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2 Genotyping of Polyomavirus BK by Real Time PCR for VP1 Gene

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8 **Abstract** Polyomavirus BK latently persist in different
9 sites, including the renourinary tract, and may reactivate
10 causing nephropathy in renal transplant recipients or
11 hemorrhagic cystitis in bone marrow recipients. Based on
12 the sequence of the VP1 gene, four genotypes have been
13 described, corresponding to the four serologically differ-
14 entiated subtypes I–IV, with different prevalence and
15 geographic distribution. In this study, the development and
16 clinical validation of four different Real-Time PCR assays
17 for the detection and discrimination of BKV genotypes as a
18 substitute of DNA sequencing are described. 379 BK VP1
19 sequences, belonging to the main four genotypes, were
20 aligned and “hot spots” of mutation specific for all the

strains or isolates were identified. Specific primers and
21 probes for the detection and discrimination of each geno-
22 type by four Real-Time PCR assays were designed and
23 technically validated. Subsequently, the four Real-Time
24 PCR assays were used to test 20 BK-positive urine speci-
25 mens from renal transplant patients, and evidenced a
26 prevalence of BK genotype I, as previously reported in
27 Europe. Results were confirmed by sequencing. The
28 availability of a rapid and simple genotyping method could
29 be useful for the evaluation of BK genotypes prevalence
30 and studies on the impact of the infecting genotype on viral
31 biological behavior, pathogenic role, and immune evasion
32 strategies.
33
34

A1 Stefano Gambarino and Cristina Costa contributed equally to this
A2 work and share first authorship.

Keywords Polyomavirus BK · Genotyping · Real-time
35 PCR · Sequencing · Prevalence
36

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Introduction 37

BK virus (BKV) belongs to the Polyomaviridae family and
38 has a circular double-stranded DNA genome of about
39 5100 bp [1]. After primary infection that usually occurs in
40 the childhood and, which is mainly asymptomatic, BKV
41 remains latent at different sites, including the renourinary
42 tract, B-cells, and brain [2]. Reactivation with viruria may
43 occur, mainly in the context of immunosuppression, and
44 potentially lead to nephropathy (BKV-associated nephrop-
45 athy—BKVAN) in renal transplant recipients [3] or hem-
46 orrhagic cystitis in bone marrow transplant patients [4].
47

BKV is the only primate polyomavirus with serologi-
48 cally differentiated subtypes (I–IV) [5]. Jin and colleagues
49 developed a genotyping method based on the amplification
50 of the epitope region of the VP1 gene by polymerase chain
51 reaction (PCR) and classified viral isolates in four different
52

53 genotypes (BKV I-IV) corresponding to the serological
54 ones [6].

55 The geographical distribution of BKV subtypes has been
56 investigated in different studies [7–10], evidencing a higher
57 prevalence for BKV I worldwide and a minor but consis-
58 tent occurrence of BKV IV, in particular in South-East
59 Asia. On the other hand, the prevalence of BKV II and
60 BKV III is very low with a higher presence of BKV III in
61 the African continent. This genotyping method is based on
62 the presence of single nucleotide polymorphisms (SNPs)
63 conserved between the various isolates belonging to each
64 of the four genotypes. Many different biomolecular pro-
65 cedures have been developed to detect and discriminate
66 SNPs [11–14]; among these, the ARMS PCR [15] uses the
67 discriminatory power of the terminal 3' nucleotide to obtain
68 a successful amplification only for the DNA strand with a
69 correct complementarity.

70 In this article, we describe the design of four different
71 Real-Time PCR assays which can detect and discriminate
72 among the four main BKV genotypes (I–IV) as a valid
73 substitute of DNA sequencing.

