Journal of Clinical Virology 121 (2019) 104210

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Quantification of plasma BK polyomavirus loads is affected by sequence variability, amplicon length, and non-encapsidated viral DNA genome fragments

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1. Background

BK polyomavirus (BKPyV), a member of *Polyomaviridae*, is an opportunist pathogen that infects > 90% of humans and establishes asymptomatic life-long persistence in the kidneys [1]. While BKPyV remains subclinical in the general population, significant diseases occur in immunocompromised hosts, particularly after kidney transplantation (KT) and allogeneic hematopoietic cell transplantation (HCT) [2,3]. BKPyV is associated with late-onset hemorrhagic cystitis in 5%–20% of HCT recipients [4] and causes BKPyV-associated nephropathy (BKPyVAN) in 1%–15% of KT patients [5], and may cause urothelial cancer [6]. As no antiviral drugs are currently available, the paradigm of high-level viruria above 7 $\log_{10}$ copies (c)/mL or "decoy cells" followed by viremia and histologically proven viral allograft nephropathy has been adopted for patient management [7]. Indeed, BKPyV-load testing has become widely available since, and current guidelines recommend screening of plasma for BKPyV-genomes in the first 2 years after KT. In KT patients with plasma BKPyV-loads above 1000 c/mL for more than 2 weeks or increasing above 10,000 c/mL, reducing immunosuppression is advised in order to regain immune control over BKPyV replication [5,8]. BKPyV-loads are also used for diagnosis and management of hemorrhagic cystitis in HCT patients [4]. Thus, BKPyV quantitative nucleic acid testing (QNAT) is key for clinical decisions in transplant patients.

Similar to cytomegalovirus (CMV) load quantification [9,10], the clinical use of BKPyV-QNAT requires technically robust, reproducible, and standardized laboratory procedures. However, external quality assurance testing and laboratory trials suggest variability of BKPyV-loads of > 0.5 $\log_{10}$ c/mL among some of the participating diagnostic laboratories using commercial or laboratory-developed assays [11,12]. Potential reasons for the observed variability are differences in quantification standards, extraction methods, instrumentation, QNAT reagents, and in primer and probe design. The observed variability may be critical given that errors in BKPyV-quantification may seriously impact management decisions. Specifically, if BKPyV-loads are underquantified or undetectable, the timely reduction in immunosuppression might be missed or delayed, thereby increasing viral allograft damage and prolonging the time to plasma clearance [13–15]. Conversely, overestimation of BKPyV-loads may lead to inappropriate reduction of immunosuppression risking excess allograft rejection and biopsy work-up.

In order to improve harmonization of BKPyV-QNAT among diagnostic laboratories, a WHO-approved international calibrator for BKPyV from the National Institute for Biological Standards (NIBSC) in the United Kingdom has been released [16,17]. However, recent studies showed that the BKPyV NBISC-calibrator consists of multiple genome subpopulations with deletions in the T antigen-encoding sequences [18]. Furthermore, as new BKPyV sequences become available, regular update of primer and probe design is desirable to accommodate relevant BKPyV sequence variation and thereby assure optimal QNAT assay performance.

2. Objectives

As accurate and precise BKPyV-load quantification is pivotal for the clinical management of KT patients, we wished to update and validate a previously published QNAT assay targeting conserved sequences encoding the BKPyV large tumor antigen (*LTAG*) [7,19,20]. We also investigated the role of amplicon length and DNA encapsidation using DNase-I susceptibility for the newly designed assays.

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https://doi.org/10.1016/j.jcv.2019.104210
Received 9 August 2019; Received in revised form 31 October 2019; Accepted 3 November 2019
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3. Materials and methods

3.1. Phylogenetic analyses

Complete genome sequences, the *LTAG* target sequence (239 ± 200 bp; corresponds to nucleotide positions 4009–4647 in the BKPyV-WW reference genome [acc. no. AB211371.1]) and the *VP1*-target sequence (328bp ± 200bp; corresponds to nucleotide positions 1309–2036 in the BKPyV-WW reference genome [acc. no. AB211371.1] were downloaded from GenBank. In addition, *LTAG* target sequence analyses (239 ± 100bp, 239 ± 5bp, and 88 ± 5bp) were performed (see also Supplemental data; Table S1). Sequence alignments were performed with the CLC Genomic Workbench software (version 12; QIAGEN, Hilden, Germany), using default parameters (gap cost settings: gap open cost = 10; gap extension cost = 1.0). Divergences were estimated by the Jukes-Cantor method, and neighbor-joining trees were visualized with the CLC Genomic Workbench software using JC polyomavirus as outlier (acc. no. NC_001699).

3.2. Plasmids, patient samples and DNase I digestion

Plasmids harboring the *LTAG* target sequence from nucleotide position 3859–4797 of BKPyV-strain WW (acc. no. AB211371.1) or containing single nucleotide polymorphisms (SNPs) at the primer/probe binding sites were chemically synthesized into the pUC57 plasmid (Eurogentec, Belgium) and called BKPyV-WW, BKPyV-var1, BKPyV-var2, BKPyV-var3 and BKPyV-var4 (Table S2).

In order to determine the proportion of non-encapsidated BKPyV-DNA in patient samples, plasma from KT recipients were treated with DNase-I prior to DNA extraction. DNase-I digestion was performed as described previously [10]. All plasmids were diluted in TE-buffer (10 mM Tris-HCl, 1 mM EDTA; pH = 8) with salmon sperm (10 µg/µL). The BKPyV NIBSC-calibrator (Hertforshire, UK) was diluted in 1 mL nuclease free H_2O and 900 µl were added to 6.21 mL negative human plasma (final concentration of 10^6 IU/mL).

3.3. DNA extraction from patient samples and QNAT assays

Plasma samples were vortexed and aliquots of 200 µL were extracted on the QIASymphony (QIAGEN) or the MagNA Pure 96 system (Roche, Rotkreuz, Switzerland) and eluted in 100 µL elution buffer. All QNAT assays were performed with an ABI7500 Fast Real-Time PCR System (Thermo Fisher Scientific) using a reaction volume of 25 µL and 5 µL of extracted DNA.

All QNATs were done with the qPCR MasterMix Plus Low-ROX containing uridine and the uracil-*N*-glycosylase (UNG; Eurogentec), and 300 nmoles end concentration of the primers and probes (Eurogentec). The denaturation and cycling conditions for all QNATs were: 50 °C for 2 min for UNG activation; 95 °C for 10 min to inactivate UNG and activate the HotGoldStar polymerase; 45 cycles of 95 °C for 15 s and 60 °C for 60 s for annealing and extension. This applied also to quantification of the human diploid gene aspartoacylase (ACY) modifying the protocol in [21], with primer-f (5′ – CCCTGCTACGTTTATCTGATTGAG-3′), primer-r (5′ – CCCACAGGATACTTGGCTATGG-3′), and probe (5′-FAM – CCTTCCCTCAAATATGCGACCACTCG-TAMRA-3′) [6].

