories using the R-gene kit, suggesting that the better performance of commercial assays was not biased by the high representativeness of the R-gene kit in our study (see Fig. S1 in the supplemental material). Interestingly, in a comparison of the results reported by laboratories using the same qPCR assay, we still observed differences up to 2 $\log_{10}$, suggesting that pre-qPCR steps, i.e., DNA purification, may influence interlaboratory variability.

Three DNA extraction instruments, NucliSENS easyMAG (bioMérieux, Marcy l'Etoile, France) (37%), QIAsymphony SP (Qiagen GmbH, Hilden, Germany) (19%), and MagNA Pure LC 2.0 (Roche Diagnostics, France) (15%), were predominantly used by the participants. The results obtained from 12 and 15 users of the R-gene qPCR assay in 2013 and 2014, respectively, were analyzed depending on the extraction method. The performance of DNA extraction was significantly different between the 3 platforms ($P < 0.001$, Kruskal-Wallis test). The MagNA Pure LC showed lower performance for DNA extraction in urine samples than that with both QIAsymphony and easyMAG extractors ($P = 0.02$ and <0.001, respectively, Dunn's multiple-comparison test) and for DNA extraction in plasma samples compared to QIAsymphony ($P = 0.03$, Dunn's multiple-comparison test). The QIAsymphony seemed to show a higher performance than the easyMAG extractor for WB sample DNA extraction; however, this difference was at the limit of statistical significance ($P = 0.06$, Dunn's multiple-comparison test) (see Fig. S2 in the supplemental material).

To investigate whether polymorphisms in amplification target genes might lead to inaccurate BKVL measurement, the *StAg*, *LTAg*, and *VP1* gene sequences from samples harboring a single genotype were aligned with the primers and probes of the in-house assays. One mismatch was detected in the probe-annealing site of the assay targeting *LTAg* (positions 4392 to 4567) (26) for

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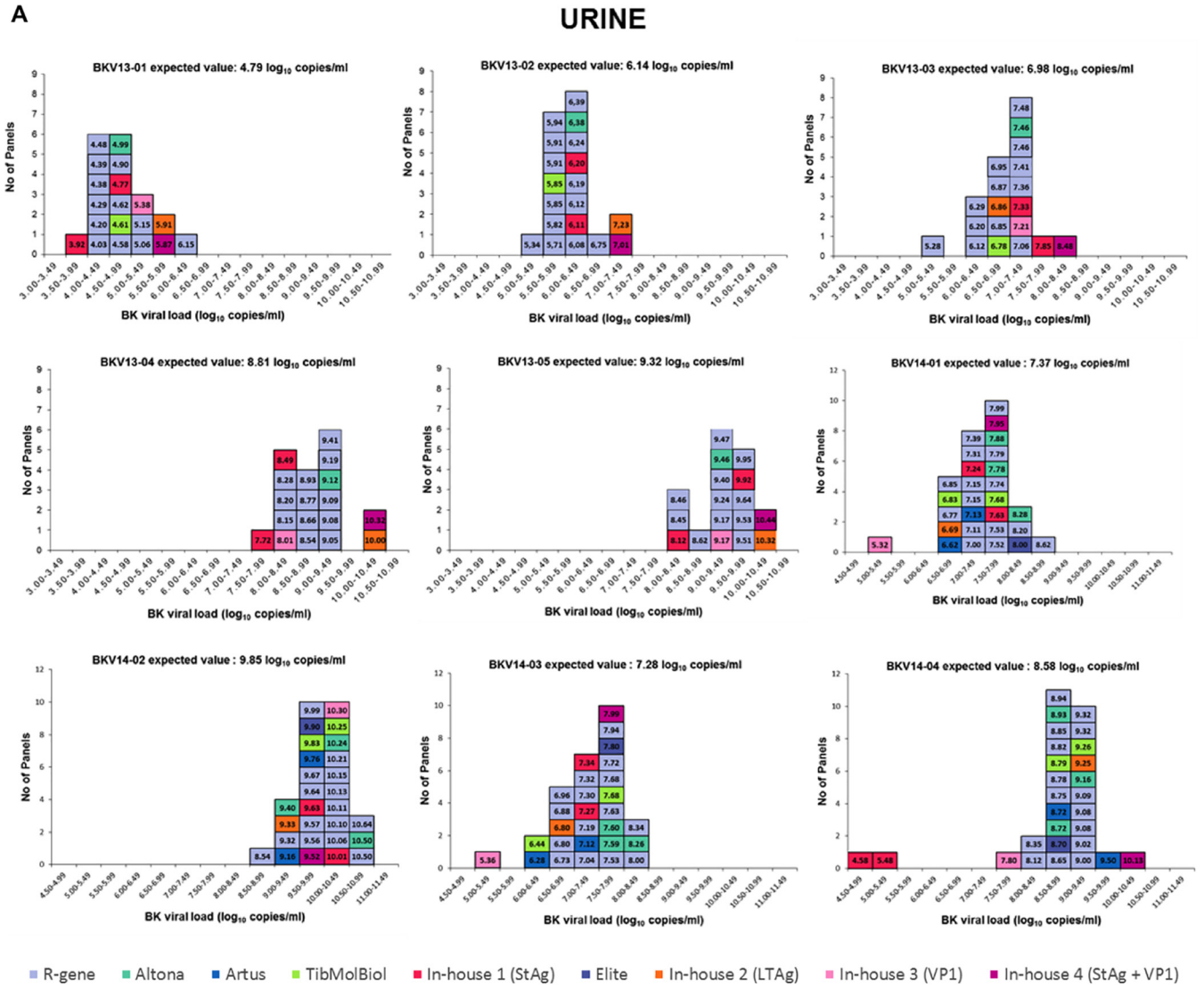


