Importance of molecular diagnostic of viral infections in renal transplant recipients
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CHAPTER 2

A Real Time genotyping PCR assay for Polyomavirus BK

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Chapter 2

ABSTRACT

Background
Polyomavirus BK (BKV) may cause nephropathy in renal transplant recipients and hemorrhagic cystitis in bone marrow recipients. We developed Real-Time PCRs (RT-PCR) to determine easily and rapidly the different BKV genotypes (BKGТ) (I-IV).

Methods
On the VP1 gene a duplex of RT-PCRs was developed and validated to differentiate the four main BKGТ. 212 BKV positive samples (21 plasma, 191 urine) were tested with these specific PCRs. Of these 212 samples, 55 PCR results were additionally confirmed by sequencing a VP1 gene fragment (nucleotide 1630-1956).

Results
For every genotype, a highly specific, precise and internally controlled assay was developed with a limit of detection of log 3 copies per ml. In 18 (8.5%) of these samples genotyping was not successful due to a low viral load. By sequence analysis, the genotype of 46 out of 55 and 2 out of 4 samples with double infection could be confirmed.

Conclusions
This study describes RT-PCRs for detection of the main BKGТ. It proved to be rapid, cheap and sensitive compared to sequencing. Double infections can also be detected. This method will be of value to investigate the role of BKV infection in relation to the genotype.
INTRODUCTION

In the early 1970s, Polyomavirus BK (BKV) was first isolated from the urine of a renal transplant patient by Gardner et al. 1 It is a non-enveloped double stranded DNA virus belonging to the Polyomavirus family. BKV is ubiquitous and widely spread in the adult human population, with sero-prevalence rates up to 80%. Infection results in a lifetime persistence of the virus in which BKV establishes latency in the reno-urinary tract.1-3 BKV can be classified into four genotypes (I-IV) using serological or genotyping methods.2,4 Several studies investigated the geographical distribution of BKV. Genotype I is the most prevalent throughout the world, followed by genotype IV, which is mainly seen in Europe and Asia. Genotypes II and III occur rarely, while genotype III is mostly seen in Africa 5-7.

Through the diversity of humans origin within the different continents, it is most likely that genotypes II, III and IV also occur in humans of different origin. So far, more than twelve other human polyomaviruses have been described. As far as known, only JC virus (JCV), Merkel cell virus and Trichodysplasia spinulosa virus can be associated with a human clinical disease. 8 Although the genome of BKV is closest related to JCV, both do cause different clinical features in immunocompromised patients. In bone marrow transplant recipients, BKV is associated with hemorrhagic cystitis whereas in renal transplantation patients it may cause nephropathy (BKVAN) with subsequent allograft loss. 9

Predictive markers for developing BKVAN are high titers in urine and plasma 10. At present reduction, changing or discontinuation of the immunosuppressive regimen is recommended, in patients with BKV viremia, 11,12 through the lack of antiviral treatment for BKV diseases 13.

Relating to the development of BKVAN, much is still unknown, like the association among the various BKV genotypes (BKGT) with their causative role in the development of clinical syndromes. Krautkramer et al. showed that high BKV viral load is independent of the mutations in BKV VP1 genes. 14 On the other hand, Boldorino et al found a correlation between BKVAN and the frequency of single base-pair mutations in BKV DNA.15

The availability of a rapid, simple and cheap genotyping Real-Time PCRs (RT-PCRs) will be useful for studies to investigate the impact of the role of BKV infection in relation with BK genotype. Hence the aim of this study was to develop easy to use RT-PCRs.
Chapter 2

MATERIALS & METHODS

Clinical Specimens and plasmid
For this study, clinical specimens were tested for BKV DNA in the period January 2008 until September 2013 using an in-house developed quantitative BKV RT-PCR on the VP2 gene. From this period, 212 BKV positive (21 plasma and 191 urine) samples from 191 different patients (171 kidney Tx, 2 liver Tx, 16 hematology, 1 pancreas Tx and 1 other) were randomly selected with a DNA load ranging between log 2 copies per ml (cp/ml) and log 11cp/ml. Subsequently, 50 of these urine and plasma samples were randomly selected and sequenced for the determination of the BKGT.

For the validation of the assay urine and plasma from three patients with either genotype I, II or IV were used to prepare 10-fold dilution series. Since there was no clinical sample for genotype III a plasmid, using pDONR as vector, was constructed (Gene Art Life Technology, Regensburg, Germany) using the VP1 gene sequence from Genbank accession number M23122.