74 Materials and Methods

75 BKV Sequences and Phylogenetic Analyses

76 379 BKV VP1 sequences, belonging to the main four
77 genotypes (I–IV), were obtained from three different arti-
78 cles [8–10] by extrapolation from the server Nucleotide
79 (www.ncbi.nlm.nih.gov/nucleotide/). Nucleotide positions
80 from 1663 to 1912 (Dunlop strain complete genome,
81 GeneBank Access no. V01108) were considered to obtain
82 250-bp length DNA sequences. Subsequently, the VP1
83 DNA sequences were aligned using the software Clustal X
84 (i.e., the graphical version, with windows interface, of the
85 bioinformatic software Clustal W). This software, that
86 incorporates a novel position-specific scoring scheme and a
87 weighting scheme for down weighting over-represented
88 sequence groups, is used for multiple sequence alignment

89 and phylogenetic analysis, with the possibility to draw
90 phylogenetic trees. The software BioEdit was used to
91 manipulate the alignment for a clearer sight of differences
92 between the aligned sequences. BioEdit software is a user-
93 friendly tool for post-alignment modifications, with the
94 possibility to emphasize few sequences, cut and paste
95 them, and make other evaluations, such as restriction
96 mapping. The BKV VP1 sequences were then used for
97 Phylogenetic analyses. Clustal X, that was utilized to make
98 a neighbor-joining phylogenetic tree, and the free software
99 NJplot were employed to display the resulting tree. The
100 confidence of branching patterns of the neighbor-joining
101 tree was determined by bootstrap analysis, using a set of
102 1,000 replicates.

103 Primers and Probes Design

104 The alignment was screened to find “hot spots” of muta-
105 tion specific for all the strains or isolates belonging to each
106 genotype (Fig. 1). In the absence of a specific single
107 nucleotide mutation, the occurrence of a different nucleo-
108 tide combination between the genotypes was considered.
109 Every mutation was evaluated as a possible target for
110 designing of a set of primers able to recognize all the BKV
111 strains belonging to a specific genotype and to discriminate
112 between the others by a mismatch at the 3' primer ending.
113 The parameters that were taken into consideration for
114 selecting the “hot spots” and discriminating the primers
115 design were the presence of a unique sequence shared
116 between the strains belonging to one genotype but not to
117 the others, the 60°C melting temperature, and the possi-
118 bility to design a Real-Time PCR probe in the most con-
119 served nucleotide region inside the hypothetical amplicon.

120 Primers and probes were designed with the help of the
121 software Primer Express® v3.0 (Applied Biosystem,
122 Cheshire, UK). The primers obtained were then analyzed
123 with the Autodimer Software, an open-source tool that
124 evaluates the hairpin and primer–dimer formation, with the
125 aim of excluding the presence of factors that could alter the
126 amplification efficiency. In order to include all the strains

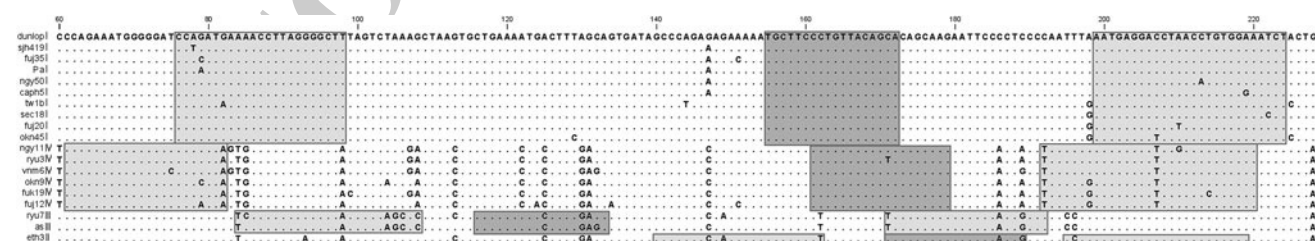


Fig. 1 Regions considered for the primer and probe design. Identical residues are represented as dots. Light squares indicate the primer regions. Dark squares represent the probe regions