FIG 2 Histograms of reported quantitative values for the positive samples from both panels. BK viral loads are represented in 0.5-$\log_{10}$ copies/ml intervals. Each rectangle refers to the result of one data set. Each color represents a different technique. The results obtained using commercial techniques are shown in blue and green, whereas results from in-house methods are colored in orange, red, and purple. The expected value is determined for each sample by calculating the geometric mean of all reported data set values. Results were obtained for urine (A), whole-blood (B), and plasma (C) specimens.

samples BKV14-01/03, BKV14-01/02, BKV14-01/04, and BKV14-01/08, with no noticeable effect observed on BKVL measurement. In contrast, the assay targeting *VP1* (positions 2227 to 2409) underestimated BKVL values by 1.98 and 0.79 $\log_{10}$ for BKV14-01/03 (genotype IV) and BKV14-04 (genotype II) samples, respectively. Alignment of the VP1 primers and probe with VP1 sequence within the BKV14-01/03 sample revealed 5 mismatches, including 3 (G-T, T-T, and T-T) in the center of the forward primer, 1 (T-C) in the center of the reverse primer, and 1 mismatch (C-T) in the probe. Four mismatches were detected within the BKV14-04 sample, including 2 in the center of the forward primer, 1 at the 3′ end of reverse primer, and 1 in the probe. In the assay targeting *StAg* (positions 4811 to 4895) (27), a sequence analysis of the BKV14-04 sample (genotype II) revealed 1 (G-T) and 2 (C-A and C-A) mismatches at the 3′ ends of the forward and reverse primers, respectively, and an additional mutation (A-T) at the 5′ end of the probe. These mismatches are likely to significantly hinder annealing of the primers and amplification of the target DNA, and they led to BKVL underestimation by 2.61 $\log_{10}$.

Interestingly, 1 laboratory provided results close to the expected values for all but 1 sample of the 2014 panel. The BKV load for BKV14-04 (genotype II) was indeed overestimated by 1.55 $\log_{10}$. This participant used an assay adapted from Hoffman et al. (17) that was composed of a mixture of primers and probes targeting *VP1* (V3A) (positions 2458 to 2525) and *StAg* (T3A) (positions 4673 to 4770) simultaneously, in which the *StAg* primers and probe were slightly modified to improve amplification of BKV genotype II/III. As a reference material, this in-house assay used the ABI control corresponding to the BKV MM strain (GenBank accession no. V01109). Sequence analysis found no mutation in the *VP1* target gene, while 2 mutations (C4724G and A4726G)