BKV RT-PCR
Isolation of the BKV DNA was performed using the MP96 isolation station (Roche Diagnostics, Germany) according to the manufactures instructions. The DNA viral NA Blood SV 2.0 protocol was used with input of 200µl plasma or urine and elution in 100µl.

An internally controlled in-house developed quantitative BKV RT-PCR was used for the detection of BKV DNA. Currently, no international standard for BKV is available, therefore the validation and quantification of this BKV RT-PCR was done using the EZ Validation software (Life Technology, USA) using the AcroMetrix quality control materials. Furthermore, patient samples ranging from < log 2 to log 11 copies/ml were compared with the RealStar BKV PCR Kit (Altona Diagnostics, Germany) and the BKV Q-PCR Alert-kit (ElitechGroup, France) to confirm that samples with a load of < log 2 with the in-house BKV RT-PCR are not false-negative (data not published). In short, our BKV RT-PCR is an internally controlled duplex based on the VP2 gene, with a detection limit of log 2 cp/ml. The reaction mixture was the same as described below for the specific BKGT RT-PCR. It consist of 300nM of each primer (Forward:5’- ATGGGTGCTGCTCTAGCACTTT-3’, Reverse 1:5’-GGATGCAATTGGAACTTCTATGGC-3’, Reverse 2: 5’-GGATGCAATTTGAACTTCTATAGCAG-3’) and 100nM of the probe (5’-FAM-CAGTGTATCTGAGGCTG-MGB-3’), which amplifies a 131bp of the VP2 gene from nucleotide position 624 to 755 using Genbank accession number NC_001538. As internal control, Phocine herpesvirus (PhHV) with minor modifications in the probe sequence (5’-Cy5-TTTTTATGTGTCCGCCACCATCTGGATC-BBQ-3’) was used as described by van Doornum et al. 16
**BKGT specific RT-PCR**

**Primers and probe design**

In total, 304 complete BKV genomic sequences that were available, up to February 2015, present in the Genbank database were aligned using Genedoc software to identify highly conserved regions in each BKGT. Primers and probes were designed on the VP1 gene, in a region with low variability between the subtypes of a genotype, but with high variability between the genotypes. The design was performed with unique sequences for the BKV subtypes I and IV and genotypes II and III using Primer Express v3.0 (Applied Biosystem, USA) and with consideration of the primer and probe design guidelines. Confirmation of the specificity as well as their homology was checked using BLAST software on the NCBI site. MGB (minor groove binding) probes were used for the discrimination between the 4 BKGT.

**Validation**

The four BKGT RT-PCRs are internally controlled and optimized for the primer/probe concentrations. PhHV, (see paragraph 2.3.1), is used as an internal control. In all experiments, PCR reactions were performed in a 50µl reaction containing 20µl of isolated DNA, 2x Taqman Universal Mastermix (Life Technologies, USA), 5mg/ml Bovine serum albumin (Roche, Germany), 300nM of each PhHV primer, 100nM PhHV probe and primers and probes for the specific BKGT (Life Technologies, USA). Table 1 shows the concentration of the specific BKGT primers and probes.

The 4 internally controlled BKGT RT-duplex PCRs were performed on the ABI PRISM 7500 (Life Technologies, USA), with the following thermal conditions: 50°C for 2min, 95°C for 10min followed by 42 cycles of 95°C for 15sec, 60°C for 1 min.

**Precision, Sensitivity and Specificity**

The precision is validated by the intra/inter assay variation, which was assessed by testing 10-fold serial dilutions in triplicate of each genotype. Sensitivity was determined by using 10-fold serial dilutions of BKGT I to IV. All dilutions were tested in 10fold for the in-house BKV RT-PCR and the specific BKGT RT-PCRs. For the specificity, 10-fold serial dilutions (ranging from log 2 to log 8 cp/ml) of each genotype were tested in every specific BKGT RT-PCR and the following viruses were tested with a dilution of log 4-5 cp/ml: B19, CMV, EBV, HHV6, HHV8, HSV 1, HSV 2, JCV and VZV. All viruses origins from patient samples Furthermore, the accuracy was determined by comparing the results of 55 positive BKV samples in the specific BKGT RT-PCRs with the sequence analysis of the 1630-1956 VP1 nucleotide fragment as described by Jin et al. 4
Sequence analysis
In total, 55 samples of the 212 samples were also sequenced, in order to confirm the RT-PCR genotype results. These samples were randomly selected with a mix of urine and plasma samples and varying in load from log 2 cp/ml to log 11 cp/ml.