Table 1 Primers and probes for the four Real-Time PCR assays for BK genotyping

	Forward primers	Reverse primers	Probes
BK I	5'-CCAGATGAAAACCTTAGGGGCTT-3' 5'-CCTGATGAAAACCTTAGGGGCTT-3' 5'-CCACATGAAAACCTTAGGGGCTT-3' 5'-CCAAATGAAAACCTTAGGGGCTT-3' 5'-CCAGATAAAAACCTTAGGGGCTT-3'	5'-AGATTTCCACAGGTTAGGTCCTCAIT-3' 5'-AGATTTCCACATGTTAGGTCCTCAIT-3' 5'-AGATTTCCACACAGGTTAGGTCCTCAIT-3' 5'-AGATTTCCACAGGTTAGATCCTCAIT-3' 5'-AGGTTTCCACAGGTTAGGTCCTCAIT-3' 5'-AGATTTCCACAGGTTAGGTCCTCAIT-3' 5'-TCCACAGGTTAGGTCCTCAITTAGA-3' 5'-GGCAGTGGAAITCTTGCTGTA-3'	5'-FAM-TGCTTCCCTGTACAGCA-MGB-3' 5'-FAM-CACAGCAAGAATTCCTCACTG-MGB-3' 5'-FAM-CTGAAAATGCCTTTGASA-MGB-3' 5'-FAM-CTGTTTACAGYACAGCAAG-MGB-3'
BK II	5'-GCCAGACAAAAAATGCTTCCT-3'	5'-TTCCACAGGTTAGATCCTCAITTAATA-3'	
BK III	5'-TMACCTTAGGGCTATAGTCAGCAC-3'	5'-TTCCACAGGTTAGATCCTCAITTAATA-3'	
BK IV	5'-CCAGAAAATGGGGATCCAGATA-3' 5'-CCAGAAAATGGGGACCCAGATA-3' 5'-CCAGAAAATGGGGATCCAAATA-3' 5'-CCAGAAAATGGGGATCCACATA-3'	5'-TTCCACAGGTTAGATCCTCAITTAATA-3' 5'-TTCCACAGGTTAGATCCTCAITTAATA-3' 5'-TTCCACAGGTTAGATCCTCAITTAATA-3' 5'-TGGTAATGGACTAAGTGTGCGTTATTTTC-3'	
CLONING	5'-GACAATCACATGCCTGGATAATG-3'		

belonging to one genotype, many pairs of primers were designed and produced. Four Taqman[®] probes MGB (minor groove binding) labeled at the 5' end with FAM (6-carboxyfluorescein), each being able to recognize one of the four target regions amplified by the different pairs of primers, were also designed. The list and sequences of primers and probes are reported in Table 1.

Real-Time PCR Optimization

Four different mixes for the amplification of each genotype were prepared. Different concentrations of primers and probe were evaluated: in particular, 200/200, 200/100, 100/200, 200/50, and 50/200 nM for primer forward/reverse, and 250 or 100 nM for probe. The reaction mixture contained 1× Master Mix (Platinum qPCR supermix—UDG with ROX [Invitrogen, Carlsbad, CA]). For this evaluation, isolates from BKV I (Dunlop strain) and BKV IV (kindly provided by Dr. Andi Krumbholz and Dr. Ellen Krautkramer), and ultramers that simulated BKV II and III sequences (comprehending a portion of isolates ETH3 and NEA27, respectively [Tema Ricerca, Bologna, Italy]), were used. Fifteen microliters of amplification mix were added to 5 µl of BKV DNA, obtaining a final volume of 20 µl. The procedure was performed and optimized on the 7300 Real-Time PCR System (Applied Biosystems). The following thermal profile was used: one cycle of decontamination at 50°C for 2 min, one cycle of denaturation at 95°C for 10 min, and followed by 45 cycles of amplification at: 95°C for 15 s, and 60°C for 60 s. The following parameters were considered for the evaluation: quick observation of the amplification, and good discrimination between the specific and aspecific amplification.