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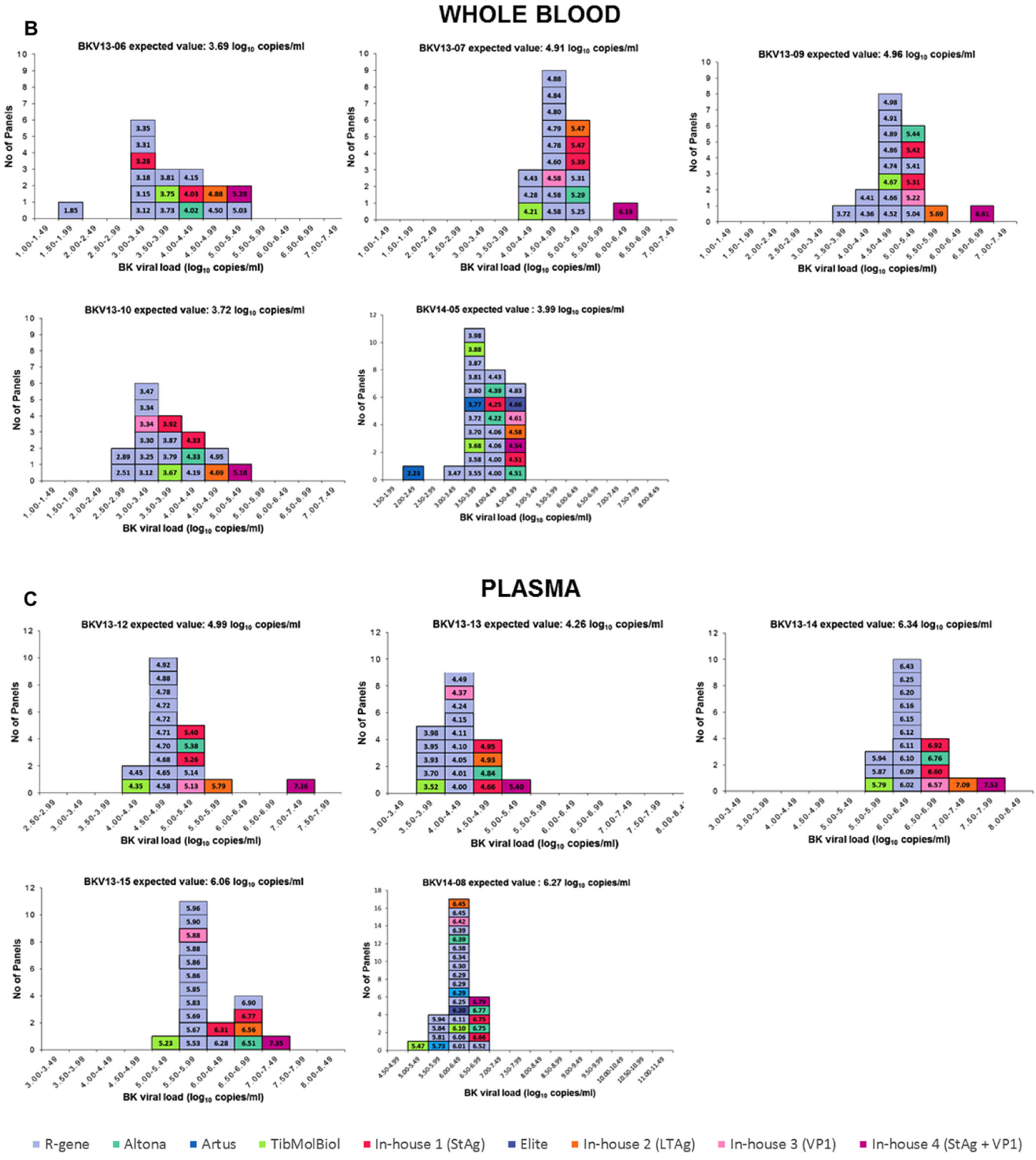
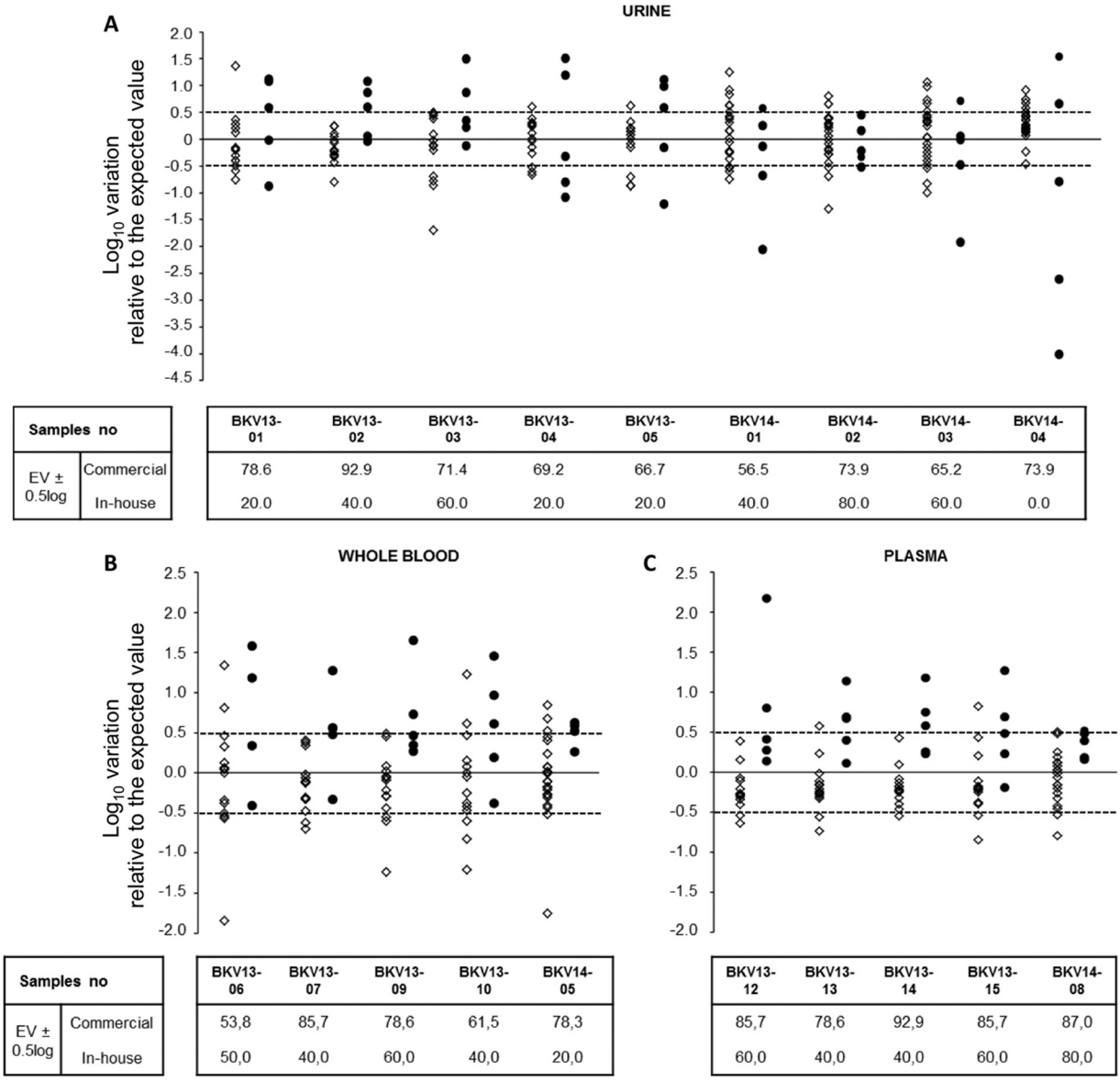