3.4. Sequencing of the LTAG target region

The *LTAG* target region was amplified with primers BKPyV-*LTAG*-(3883–3902): 5′-GTATTCCTTATTAACACCCTTAC-3′ and BKPyV-*LTAG*-(4564-4545): 5′-GTGGGTCCAAATAATTGGAG-3′. The amplicons were verified on a 1% agarose gel and purified using Illustra ExoProStar 1-Step (GE Healthcare, England). Sequencing was performed using the BigDye Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific), purification with Sephadex G-50 Superfine (GE Healthcare, Little Chalfont, England) and capillary electrophoresis on a 3500 Genetic Analyzer (Applied Biosystems, Rotkreuz, Switzerland). The sequences were analyzed using the CodonCode Aligner software

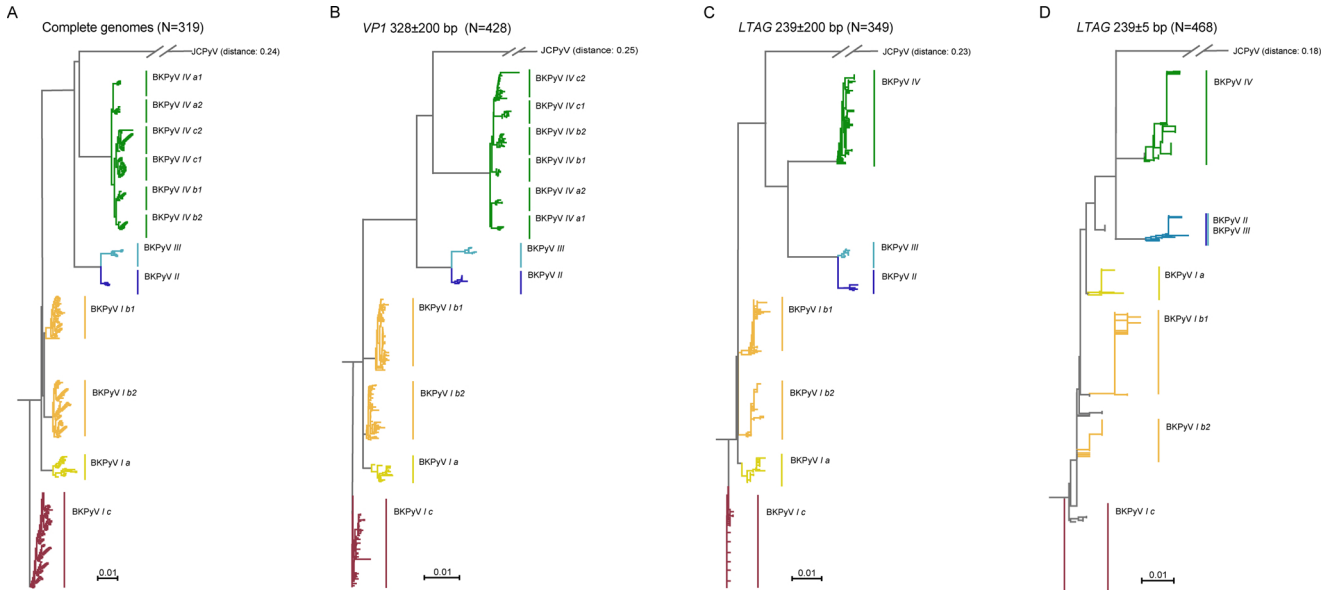

Fig. 1. Phylogenetic analysis using complete BKPyV-genome sequences or the *VP1* and *LTAG* target region sequences.
Divergences were estimated by the Jukes-Cantor method and neighbor-joining trees were constructed with the CLC Genomic Workbench software and the closely related JC polyomavirus (JCPyV) was used as outlier (acc. no. NC_001699.1).
A.Phylogenetic analysis of 319 complete BKPyV-genome sequences.
B.Phylogenetic analysis of the 328 bp *VP1*-target region (± 200bp; corresponds to nucleotide positions 1309–2036 in the BKPyV-WW reference genome [acc. no. AB211371.1]; N = 428 BKPyV GenBank entries as of October 2019).
C.Phylogenetic analysis of the 239 bp *LTAG*-target region (± 200bp; corresponds to nucleotide positions 4009–4647 in the BKPyV-WW reference genome [acc. no. AB211371.1]; N = 349 BKPyV GenBank entries as of January 2019).
D.Updated phylogenetic analysis of the 239 bp *LTAG*-target region (± 5bp; corresponds to nucleotide positions 4204–4452 in the BKPyV-WW reference genome [acc. no. AB211371.1]; N = 468 BKPyV GenBank entries as of October 2019).

(CodonCode Corporation, MA, USA).

3.5. Statistical analysis

All statistical data analysis was done in R (version 3.6.1; https://cran.r-project.org), and Prism (version 8; Graphpad Software, CA, USA) was used for data visualization. Statistical comparison of parametric variables was done using t-test. For non-parametric data, Wilcoxon tests were used. If multiple comparisons were done, Bonferroni correction was applied.

4. Results

In order to assess the genetic diversity of BKPyV, a phylogenetic tree was constructed including all full-length BKPyV-genome sequences available in the NCBI-GenBank database (N = 319) (Fig. 1A). Using the Jukes-Cantor method, the complete BKPyV-genomes clustered into the previously defined BKPyV-subtypes *I*, *II*, *III* and *IV*. Subtypes *II* and *III* were more closely related than subtypes *I* and *IV*, and permitted subtyping of BKPyV *Ia*, *Ib1*, *Ib2*, *Ic* and BKPyV *IVa1*, *IVa2*, *IVb1*, *IVb2*, *IVc1* and *IVc2*. When restricting the search to smaller BKPyV-QNAT *VP1* or *LTAG* target sequences, 428, 349 and 468 complete entries were found (Fig. 1).

Using the basic local alignment search tool analysis (BLAST; taxid:1891762) [22], 37 SNPs were identified in the BKPyV 239bp ± 200bp *LTAG*-target region with 20 SNPs in the primer/probe binding sites as of January 2019. Repeating this approach in 20 October 2019, 44 SNPs were identified in *LTAG*-target sequences with one additional SNP in the primer/probe binding sites (A4327 G, frequency 0.2%; Table 1). Moreover, no insertions or deletions were found in the 239bp *LTAG*-target sequence. Based on the standard *LTAG*-(2.1)-133bp QNAT [20], new assays were designed that covered all SNPs occurring with a frequency of at least 5%, 1.5%, and 0.3% (Table 1). In addition, three different reverse primers were designed that generated amplicons of 88, 133 and 239 bp. The SNPs, primers and probe sequences of the four new *LTAG*-QNAT assays are summarized in Fig. 2.