Plasmids and Standards

The BKV I Dunlop standard plasmid was kindly provided by Prof. Tiziana Musso. The BKV II and BKV III standard plasmids were produced starting from the ultramers (Tema Ricerca), then amplified with cloning primers (Table 1). The BKV IV standard plasmid was prepared starting from the BKV IV isolate J/2296/04 (kindly provided by Dr. Andi Krumbholz). The primers were used for producing a PCR product, then cloned using the pTOPO-TA cloning system (Invitrogen) and propagated in competent *Escherichia coli* TOP10 cells. After overnight culture, selected transformed clones were amplified by culture in liquid LB medium (10 g BACTOTRYPTONE, 5 g yeast extract, 171 mM NaCl, and 15 g/l agar, pH 7.5) containing 50 µg/ml of ampicillin. Plasmid DNA was extracted and purified using the Wizard Plus SV Miniprep DNA Purification System (Promega, Madison, WI). The plasmid concentration was estimated on spectrophotometric reading at OD260.



176 *Real-Time PCR Evaluation (Linearity, Efficiency,*
177 *Interference Limit, Limit of Detection, Precision)*

178 The four genotyping assays were evaluated using serial
179 10-fold dilutions of the target plasmid (ranging from 10^7 to
180 10 copies per reaction). The linearity was assessed by the
181 correlation coefficient (R^2) of the standard curve obtained by
182 plotting 10^7 –10 standard copies per reaction with the four
183 different tests. The efficiency was evaluated by the slope of
184 the standard curve, using the formula $E = 10^{(-1/\text{slope})-1}$.

185 In order to discriminate between specific and aspecific
186 amplifications, three repetitions of 10^7 , 10^5 , and 10^3 , and 10
187 copies per reaction of the four standards were amplified with
188 the four different genotyping mixes. The average threshold
189 cycle (C_t) values of specific and aspecific amplifications
190 were obtained. The background C_t was evaluated by the
191 formula $C_{t_{lim}} = C_{t_a} - t_{0.01} \cdot \sigma_a / N$, where N is the number of
192 observations, and $t_{0.01}$ is the tabulated value of Student's t for
193 the 1% probability level and $N-1$ degree of freedom [16].
194 For the evaluation of the lowest genotype concentration that
195 could be distinguished from the aspecific amplification of the
196 other three genotypes, the formula $IL = 100 / (2^{(C_{t_s} - C_{t_{lim}})} +$
197 $1)$ was used, where C_{t_s} represents the specific C_t [17].

198 The limit of detection, defined as the lowest target
199 quantity detectable, was also estimated by serial 10-fold
200 standard dilutions. The precision of each test was calcu-
201 lated by the intra- and inter-test coefficients of variation,
202 according to the formula $CV = 100 \cdot (\sigma / MC)$, where MC is
203 the average measured concentration, and σ the correspon-
204 dent standard deviation. Three repetitions for each standard
205 dilution were considered in the same run or in different
206 experiments, for the intra- and the inter-assay coefficients
207 of variation, respectively.

208 *Clinical Specimens*

209 Twenty urine specimens resulting positive to a reference
210 amplification assay (BKV Q-PCR Alert Kit [Nanogen,
211 Buttigliera Alta, Turin, Italy]) were tested with the four
212 amplification assays to assess the genotyping procedure.
213 Automated DNA extraction was performed with the
214 NucliSens EasyMAG platform (bioMérieux, Marcy l'Etoile,
215 France) from 1 ml of urine, according to the manu-
216 facturer's instructions, and eluted to a final volume of
217 50 μ l. Five microliters of extracted specimen were then
218 added to 15 μ l of each genotyping mix. The results were
219 analyzed using the System SDS software.