FIG 2 continued

were detected in the center of the probe of the *StAg* target gene for genotype I and IV samples. Sequence analysis of the ABI control found a deletion of 271 bp, including the *StAg* target gene. Hence, the *VP1* primers and probe perfectly match genotype I samples, genotype IV samples, and the ABI control, while mismatches in these samples and the deletion in the ABI control hinder *StAg* primers and probe annealing. In contrast, both *VP1* and *StAg* target genes match the genotype II sample, leading to an overestimation of the BKVL. It should be noted that an erroneous information is reported for the ABI control sequence in the supplier's

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Samples no		BKV13-01	BKV13-02	BKV13-03	BKV13-04	BKV13-05	BKV14-01	BKV14-02	BKV14-03	BKV14-04
EV ± 0.5log	Commercial	78.6	92.9	71.4	69.2	66.7	56.5	73.9	65.2	73.9
	In-house	20.0	40.0	60.0	20.0	20.0	40.0	80.0	60.0	0.0

Samples no		BKV13-06	BKV13-07	BKV13-09	BKV13-10	BKV14-05
EV ± 0.5log	Commercial	53,8	85,7	78,6	61,5	78,3
	In-house	50,0	40,0	60,0	40,0	20,0

BKV13-12	BKV13-13	BKV13-14	BKV13-15	BKV14-08
85,7	78,6	92,9	85,7	87,0
60,0	40,0	40,0	60,0	80,0

FIG 3 Comparison of reported results using in-house and commercial assays. The $\log_{10}$ variation in the reported results relative to the expected value (EV) was calculated for each positive sample of the panel. The results for commercial and in-house assays are depicted by open diamonds and filled circles, respectively. The dashed lines indicate a difference of ± 0.5 $\log_{10}$ relative to the EV. The tables specify the percentages of results in the interval EV $\pm$ 0.5 $\log_{10}$ for commercial and in-house assays. Results were obtained for urine (A), whole-blood (B), and plasma (C) specimens.

instructions online (tebu-bio, Le Perray-en-Yvelines, France). Indeed, the BKV MM plasmid is linearized in the *StAg* gene and not the *VP1* gene.

Infection with multiple BKV genotypes was previously described for KTR (31, 32). To further investigate whether this might impact BKVL quantification, variability between samples harboring a single genotype and those harboring multiple genotypes was analyzed by comparing their respective SD (Table 1). No significant difference was observed between the 2 groups ($P = 0.11$, Mann-Whitney test).

As several standard materials were used, there were not sufficient numbers in each group to allow any statistical comparison for all the data sets. In particular, each commercial kit used by the study participants contains its own standard material, the composition of which is proprietary. Nevertheless, we were able in our laboratory to test both panels by 4 out of the 5 commercial assays used in this study. For this intralaboratory comparison of commercial kits, assays were strictly conducted according to the manufacturers' recommendations. In particular, the calibration of each assay was performed using the standard material included in the kit. DNA was purified using a single extraction platform. The standard materials included in the commercial kits did not require DNA extraction. The percentage of results within the acceptable range increased from 75% when the assays were performed in different laboratories to 100% when they were tested by the same laboratory (see Fig. S3 in the supplemental material), suggesting that pre-qPCR steps significantly contribute to interlaboratory variability, more so than standard materials.

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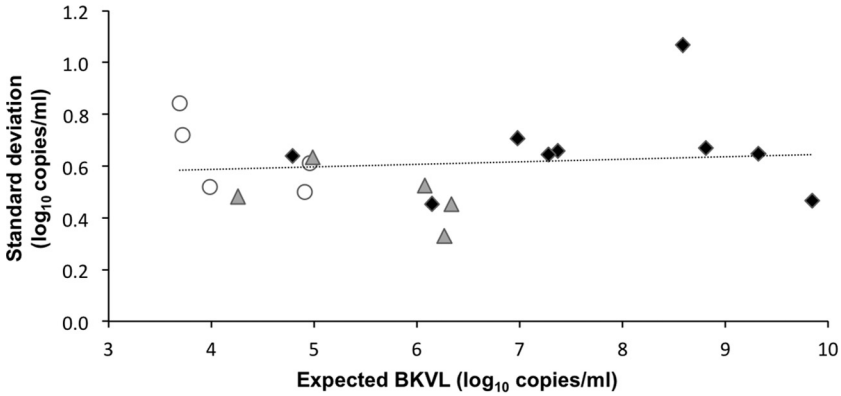


FIG 4 Distribution of the standard deviations according to the expected BKVL. Standard deviations (SD) are represented by black diamonds for urine samples, gray triangles for plasma samples, and open circles for the whole-blood samples. The SD ranged from 0.33 to 1.07 $\log_{10}$ copies/ml. No difference in variability was found across the analytical measuring range. The variability of BKVL in the 3 matrices (urine, WB, and plasma) was not statistically different ($P = 0.08$, Kruskal-Wallis test). The greatest SD (>1 $\log_{10}$) was observed for the BKV genotype II sample.