First, the diagnostic performance of the standard *LTAG*-(2.1)-133bp was compared with the *LTAG*-(3.1)-88bp. Good amplification efficiency with a slope of -3.4 and a correlation coefficient above 0.99 was found. A sensitivity analysis of 20 replicas of 2-fold indicated a 50 percent detection threshold at 3 copies/reaction (data not shown). Next, 58 plasma samples including 3 patients with biopsy-proven nephropathy were prospectively analyzed (Fig. 3) Significantly higher BKPyV-loads were obtained with the new *LTAG*-(3.1)-88bp compared to the standard *LTAG*-(2.1)-133bp ($P < 0.001$; Fig. 3A). Bland-Altman analysis showed 0.56 $\log_{10}$ c/mL higher BKPyV-loads in the *LTAG*-(3.1)-88bp (Fig. 3B).

In order to assess the contribution of non-encapsidated viral DNA-genome fragments to the viral load results, BKPyV-quantification was performed prospectively in parallel without and with DNase-I treatment prior to nucleic acid extraction. The results indicated that DNase-I digestion significantly reduced the plasma BKPyV-loads by approximately 90% in the *LTAG*-(2.1)-133bp and the *LTAG*-(3.1)-88bp (1.0 ± 0.6 $\log_{10}$ c/mL and 2 ± 0.6 $\log_{10}$ c/mL, respectively; $P < 0.001$; Fig. 3C).

Table 1
Frequency of single nucleotide polymorphisms in the BKPyV LTAG target region.

Position	SNP[1]	Frequency (%)	Frequency (%)[2]			Position	SNP	Frequency (%)	Frequency (%)		
		239bp ±200bp	239bp ±100bp	239bp ±5bp	88bp ±5bp			239bp ±200bp	239bp ±100bp	239bp ±5bp	88bp ±5bp
4210	G → A	5.1	[5.1]	[6.2]	[6.2]	4327	A → G	0.0	[0.2]	[0.2]	-
4216	A → G	5.9	[6.7]	[6.6]	[6.6]	4331	G → C	2.6	[1.6]	[1.5]	-
4219	G → A	20.7	[23.7]	[23.3]	[23.2]	4338	C → T	0.3	[0.4]	[0.4]	-
4219	G → T	5.0	[5.1]	[6.2]	[6.2]	4339	T → C	21.2	[23.5]	[23.1]	-
4231	A → G	0.3	[0.2]	[0.2]	[0.2]	4342	T → C	3.5	[23.5]	[23.1]	-
4234	T → C	0.3	[0.2]	[0.2]	[0.2]	4351	G → A	1.2	[1.6]	[1.5]	-
4240	T → A	0.3	[0.2]	[0.2]	[0.2]	4356	A → G	0.3	[0.2]	[0.2]	-
4243	A → G	1.5	[2.7]	[2.6]	[2.6]	4363	T → C	4.9	[5.1]	[6.2]	-
4252	C → A	0.3	[0.2]	[0.2]	[0.2]	4372	A → G	7.2	[27.5]	[28.2]	-
4252	C → T	0.3	[0.2]	[0.2]	[0.2]	4375	T → C	0.0	[0.2]	[0.2]	-
4260	G → C	0.3	[0.0]	[0.2]	[0.2]	4378	A → G	6.1	[6.0]	[6.0]	-
4265	G → C	1.5	[1.1]	[1.1]	[1.1]	4381	T → G	0.3	[0.2]	[0.2]	-
4267	A → G	14.5	[13.3]	[14.7]	[14.9]	4390	T → C	0.0	[0.2]	[0.2]	-
4293	T → C	0.3	[0.2]	[0.2]	[0.2]	4402	G → A	0.9	[0.9]	[0.9]	-
4297	C → T	82.8	[76.3]	[76.5]	-	4405	G → A	50.4	[48.1]	[47.7]	-
4303	C → T	29.6	[29.9]	[31.0]	-	4405	G → C	0.0	[14.4]	[14.3]	-
4306	G → T	0.3	[0.2]	[0.2]	-	4406	C → T	0.3	[0.2]	[0.2]	-
4306	G → C	0.0	[0.2]	[0.2]	-	4414	G → A	2.3	[2.7]	[2.8]	-
4312	T → C	1.8	[1.3]	[1.3]	-	4414	G → T	0.0	[0.2]	[0.2]	-
4315	G → A	1.5	[3.3]	[3.2]	-	4417	C → T	0.0	[0.2]	[0.2]	-
4315	G → T	81.7	[73.0]	[73.3]	-	4438	G → T	0.3	[0.2]	[0.2]	-
4324	A → G	27.8	[28.8]	[29.5]	-	4444	C → T	0.3	[0.2]	[0.2]	-

[1] SNP (single nucleotide polymorphism) and frequency among 349 BKPyV (taxid 1891762) *LTAG* GenBank sequences compared to BKPyV WW as reference genome (acc. no. AB211371).
Colored backgrounds indicate a SNP at the primer or probe binding sites.
(forward primer-yellow; probe-grey; reverse primers: green- 88bp, orange- 133bp and blue- 239bp).

Fig. 2. Sequence of *LTAG* target sequence and position of BKPyV-QNAT primers and probes.
Frequencies of single nucleotide polymorphisms (SNPs) are indicated by color (green ≥5.0%, yellow ≥1.5%, and red ≥0.3%). SNPs identified in the repeated analysis as of October 2019 are marked in blue. Top: Overview of SNPs identified in the *LTAG* target sequence of 349 BKPyV GenBank entries. Forward primer (yellow) and probe (grey) were used for all assays, reverse primers generated different amplicon lengths (88 bp, 133 bp and 239 bp). Bottom: *LTAG* target sequence is displayed (nucleotide positions 4209–4447 in the BKPyV-WW reference genome [acc. no. AB211371.1]). The standard *LTAG*-(2.1)-133bp and the new *LTAG* QNAT assays as detailed in Table 1.

Of 58 plasma samples, BKPyV-loads were undetectable after DNase-I digestion in 14 (24.1%) by the *LTAG*-(2.1)-133bp and 11 (19.0%) by the *LTAG*-(3.1)-88bp (Fig. 3C). Similarly, cell-free human genomic DNA levels in plasma measured by the ACY QNAT was significantly reduced by prior DNase-I digestion by 1.3 ± 0.6 $\log_{10}$ c/mL (Fig. 3C). The susceptibility to DNAse-I digestion (defined as a reduction in viral load of > 1 $\log_{10}$ c/mL) was not dependent on the initial plasma BKPyV-loads (Fig. 3D).

Taken together, the updated version *LTAG*-(3.1)-88bp with a smaller amplicon, but accommodating all reported SNPs with frequencies of at least 5% performed equally well or better than our present standard *LTAG*-(2.1)-133bp assay. Moreover, DNase I-sensitive non-encapsidated BKPyV-DNA accounted for more than 90% of the plasma BKPyV-loads in transplant patients and could be quantified together with cell-free human DNA.

To cover SNPs present at lower frequency of at least 1.5% or 0.3% reflecting 5 and 1 reported SNPs in the GenBank sequences, respectively (Table 2), nine further assays were designed (Fig. 2). Plasmids served as targets containing the BKPyV-WW reference as well as four chemically synthesized SNP-derived *LTAG*-variants (Table S1).