220 *Sequencing Procedure*

221 To confirm the results obtained by the Real-Time ampli-
222 fication, sequencing procedure was performed. The 20

specimens were amplified with primers BKV-1 (5'-GAA 223
GTT CTA GAA GTT AAA ACT GGG-3') and BKV-2 224
(5'-GTG GAA ATT ACT GCC TTG AAT AGG-3') [9], 225
obtaining an amplicon of 354 nucleotides within the VP1 226
gene (from 1663 to 2016, Dunlop reference numbering). 227
The amplification mix contained 6 μ l of GoTaq[®] HotStart 228
Polymerase buffer 5 \times (Promega), 200 μ M of each dNTP, 229
6 mM of MgCl₂, 1 unit of GoTaq[®] HotStart Polymerase 230
(Promega), and 20 pmol of BKV-1 and BKV-2 primers, 231
respectively. The resulting amplicons were run on agarose 232
gel (2% w/v) by electrophoresis, the gel was observed on 233
an UV transilluminator UV and the bands cut. Subse- 234
quently, the bands were purified using the Nucleospin[®] 235
Extract II (Macherey–Nagel, Düren, Germany). The purified 236
PCR products were sequenced using the BigDye[®] 237
Terminator v1.1 Cycle Sequencing Kit (Applied Biosys- 238
tem). In brief, a mix containing 8 μ l of Terminator Ready 239
reaction mix, 2 μ l of PCR product, 3.2 pmol of inner pri- 240
mer (BKV-1 or BKV-2), and deionized water to obtain 241
20 μ l final volume was prepared. Then, the Cycle 242
sequencing was carried out on 9800 Fast Thermal Cyclers 243
(Applied Biosystem) with an initial denaturation step at 244
96°C for 1 min, followed by 25 cycles of 96°C for 10 s, 245
50°C for 5 s, and 60°C for 4 min. 246

The Cycle sequencing product was then purified by 247
Ethanol/EDTA/Sodium Acetate precipitation and loaded 248
onto the ABI PRISM 31 Genetic Analyzer (Applied Bio- 249
system). Forward and reverse sequences were aligned with 250
the ClustalX software and then compared to BKV 251
sequences. 252

253 **Results**

The best amplification was obtained with the following 254
concentrations of primers forward/reverse: 100/200, 255
200/200, 200/200 and 200/100 nM for genotypes I, II, III, 256
and IV, respectively, and with a probe concentration of 257
250 nM; however, the amplification efficiencies were 104, 258
104, 97, and 88%, respectively. The R^2 coefficient was 259
0.996, 0.999, 0.995, and 0.999 for BK I, II, III, and IV 260
genotype amplifications, respectively. 261

Subsequently, the Real-Time PCRs were tested for the 262
discrimination of the four genotypes. Three repetitions of 263
 10^7 , 10^5 , and 10^3 , and 10 copies/reaction of the target 264
sequence of the main four BKV genotypes were employed to 265
compare the amplification and distinguish between specific 266
and aspecific amplifications using the four mixes (Table 2). 267
As regards the BKV I genotype mix, this resulting product 268
was able to amplify the genotype I-specific target, and only 269
BKV II and IV genotype mixes cross-recognize the specific 270
genotype I target at 10^7 copies/reaction (both), and at 10^5 271
copies/reaction (only the BKV II genotype mix). However, a 272

Table 2 Aspecific and specific means Ct for each genotyping mix

	Specific mean Ct		Aspecific mean Ct		
	BK I		BK II	BK III	BK IV
10 ⁷	17.36 (0.07)		32.16 (0.34) [32.06]		
10 ⁵	24.12 (0.07)		38.8 (0.16) [38.75]		38.13
10 ³	30.9 (0.51)				
10	36.12				
	BK II	BK I	BK III	BK IV	
10 ⁷	16.85 (0.04)		36.57 (0.62) [36.39]	32.2 (0.36) [32.10]	
10 ⁵	23.21 (0.04)			35.94 (0.65) [35.75]	
10 ³	29.97 (0.19)	36.14	37.74	36.51 (0.94) [36.24]	
10	36.03		40.89	37.15	
	BK III	BK I	BK II	BK IV	
10 ⁷	17.78 (0.16)			35.62 (0.9) [35.36]	
10 ⁵	24.2 (0.16)				
10 ³	30.84 (0.03)				
10	37.62 (0.63)		36.91	38.61 (0.74) [38.40]	
	BK IV	BK I	BK II	BK III	
10 ⁷	17.67 (0.03)		36.12 (0.81) [35.89]	35.88 (0.59) [35.71]	
10 ⁵	24.74 (0.01)				
10 ³	32.3 (0.19)	36.78			
10					

The values in the round brackets are the standard deviations of the repetitions for each dilution; in the square brackets the C_{lim} are reported (see text for explanation)

273 gap of approximately 14.8–14.68 C_{ts} separated the specific
274 and aspecific amplifications; moreover, no aspecific ampli-
275 fication was evidenced for lower dilution.