Analysis of the BKVL SD did not show any significant difference between the 3 matrices ($P = 0.08$, Kruskal-Wallis test). More specifically, no significant difference was observed between the 2 blood matrices, plasma and WB ($P = 0.11$, Mann-Whitney test) (Table 1 and Fig. 4). Last, it has been suggested that levels of viral DNA, as measured by qPCR, may be dependent on the amplicon size (19, 33). However, there were no sufficient data in each group to allow any statistical comparison.

DISCUSSION

BKVL quantification needs to be reproducible and comparable across time and techniques to ensure appropriate patient care. In this work, we demonstrate important interlaboratory variability for the monitoring of BKVL. We believe that this variability limits interinstitutional comparison and may lead to inadequate BKV infection monitoring and BKVN diagnosis and management.

The leading event of BKVN seems to be viral replication starting in tubular epithelial cells, followed by urothelial amplification and cross-feeding back to the tubular compartment (34). The urine loads of BKV measured in renal allograft recipients result more from urothelial replication (>95%) than from tubular epithelial replication (34). When viral replication can be controlled by BKV-specific cytotoxic T cells, intragraft inflammation remains limited, and no progression to BKVN ensues (35). BKVL measured in the blood illustrates persistent viral replication due to inefficient BKV-specific immunity and persistent inflammation, which lead to continued tubular epithelial damage. Thus, BKVL monitoring plays a substantial role in the early detection of BKV replication in immunocompromised patients and in improvement of the outcome of BKVN.

In this study, we elected to use urine and blood samples with different BKV genotypes (mono- and multiple infections) for interlaboratory BKVL comparison to mimic the clinical practice.

The qualitative results were overall satisfactory. The only sample with a false-negative result by a laboratory using an in-house assay was a WB specimen with a low expected BKVL. Point mutations were detected in the primers and probe, suggesting their impact on BKV detection. Three laboratories reported false-positive results for 3 different blood samples. These laboratories used different commercial qPCR kits and DNA extraction techniques. However, they performed DNA extraction of both urine and blood samples on the same platform. A major concern when extracting nucleic acids for use in amplification reactions is the risk of cross-contamination, especially when extracting BKV-strongly positive urine samples interspersed with BKV-negative samples. The urine samples within our panels harbor high BKVL, suggesting that intersample contamination occurred in the 3 laboratories.

Quantitative results showed important variability, although 68% of the reported results fell within the acceptable range. Compared to other opportunistic viruses in transplant recipients, such as CMV and EBV (18, 19, 22), it is actually encouraging that two-thirds of the reported results in our study fell within the acceptable range. This may be explained by the high number of commercial assays used in our study that had received approval in Europe. As the development of BKV assays is more recent, we believe it has benefited from important progress made in qPCR techniques in the last decade. This is illustrated by the significant difference in performance when commercial and in-house assays were compared (76% versus 44%, $P < 0.0001$).

The differences between the highest and the lowest reported BKVL averaged 2.61 $\log_{10}$ and reached 5.55 $\log_{10}$. BKV genetic polymorphisms have been described as the most significant source of error between qPCR assays (17, 22, 30). The composition of our panels, including different BKV genotypes and the multiplicity of evaluated qPCR assays, provided a unique opportunity to define for the first time the effects of mutation frequency and position on BKVL measurement. All positive samples were sequenced, and sequences were aligned with the primers and probes of the in-house assays. Mismatches were found at the primer and/or the probe annealing-sites in *VP1* (positions 2227 to 2409), and *LTAg* (26), and *StAg* (27), while the amplification target region *VP1* (positions 2458 to 2532) (17) was highly conserved in the GenBank database and in our KTR cohort (data not shown). The number of mismatches in the primers and/or probes and their distance from the 3′ end of the primer highly impacted BKVL measurement. For example, 3 mismatches at the 3′ end position of primers led to BKVL underestimation by 2.61 $\log_{10}$, while 3 other primer mismatches (1 at the 3′ end position and 2 located ≥8 bp from it) led to BKVL underestimation by 0.79 $\log_{10}$. Furthermore, no specific effect was observed with a single mismatch in the probe or at the 5′-end position of the primers. A compar-