The general performance was evaluated using plasmid concentrations of 100, 10000 and 1 million copies per reaction (Fig. 4A). Overall, the *LTAG*-(3.2)-88bp assay performed best with all variant SNP-plasmids except the BKPyV-var4. The standard *LTAG*-(2.1)-133bp showed intermediate detection characteristics, but also failed to quantify the BKPyV-var4. Finally, assays with a larger amplicon size tended to have higher cycling threshold (Ct) values. In detail, the *LTAG*-(3.2) QNAT assays showed a higher amplification efficiency for the BKPyV-WW archetype and the BKPyV-var1 sequence, compared to the *LTAG*-(3.3) and the *LTAG*-(3.4) assays, for which the Ct values were 3–5 cycles higher in order to identify the same target load (Fig. 4A). When using the BKPyV-var2 and the BKPyV-var3 targets, the *LTAG*-3.2 QNAT assays still showed the highest amplification efficiency; although, the difference to the *LTAG*-(3.3) and *LTAG*-(3.4) QNAT assays was now reduced to only 1–2 Ct values. When using the BKPyV-var4 sequence that synthetically combined all possible, but not naturally observed SNPs in one target sequence, only the *LTAG*-(3.3) and *LTAG*-(3.4) assays showed a fluorescence signal (Fig. 4A). The QNAT assays having the 239bp-amplicons required on average 3 Ct values more to detect the same target load than the 133bp and 88bp assays (Fig. 4A). The performance of the *LTAG*-(3.2) assays was further characterized by the limiting dilution sensitivity analysis performing equally well or better

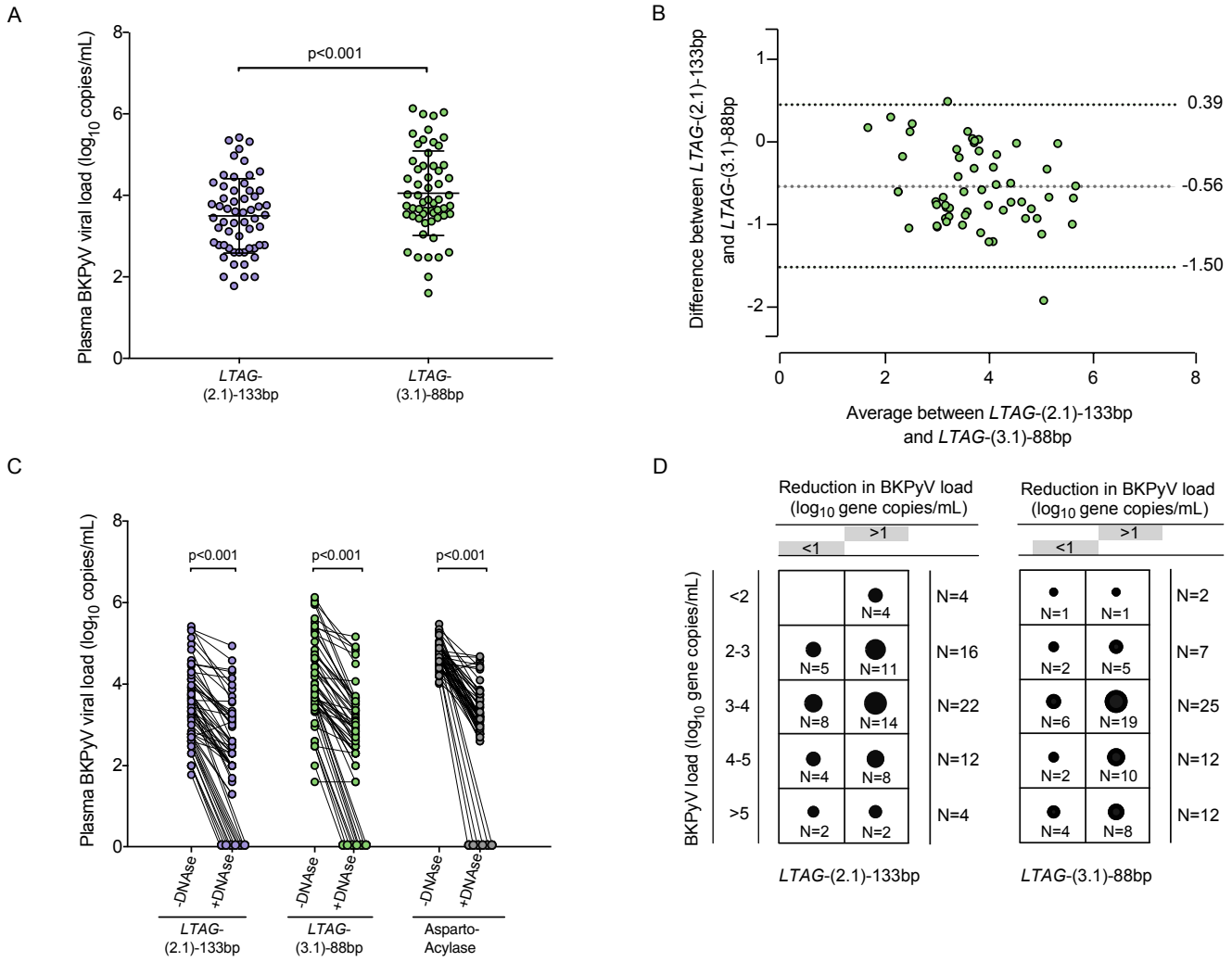

Fig. 3. Prospective comparison of the standard *LTAG*-(2.1)-133bp and the new *LTAG*-(3.1)-88bp QNAT assays.
Plasma samples from transplant patients submitted for routine testing using standard *LTAG*-(2.1)-133bp as described previously were tested in parallel as detailed below.
A. Plasma BKPyV-loads determined with the indicated BKPyV-QNAT (mean ± standard deviation; t-test).
B. Bland-Altman analysis indicating a mean bias of -0.56 $\log_{10}$ c/mL (95% limit of agreement −1.5 and 0.39).
C. DNase-I sensitivity of plasma BKPyV-loads using the indicated BKPyV-QNATs as well as of cell-free human genomic DNA using the aspartoacylase QNAT (Wilcoxon test).
D. Association of initial BKPyV-load (vertical category) and DNase-I susceptibility defined as a reduction in BKPyV-load of > 1 $\log_{10}$ c/mL (horizontal category) was assessed by X^2-test. The number of patient samples in each category are reflected by the dot size in the bubble blot.

than the ones found for the other assays (Fig. 4B, and data not shown). Based on the performance and SNP coverage of at least 1.5%, the *LTAG*-(3.2) assays were further evaluated on clinical samples.