276 BKV II genotype mix that resulted was able to amplify
277 the specific BKV II target sequence; however, this was
278 also amplified by the other three BKV discriminating
279 mixes. Only the amplification of the BKV II-specific target
280 with the corresponding mix was linear, and the threshold
281 crossing appeared at least 15 cycles earlier for BKV II mix
282 at 10⁷ copies/reaction compared to the other amplification
283 mixes, decreasing to six cycles at 10³ copies/reaction. The
284 amplification with BKV II mix at lower BKV II genotype-
285 specific target concentration was considered not discrimi-
286 nating, because of the overlapping Ct with BKV III and IV
287 amplification mixes. The BKV III target was recognized by
288 the corresponding mix with a linear amplification; how-
289 ever, 10⁷ copies/reaction were recognized also by the BKV
290 I mix, although a mean gap of 17.84 C_{ts} was observed
291 between specific and aspecific amplifications. An undis-
292 tinguishable specific amplification was seen with a con-
293 centration of BKV III of 10 copies/reaction, because of the
294 simultaneous amplification with BKV II, III, and IV mixes
295 with a similar Ct. The BKV IV target showed a specific

296 recognition using the corresponding mix, although the
297 target was also amplified with BKV II and III mixes at 10⁷
298 copies/reaction, with a mean gap of 18.21 and 18.45 C_{ts}
299 between specific and aspecific amplifications for BKV II
300 and III mixes, respectively. However, at a concentration of
301 10³ copies/reaction, the target was also recognized by BKV
302 I mix with a mean gap of 4.48 mean C_{ts} .

303 Using the same data to evaluate the specific and aspe-
304 cific amplifications, a C_{lim} was calculated for each geno-
305 typing amplification, as an interference limit (Table 3). The
306 precision was calculated with the intra- and inter-test
307 coefficients of variation (Table 4).

308 In order to establish the limit of detection for the four dif-
309 ferent genotyping amplifications, 10-fold dilutions of the
310 targets (ranging from 10⁷ to 10 copies/reaction) were ampli-
311 fied with the above four methods. The limit of detection dif-
312 fered between the four genotyping amplification procedures
313 and was as follows: 10² copies/reaction for BKV IV and 10
314 copies/reaction for BKV I, II, and III, each. However, only
315 BKV III procedure showed a sensitivity of 10 copies/reaction,
316 while for the others assay, it was of 100 copies/reaction.

317 Results for the clinical specimens are reported in Table 5.
318 In brief, the amplification procedures yielded positive results

Table 3 Interference limit

	10 ⁷ (%)	10 ⁵ (%)	10 ³ (%)	10 (%)
I–II	0.00375	0.00393		
II–III	0.00013			
II–IV	0.00257	0.01674	1.27874	
III–IV	0.00051			36.83754
IV→II	0.00033			
IV–III	0.00037			

The lowest limit of quantity of BKV-specific genotype, expressed in percentage, distinguishable from the background (aspecific amplification). In the case of no reported percentage, sporadic or no background amplification was observed

Table 4 Intra- and inter-assay coefficients of variation for dilutions of 10⁷, 10⁵, and 10³, and 10 copies/reaction