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ison of the BKV sequences of our panel samples with sequences from the GenBank database and from our local KTR cohort revealed that these mutations were genotype specific (66 to 100% for genotype II/III and 100% for genotype IV) (data not shown). BKV genotype III is not represented in our study, since none of our patients were infected by BKV genotype III (genotype III represents <1% of the BKV isolates in Europe). However, several studies using phylogenetic and single-polymorphism analyses of BKV DNA sequences suggest that BKV genotypes II and III may belong to the same genotype, since only a few nucleotides differ between them (36–38). Furthermore, a comparison of the BKV sequences of our panel samples with sequences from the GenBank database revealed that all the mutations observed in the genotype II samples were specific not only to this genotype but also to genotype III strains.

Our BKV sequence analyses suggest that the primers and probes of most in-house assays were designed solely against genotype I, while commercial assays allow the amplification of the major BKV genotypes, as claimed by the manufacturers. Due to the proprietary nature of commercial reagents, we could not analyze the presence of mutations in the amplification target genes of commercial assays that may impact qPCR amplification. However, the high performance of commercial assays does not support the presence of such mutations. Altogether, our data suggest that commercial external quality controls for BKVL assessment should include all the major BKV genotypes to allow correct evaluation of BKV assays.

BKVL quantification may also be influenced by polymorphisms in standard materials. Henriksen et al. (39) recently reported the presence of point mutations in the plasmid pBKV Dunlop (ATCC 45025) in comparison to the published sequence and indicated that these mutations may alter BKVL quantification, since pBKV is used as a positive control in many qPCR assays (39). In our study, we demonstrate the impact of the sequence variation of a BKV control used as a standard material for quantification. Due to a deletion in this plasmid, BKVL was overestimated up to 1.55 $\log_{10}$. Our data strongly suggest that the sequence of BKV commercial standards should be provided to users to verify the absence of mismatches with the primers and probes of their BKV assays. Alternatively, virologists should sequence the commercialized control they intend to use as standard material.

The standard material used to establish the calibration curves in qPCR is an important source of interlaboratory disagreements (23). We recently demonstrated that recalibration with a common standard material significantly reduced the bias between a commercial assay and an in-house assay (23). Here, by retesting BKV panels by 4 commercial assays in a single laboratory, we show that commercial standard materials are equivalent, suggesting that this factor only poorly contributes to interlaboratory variability, at least for commercial assays. Moreover, the development of the WHO international standard will probably decrease the impact of this interlaboratory variability factor, provided the publication of the nucleotide sequence will occur to allow each laboratory and commercial assay supplier to verify the absence of mismatches with the primers and probes of their BKV qPCR assays.

The use of plasma or WB for viral load monitoring is still debated, although WB remains the preferred clinical sample type for detecting CMV and EBV in numerous diagnostic laboratories (40, 41). We recently demonstrated that the BKVL measured in plasma and WB are highly correlated (23). In the present multisite study, we further show that the variability within each matrix is not significantly different. We thus provide evidence of equivalence between plasma and WB as suitable matrices for BKV detection and quantification in peripheral blood. In the case of CMV and EBV, viral loads measured in WB reflect both free DNA and leukocyte-associated DNA (18, 19). In contrast, BKV viremia mostly reflects free DNA, although BKV DNA has been detected in peripheral blood mononuclear cells (42).

Every year, more than 3,000 patients undergo kidney transplantation in France, and most of them (~90%) are routinely screened for BKV (more than 45,000 tests/year) in the laboratories participating in this study. These laboratories use a variety of extraction techniques and qPCR assays, whether published in house (26, 27) or as commercial assays. We therefore believe that the results of this study are representative of the current status of BKV monitoring in Europe and the United States.

In summary, the results of this comparative study demonstrate that significant interlaboratory variability in BKVL quantification exists and may significantly impact patient care. Genotype II- and IV-specific polymorphisms and a range of DNA purification methods arose as major sources of interlaboratory disagreement. The optimization of primer and probe design and the standardization of DNA extraction methods may substantially decrease interlaboratory variability and allow interinstitutional studies to define a universal cutoff for presumptive BKVN and, ultimately, to ensure adequate patient care.

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