For a retrospective analysis, 40 frozen plasma samples from KT patients were analyzed with the *LTAG*-(3.2) QNAT assays. These samples had been initially quantified using the standard *LTAG*-(2.1)-133bp assay in diagnostic routine and had been stored for < 6 months at −20 °C. Notably, 6 samples with low plasma BKPyV-loads ranging from 1.8 to 2.8 $\log_{10}$ c/mL in the standard *LTAG*-(2.1)-133bp were now undetectable with the *LTAG*-(3.2)-133bp and the *LTAG*-(3.2)-239bp, while the *LTAG*-(3.2)-88bp still detected BKPyV-DNA in four of them with loads ranging from 0.7 to 2.7 $\log_{10}$ c/mL (Fig. S1). In the remaining samples, significantly lower BKPyV-loads were obtained with the *LTAG*-(3.2)-133bp and the *LTAG*-(3.2)-239bp assays (P < 0.001). Despite the smaller amplicon, the BKPyV-loads obtained by the *LTAG*-(3.2)-88bp assay were statistically not different to the ones obtained by the standard *LTAG*-(2.1)-133bp assay (P = 0.55). This was also reflected in the Spearman rank correlation and Bland-Altman analyses (Fig. S2). Following DNase-I digestion, the BKPyV-loads were significantly reduced for all assays by more than 90% and the rate of undetectable results after DNase-I digestion was approximately twice that of the prospective analysis (Fig. S1)

To circumvent the potential role of freezing and thawing on the non-encapsidated BKPyV-DNA, 34 plasma samples were prospectively analyzed in parallel. Indeed, plasma BKPyV-loads were significantly higher using the *LTAG*-(3.2)-88bp compared to the standard *LTAG*-(2.1)-133bp assay (P < 0.05; Fig. 5A). Accordingly, Spearman rank correlation and Bland-Altman analyses indicated higher values across a range of BKPyV-loads (mean difference of -0.72 $\log_{10}$ c/mL; Fig. 5B). The analysis also showed partially discordant results between the old and the new assay for three different samples having very low viral loads at the limit of detection. The Ct-difference between the duplicates suggests that quantification for low BKPyV loads is not reliable probably due to the stochastic distribution of the targets in the extracts.

No difference was observed for *LTAG*-(3.2)-133bp and the standard *LTAG*-(2.1)-133bp having the same amplicon size, whereas lower

Table 2
Primer and probe sequences used in the *LTAG* QNAT assays and their positions in the BKPyV-WW reference genome (acc. no. AB211371.1).

Primer/ Probe Set	SNP Frequency	Primer/Probe	Sequence[1]	Position[2]	Primer Length (nt)	Amplicon Length (bp)
LTAG-(3.1)	≥1.5%	BKPyV-*LTAG*-4209-f	ARC AGG CRA GDG TTC TAT TAC TAA AT	4209 – 4234	25	
	≥5.0%	BKPyV-*LTAG*-4238-p	6-FAM-TCC YTC TGA TCT ACA CCA GTT TCT TAG TCA AGC-TAMRA	4270 – 4238	32	
	≥1.5%	BKPyV-*LTAG*-4296-r	AGA AAG GTA GAA GAC CCT AAA GAC	4296 – 4273	23	88
LTAG-(3.2)	≥1.5%	BKPyV-*LTAG*-4209-f	ARC AGG CRA GDG TTC TAT TAC TAA AT	4209 – 4234	25	
	≥1.5%	BKPyV-*LTAG*-4238-p	6-FAM-TCC YTS TGA TCT ACA CCA GTT TCT TAG YCA AGC- TAMRA	4270 - 4238	32	
	≥0.3%	BKPyV-*LTAG*-4296-r	AGA RAG GTA GAA GAC CCT AAA GAC	4296 – 4273	23	88
	≥0.3%	BKPyV-*LTAG*-4341-r	GAR RCA ACA GSA GAT TCYCAA CA	4341 – 4319	22	133
	≥0.3%	BKPyV-*LTAG*-4447-r	GGT RCC AAC MTA TGG AAC AGA A	4447 – 4428	19	239
LTAG-(3.3)	≥0.3%	BKPyV-*LTAG*-4209-f	ARC AGG CRA GDG TTC TAT TAC TRA AY	4209 – 4234	25	
	≥0.3%	BKPyV-*LTAG*-4238-p	6-FAM-TCC YTS TGA TST ACA CCA RTT TCT TAG YCA-TAMRA	4270 – 4235	32	
	≥0.3%	BKPyV-*LTAG*-4296-r	AGA RAG GTA GAA GAC CCT AAA GAC	4296 – 4273	23	88
	≥0.3%	BKPyV-*LTAG*-4341-r	GAR RCA ACA GSA GAT TCYCAA CA	4341 – 4319	22	133
	≥0.3%	BKPyV-*LTAG*-4447-r	GGT RCC AAC MTA TGG AAC AGA A	4447 – 4428	19	239
LTAG-(3.4)	≥0.3%	BKPyV-*LTAG*-4209-f	ARC AGG CRA GDG TTC TAT TAC TRA AY	4209 – 4234	25	
	≥0.3%	BKPyV-*LTAG*-4238-p	6-FAM-TCC YTS TGA TST ACA CCA RTT TCT TAG-TAMRA	4270 – 4232	32	
	≥0.3%	BKPyV-*LTAG*-4296-r	AGA RAG GTA GAA GAC CCT AAA GAC	4296 – 4273	23	88
	≥0.3%	BKPyV-*LTAG*-4341-r	GAR RCA ACA GSA GAT TCYCAA CA	4341 – 4319	22	133
	≥0.3%	BKPyV-*LTAG*-4447-r	GGT RCC AAC MTA TGG AAC AGA A	4447 – 4428	19	239

SNP, single nucleotide polymorphism; nt, nucleotide; bp, base pair; f, forward; p, probe, r, reverse; FAM, carboxyfluorescein; TAMRA, Tetramethylrhodamine.
[1] Degenerate bases are indicated as follows: R, A or G; D, A or G or T; Y, T or C; S, G or C; M, A or C {Johnson, 2010 #17859}.
[2] Positions according to BKPyV-WW genome sequence (acc. no. AB211371.1).

plasma BKPyV-loads were observed for the *LTAG*-(3.2)-239bp assay ($P < 0.001$; Fig. 5). DNase-I treatment significantly reduced the plasma BKPyV-loads in all assays (Fig. 5C).

To investigate whether SNPs might contribute to the differences in plasma BKPyV-loads, seven patients with prospectively collected samples with > 0.5 $\log_{10}$ c/mL higher loads in the *LTAG*-(3.2)-88bp assay were identified. In samples from patients 1, 2 and 5, we identified a G4219A exchange in the forward primer and the A4267 G exchange in the probe that affected both assays. One sample from patient 6 having biopsy-proven nephropathy (PyVAN-B3; PyVL-3) showed a quantification difference of 1.0 $\log_{10}$ c/mL, where sequencing revealed relevant SNPs (Table S2).

Using the WHO-approved NIBSC-calibrator, we obtained a substantial deviation of approximately 2 $\log_{10}$ lower copy numbers per mL than expected (Fig. 6). Since a recent report indicated that a majority of BKPyV-genome reads had substantial deletions in the *LTAG*-sequences including our target region [18], the standard was spiked with a similar amount of defined BKPyV-WW plasmid and extracted without and with prior DNase-I digestion. The results demonstrated that quantification of the NIBSC-calibrator spiked plasmids yielded the expected BKPyV-loads comparable to the plasmids alone. Unlike for the plasmid controls, residual BKPyV-loads were now detected in the plasmid-spiked standard after DNase-I treatment. This suggested that the BKPyV-loads detectable in the NIBSC-calibrator were mostly derived from complete and encapsidated BKPyV-genomes.