	10 ⁷ (%)	10 ⁵ (%)	10 ³ (%)	10 (%)
Coefficient of intra-assay variation				
I	0.40	0.29	1.65	
II	0.23	0.17	0.63	
III	0.89	0.66	0.10	1.67
IV	0.16	0.04	0.59	
Coefficient of inter-assay variation				
I	0.92	2.70	0.84	1.85
II	1.19	0.15	1.45	0.99
III	1.30	1.51	0.91	2.92
IV	3.34	2.20	2.26	

in 16 cases; in particular: 12716 BKV I, 1/16 BKV II, 1716 BKV III, and 1/16 BKV IV. The DNA sequencing confirmed the results obtained with the Real-Time PCR assays for all the samples. One specimen (#4464) showed a concomitant amplification for BKV I and BKV IV, with overlapping Ct_s; sequencing identified it as BKV I genotype.

In most of the cases, the viral load found with the developed methods differed from that obtained with the reference method, with a percentage of variation ranging from –288.4 to 89.9% (Table 5). Furthermore, more specific amplifications were observed in clinical specimens. In fact, the sample #2998 showed two aspecific plots (BKV I and BKV II), while no BKV I amplification was observed with a 10⁷ copies/reaction plasmid dilution. Nevertheless, the occurrence of aspecific amplifications did not impact on the genotyping performance of the Real-Time PCRs, as the gap between the curves of amplifications remained wide.

336 Discussion

337 In this study, a Real-Time PCR-based method for geno-
338 typing of polyomavirus BK was developed. By

hemagglutination inhibition tests, four main BKV sero- 339
types were described [5], based on the differences in a short 340
amino acidic sequence on VP1 protein, probably being 341
responsible for the serotypical differences among BKV 342
variants [18]. Correspondingly, four main BKV VP1 343
genotypes were found, and their nucleotide sequences 344
analyzed [18]. On the basis of this conserved region, 379 345
VP1 sequences were collected from three different articles 346
[8–10] investigating the prevalence of BKV subtypes in 347
different countries, including Germany, Japan, and China. 348
These sequences were aligned to determine which nucle- 349
otide residues were conserved within each genotype with 350
the aim to design a Real-time PCR assay based on the 351
principle of the Amplification Refractory Mutation Assay 352
(ARMS). 353

A complete list of single nucleotide polymorphisms in 354
the total BKV genome for each genotype has been reported 355
[19]. The 379 aligned VP1 sequences presented concordant 356
residues with those previously listed by Luo and colleagues 357
[19], confirming the uniqueness and conservation of these 358
polymorphisms among the genotypes. 359

It has been reported [19] that genotyping on large T 360
antigen (LTA) is preferable, in particular, because LTA is a 361
larger region with more informative sites compared to the 362
327 bp of VP1 region considered by Jin and colleagues [6]. 363
However, in this study, we chose to use the VP1 region 364
instead of LTA as we aimed to produce powerful dis- 365
criminating Real-Time PCRs taking into consideration the 366
data published in the literature. Indeed, a higher number of 367
sequences is banked in public domain servers for VP1 368
compared to LTA, thus permitting a more detailed primers- 369
and-probes design for the detection of a higher number of 370
sequences and corresponding polymorphic residues. In this 371
study, four Real-Time PCR assays, each one that can 372
amplify specifically a specific genotype, were designed, 373
and four different amplification mixes were produced to 374
avoid a too high concentration of primers in a single well, 375
that could determine dimer formation or an increase in the 376
aspecific performance. In fact, in the presence of high viral 377
loads, aspecific amplifications might appear, and these 378
were detectable as a low quantity amplification with other 379
genotype mixes; anyway, the aspecific amplification was 380
easily recognizable by the wide gap between the specific 381
and aspecific plots. Moreover, the introduction of Ct_{lim} and 382
interference limit gave further clues about discrimination. 383
Clearer results were available for 10⁷–10⁵ copies/reaction 384
dilutions, while 10³ caused the lowest dilution with an 385
acceptable genotyping performance. For this reason, a 386
result comparable to 10³ was set as genotyping limit for all 387
the four Real-Time PCRs, and all those results with a lower 388
quantity were not accepted. 389

As regards the clinical validation of the four genotyping 390
assays, the distribution of BKV genotypes was evaluated in 391

Table 5 BK genotyping in clinical specimens

N°	ID	Reference copies/ml	BKV I (Ct)	BKV II (Ct)	BKV III (Ct)	BKV IV (Ct)	Genotype	Copies/ml
1	2998	$>5 \times 10^6$	31.63	33.82		11.59	IV	$>5 \times 10^7$
2	3068	4237203	19.57	36.36		32.78	I	$>5 \times 10^7$
3	3204	569						
4	3206	$>5 \times 10^6$	12.01	26.65		25.34	I	$>5 \times 10^7$
5	3207	2413	30.60				I	5379 (-122.9%)
6	3607	126470	29.38	36.56			I	12800 (89.9%)
7	3619	1542						
8	3965	502131	24.72	43.06		38.04	I	351153 (30.1%)
9	4011	868						
10	4063	4158	30.14				I	7458 (-79.4%)
11	4326	495	34.78				I	<500
12	4398	49606	29.73	43.09		38.53	I	9981 (79.9%)
13	4405	2472943	34.93	19.80		32.37	II	1220410 (50.6%)
14	4464	8689	36.59			36.69	I	<500
15	4674	6441	29.22	42.79		42.64	I	14342 (-122.7%)
16	4712	2393	30.58			37.65	I	5456 (-128%)
17	4714	<59	36.17				I	<500
18	4723	$>5 \times 10^6$	12.19	28.37		26.11	I	$>5 \times 10^7$
19	4758	<59						
20	4768	44562		31.50	29.14		III	173076 (-288.4%)

Viral loads (expressed in copies/ml) of positives samples for the reference method, positivity to each amplification procedure (expressed in Ct), genotype and quantities (expressed in copies/ml) are reported. *Bold* numbers shows C_t of overlapping curves

urine specimens; this type of sample was chosen for the higher mean viral load in comparison to serum samples [2]. However, clinical samples with a low reference viral load were not always detected. Most of the specimens were infected by BKV I (80%), while BKV II, III, and IV were infrequent. The prevalence of the four genotypes was concordant with the literature [20]. BKV IV, that is more prevalent in East Asia [8], is found also in Europe [9] in a percentage comparable to the one observed by us; however, notwithstanding the uncommon prevalence, both BKV II and BKV III were detected in two different samples; the results were confirmed by the subsequent VP1 DNA sequencing.

Considering viral load of different genotypes, a high divergence was observed between the genotyping assays and the reference method, with a variability ranging from -288.4 to 89.9%. These data highlighted the problem exposed by Hoffman and coll. [21]: in the presence of various genotypes, difference in probes and primers for the BKV quantification led to a substantial disagreement between assays. This was confirmed in our study, between the reference and the genotyping amplifications.

In conclusion, the four Real-Time PCRs that were produced were able to detect and discriminate the main four BKV genotypes. Compared to classical DNA sequencing,

these procedures are more rapid and simpler, since they are single PCR assays with no purification steps; results are available in approximately 2 h. One drawback could arise because of the need of at least 10^3 copies/reaction for the genotyping; in these cases, a PCR assay employing external primers, or other protocols, would be necessary to increase the target sequence amplifiable, although this should also increase the time required for genotyping.

The comparison between genotyping procedures on clinical samples and plasmid dilutions evidenced some differences, including the detection of aspecific plots during the testing of clinical samples, and the lack of similarity with the quantities reported by the reference amplification procedure. This could be caused by the primers-and-probes nature, amplifying Large T (reference method) and VP1, and by the presence of polymorphism that alter the efficiency of amplification, as previously suggested [21]. Nevertheless, the four Real-Time PCRs developed in this study should not be considered quantitative methods, as the procedures were not tested for the quantification of a broad number of isolates. The availability of a rapid and simple genotyping test could be useful for the evaluation of BKV genotypes' prevalence. and studies on the impact of the infecting genotype on viral biological behavior, pathogenic role, and immune evasion strategies.

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