5. Discussion

Sensitive and specific quantification of plasma BKPyV-loads has become pivotal for the clinical management of HCT and KT patients [4,5,23]. Accordingly, regular review of the assay performance remains a cornerstone, supported by internal and external quality assurance [10]. In addition, available viral genome sequences should be regularly reviewed for the presence of previously unknown sequence variations, which may potentially lead to under-quantification or false-negative results.

In this study, we systematically examined all BKPyV-genome sequences available in the NCBI-GenBank database with particular focus on our published viral *LTAG*-target region [20]. Although we and others have chosen the *LTAG*-target as a more conserved region across different BKPyV-genotypes [19,20,24,25], we identified 44 SNPs, of which 20 SNPs were located in the primer- or probe-binding sequences, and 1 additional one upon repeat analysis in October 2019. Thus, DNA virus sequences also represent a moving target for diagnostic detection, for which updates and quality assurance should be provided and transparently made available for both laboratory-developed and commercial tests.

Applying a SNP frequency of 5% to be covered by degenerate primer and probes, the new updated QNAT version *LTAG*-(3.1)-88bp could be technically and clinically validated in prospectively collected plasma samples from transplant patients. As expected from our previous work and that of others on CMV-QNAT [10,26], the smaller 88bp-amplicon yielded significantly higher BKPyV-load results above the technical variation coefficient. DNase-I digestion prior to extraction revealed that more than 90% of plasma BKPyV-loads represented non-encapsidated genome fragments. This interpretation was supported by the fact that cell-free human DNA was similarly DNase-sensitive using an assay, which we routinely use for estimating the amount of amplifiable human DNA in nucleic acid extractions from plasma, whole-blood and biopsies [27,28]. The DNase-I sensitivity of the BKPyV-loads was not significantly different over a range of plasma BKPyV-loads from 100 up to 1 million c/mL and included 3 patients with biopsy-proven nephropathy. Thus, our previously coined and widely used term “BKPyV-viremia” [7,19] seems no longer appropriate and should be replaced by “BKPyV-DNAemia” as suggested [5]. This terminology has also been adopted for CMV-DNAemia in recent guidelines [9].

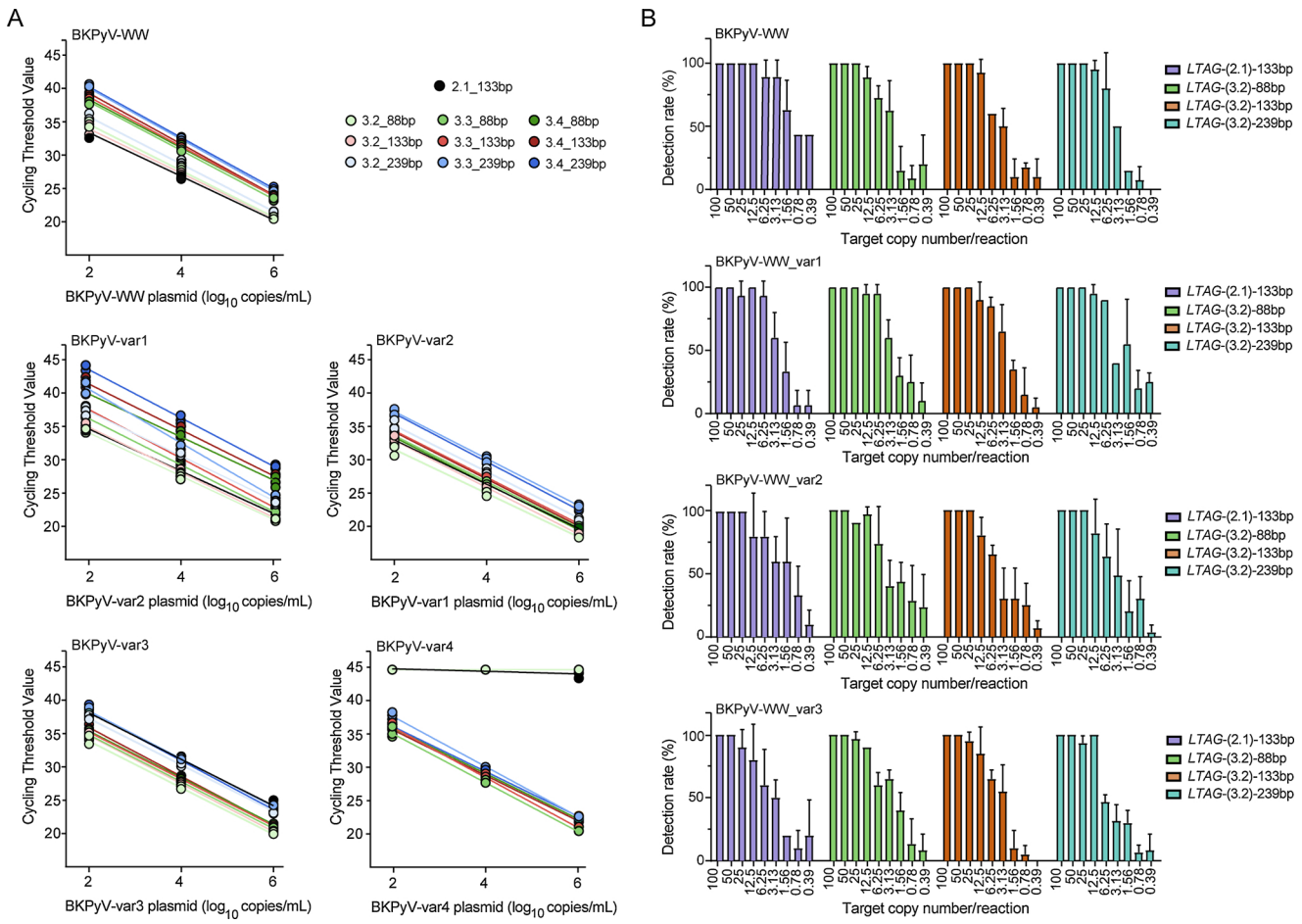

Fig. 4. Influence of primers and probe degeneration and target length on BKPyV- load quantification.
The performance of the standard *LTAG*-(2.1)-133bp assay was compared with the indicated QNAT assays detailed in Table 1 accommodating rare SNPs of less than 5% using plasmids denoted BKPyV-WW (archetype reference), as well as SNP-containing variants BKPyV-var1, BKPyV-var2, BKPyV-var3, and BKPyV-var4 (Table S2).
A. Cycle thresholds obtained with the indicated QNAT assays (color legend) for 10^6, 10^4, and 10^2 copies per reaction of the indicated plasmid targets.
B. Detection rate of limiting dilution of the indicated target plasmids for BKPyV *LTAG*-(3.2)-88bp, -133bp and -239bp, tested in 20 replicas.

Since the NCBI-GenBank entries and respective SNP frequencies are subject to change as more BKPyV-sequences are being submitted, the design of BKPyV-assays covering SNPs of lower frequencies is of interest. To this end, we designed nine further assays having amplicon sizes of 88 bp, 133 bp, and 239 bp, all of which were evaluated using plasmids harboring the archetype-WW sequence as reference or synthetic sequences with increasing number of SNPs (Fig. 2). Although our current standard QNAT *LTAG*-(2.1)-133bp appeared surprisingly robust, the quantification became insufficient or failed as SNPs increased. In contrast, the newly designed assays performed better as indicated by lower Ct numbers (i.e. 8- to 32-fold higher amplification) and the better detection rate. From these data, the *LTAG*-(3.2)-88bp covering SNPs of as low as 1.5% performed best and was taken further to the clinical evaluation together with its 133bp- and 239bp-derivatives.

In the retrospective analysis on frozen/thawed plasma samples, however, we noted that the BKPyV-loads generated by the *LTAG*-(3.2)-88bp were not significantly higher than the initial BKPyV loads generated by the *LTAG*-(2.1)-133bp assay. The *LTAG*-(3.2)-133bp and 239bp-derivatives generated 1–3 $\log_{10}$ c/mL lower values and a larger fraction of undetectable results as the amplicon size increased. This difference was not observed when using defined copy numbers of the plasmid targets. Given the fact that more than 90% of the plasma BKPyV-loads were susceptible to DNase-I digestion, we reasoned that this was best explained by the presence of unprotected non-encapsidated BKPyV-genome targets that were subject to facilitated degradation following freezing and thawing. This notion was supported by the prospective evaluation of plasma BKPyV-loads, in which the *LTAG*-(3.2)-88bp, -133bp, and -239bp assays were directly performed in parallel with the standard *LTAG*-(2.1)-133bp. Now, the *LTAG*-(3.2)-88bp yielded an approximately 0.7 $\log_{10}$ c/mL higher BKPyV-load than both, the standard *LTAG*-(2.1)-133bp- and *LTAG*-(3.2)-133bp-assay, whereas the *LTAG*-(3.2)-239bp-assay provided lower loads and detection rates. The impact of amplicon length was consistent and most pronounced for the -239bp assay in samples with low BKPyV copy numbers, leading to substantial under-quantification and even false-negative results.

Finally, we applied the new assays to the WHO-approved NIBSC-calibrator. In line with the results by Bateman and colleagues reporting substantial deletions in the T antigen region [18], all *LTAG*-assays showed 2 $\log_{10}$ lower BKPyV-loads than the presumed average copy number expressed as international units. Spiking of the NIBSC-calibrator with defined copy numbers of BKPyV-WW plasmid yielded the expected results ruling out that this observation was due to inhibition. Moreover, DNase-I digestion suggested that the residual NIBSC-calibrator loads quantifiable by the *LTAG*-assays originated in presumably encapsidated intact BKPyV-genomes. The results question the use of poorly defined BKPyV-genome targets as universal standards.

Some potential limitations of this study deserve attention. The choice of degenerated positions in the primers and probes was based on the frequency of SNPs present in BKPyV-*LTAG* sequences published in

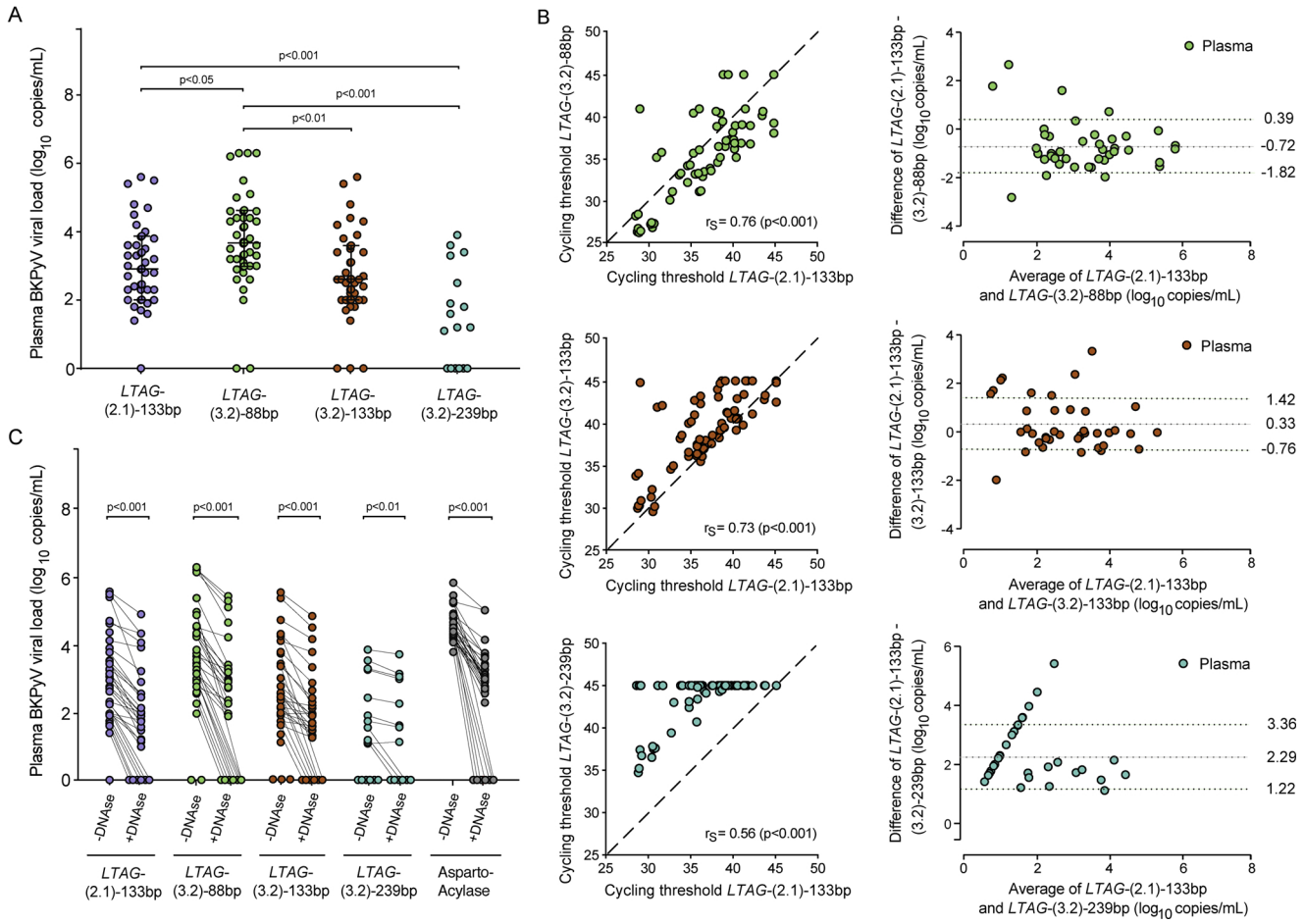

Fig. 5. Prospective comparison of BKPyV-loads in plasma using the standard *LTAG*-(2.1)-133bp and the *LTAG*-(3.2) QNAT assays.
Plasma samples from transplant patients (N = 34) submitted for routine determination of BKPyV-loads were prospectively analyzed in parallel using the standard *LTAG*-(2.1)-133bp and the indicated *LTAG*-(3.1) QNAT assays, with or without DNase-I digestion prior to nucleic acid extraction.
A. Plasma BKPyV-loads determined with the indicated BKPyV-QNAT (mean ± standard deviation; Wilcoxon test).
B. Spearman rank correlation of cycle thresholds (left panels) and Bland-Altman analysis of BKPyV-loads (right panels) of *LTAG*-(2.1)-133bp and the indicated *LTAG*-(3.2)-88bp, -133bp, and 239bp assays.
C. DNase-I sensitivity of plasma BKPyV-loads using the indicated BKPyV-QNATs as well as of cell-free human genomic DNA using the aspartoacylase QNAT (Wilcoxon test).

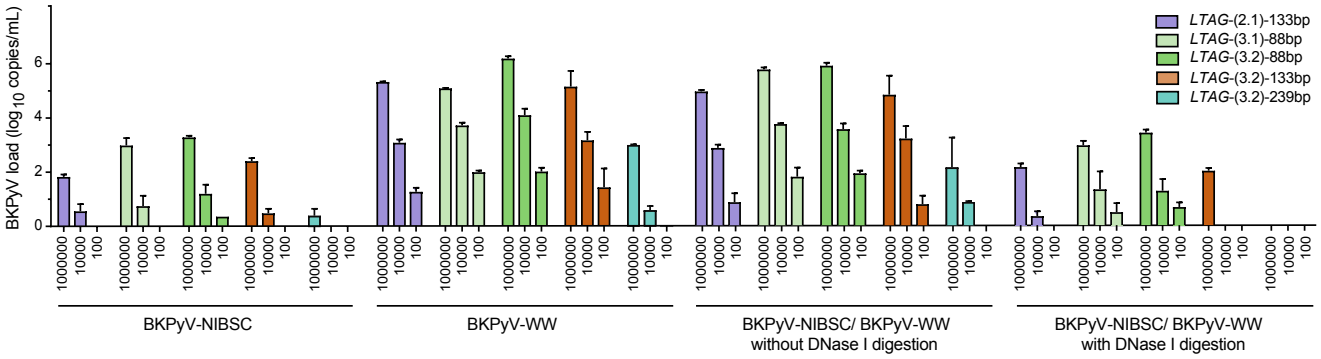

Fig. 6. Quantification of the WHO-approved BKPyV NIBSC-calibrator using the *LTAG*-QNAT assays.
The NIBSC-calibrator and plasmids were prepared as outlined in Materials & Methods, and quantified using the indicated assays. BKPyV-NIBSC, the input of the standard; BKPyV-WW, the input of archetype plasmid, BKPyV-NIBSC/BKPyV-WW, mixture of NIBSC-calibrator and plasmid, all at the indicated copy/target number, extracted either with or without prior DNase-I digestion.

the NCBI-GenBank database. Although the data are obtained from different parts and populations from different parts of the world, the collection likely does not reflect the entire epidemiological frequency of BKPyV-variants. Thus, these database entries must be considered to represent minimal estimates of genome variation. Moreover, detection bias towards the known sequences may obscure the detection rates of other variants. As shown here for a limited number of patients including those with biopsy-proven BKPyVAN, more SNPs with potential impact on quantification may emerge. Indeed, we identified two new BKPyV-variants (C4287 T and A4289C) that have not been reported previously.

Database submission of quality-controlled sequences may help to improve this diagnostic challenge and should be encouraged. Also, since production of degenerate primers may vary from batch-to-batch affecting QNAT read-outs, we recommend to manually combine the desired proportions of the single oligonucleotides with degeneration in critical base positions such as in the 3′ terminal ends. While successful tackling the SNPs and lowering the amplicon size of QNAT assays may improve detection of very low viral loads, differences between replicates may remain suggesting that detection and the lower level of quantification may be affected by the stochastic distribution of the targets in the extraction eluates, a topic to be considered when different extraction procedures are used.

Finally, use of whole-genome, massive parallel sequencing technology allows for thorough characterization of BKPyV minority variants from clinical samples and enables to study their association with the development of HCT or BKPyVAN [29]. Although very interesting and potentially relevant for clinical management of transplant recipients, this is out of the scope of the present study.

Given these results, one may raise the question whether or not urine BKPyV-loads also represent largely DNAuria. We have not studied this question at this time, since urine BKPyV-loads may be more variable in a given patient, and hence, the general clinical recommendations and decisions are based on plasma BKPyV-loads. Moreover, urine cytology showing the presence of BKPyV-infected “decoy cells” and electron microscopy studies showing the presence of polyomavirus particles and aggregates (“haufen”) indicate that substantial parts may be protected against DNase-I digestion.

6. Conclusion

This study systematically investigates the effects of amplicon size, primer/probe degeneration and DNase-sensitivity of plasma BKPyV-loads and their effects on BKPyV- quantification. Specifically, higher BKPyV-load quantification was observed with the *LTAG*-(3.1)-88bp and *LTAG*-(3.2)-88bp covering the 5% and 1.5% SNP thresholds compared to our current *LTAG*-(2.1)-133bp assay, which is mainly attributable to the amplicon size. In addition, novel SNPs in assay target sequences and the fact that plasma BKPyV-loads are mostly derived from non-encapsidated BKPyV-genomes may affect the quality of BKPyV- quantification. Thus, detection of plasma BKPyV-loads should be called “BKPyV-DNAemia “rather than “BKPyV-viremia”, a conceptual difference that may directly impact pre-analytic handling because of the potential instability of the QNAT genome target. Finally, the generation of commutable international standards should provide intact genome targets of known sequences, which provide units based on amplicon sizes of not more than 100 bp.

Ethics

The study was conducted according to good laboratory practice and in accordance with the Declaration of Helsinki and national and institutional standards. The Swiss act on medical research involving human subjects does not apply to this study.

Funding

This study was supported by the Clinical Virology Division, Laboratory Medicine, University Hospital Basel, Basel, Switzerland, and appointment grant to HHH, Department Biomedicine, University of Basel, Basel, Switzerland.

Author contributions

H.H.H. designed the study. K.B. and N.D. were responsible for the microbiological work-up of the samples. K.L., K.N., S.S, and H.H.H. supervised the study, did the data mining and analyzed the data. All authors reviewed the data and contributed to writing the manuscript.

Declaration of Competing Interest

None.

Acknowledgements

We thank the technicians of the Clinical Virology Division, Laboratory Medicine, University Hospital Basel, Basel, Switzerland, for expert help and technical assistance, especially Katia Bir, Christine Bauer, and Nadine Doppler, and Erika Hofmann for updating the electronic reference library

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2019.104210.

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