The PCR mixture consist of a total volume of 50µl, containing 5µl 10x PCR Buffer (Qiagen), 25mM MgCl₂ (Qiagen), 50x dNTP plus (Roche, Germany) 500nM of primer 327-1 and 327-2⁴, 2.5 units Hotstar Taq Polymerase (Qiagen) and 10µl template DNA. The cycling conditions were as followed: 95°C for 15 min, followed by 50 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 1 min, with an final extension of 72°C for 10 min and cooling to 4°C. According to the manufacturer instructions the PCR products were sequenced using the the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, USA) and the product was purified with the BigDye X Terminator Purification Kit (Life Technologies, USA). Sequence detection was performed on the ABI PRISM 3130 XL Genetic Analyzer (Life Technologies, USA). After alignment using the DNASTAR Lasergene 10 Core Suite software (DNASTAR, USA,) BLAST on the NCBI site was used to compare the BKV sequences.

RESULTS

Primer and probe design
Primers and probes were designed in the most conserved region for each genotype on the VP1 gene. This region was almost the same for genotypes II, III and IV, whereas for genotype I another region was used. Degenerated reverse primers were used for all assays and a degenerated probe for genotype III. Due to the melting temperature of the genotype I probe, it was not possible to use a degenerate probe, therefore the genotype I RT-PCR consist of 2 probes. Table 1 shows the sequences and locations of the primers and probes.
Table 1: Primers and Probe sequences for the BK genotyping Real-Time PCRs.

<table>
<thead>
<tr>
<th>OligoName</th>
<th>Oligosequences 5’ → 3’</th>
<th>Label</th>
<th>Concentration</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>BK-Typ1-F</td>
<td>TAACCTTCATGCAGGGTGCAAA</td>
<td></td>
<td>300nM</td>
<td>1944-1965</td>
</tr>
<tr>
<td>BK-Typ1-R</td>
<td>CTCCACCAACAGGAAAAAGTG</td>
<td></td>
<td>300nM</td>
<td>2014-2035</td>
</tr>
<tr>
<td>BK-Typ1-P1</td>
<td>AGTGCAATGACGATTGG</td>
<td>FAM-MGB</td>
<td>100nM</td>
<td>1968-1982</td>
</tr>
<tr>
<td>BK-Typ1-P2</td>
<td>TACATGAGCATGGAATTGGA</td>
<td></td>
<td>100nM</td>
<td>1970-1986</td>
</tr>
<tr>
<td>BK-Typ2-P</td>
<td>CAGTTAGCTTTTAGACTATA</td>
<td>VIC-MGB</td>
<td>200nM</td>
<td>1759-1777</td>
</tr>
<tr>
<td>BK-Typ2-F</td>
<td>GGTAGATGCTATAACAGAGGTAGATGC</td>
<td></td>
<td>600nM</td>
<td>1686-1713</td>
</tr>
<tr>
<td>BK-Typ2-R</td>
<td>TGTCAGGGCTACTACATCTAAGGGG</td>
<td></td>
<td>300nM</td>
<td>1787-1810</td>
</tr>
<tr>
<td>BK-Typ3-P</td>
<td>AATTAGGTGCTGACTATA</td>
<td>VIC-MGB</td>
<td>250nM</td>
<td>1758-1776</td>
</tr>
<tr>
<td>BK-Typ3-F</td>
<td>GGTAGATGCTATAACAGAGGTAGATGC</td>
<td></td>
<td>300nM</td>
<td>1686-1713</td>
</tr>
<tr>
<td>BK-Typ3-R</td>
<td>TGTCAGGGCTACTACATCTAAGGGG</td>
<td></td>
<td>600nM</td>
<td>1787-1810</td>
</tr>
<tr>
<td>BK-Typ4-F</td>
<td>ACTGGGGTAGATGCTATAACAGAAGTAGA</td>
<td></td>
<td>300nM</td>
<td>1681-1709</td>
</tr>
<tr>
<td>BK-Typ4-R</td>
<td>TGTCAGGGCTACTACATCTAAGGGG</td>
<td></td>
<td>300nM</td>
<td>1784-1806</td>
</tr>
<tr>
<td>BK-Typ4-P</td>
<td>CAGCAGTTAGCTTTTAGACT</td>
<td></td>
<td>100nM</td>
<td>1762-1780</td>
</tr>
</tbody>
</table>

Genbank accession number NC_001538 was used as reference.

Precision

The intra- and inter assay variation was based on the cycle time (Ct) values of serially diluted BKV DNA from the different genotypes. Urine samples were available for genotype I,II and IV. For genotype III, a 10-fold serial dilution was made from the plasmid. The coefficient of variation (CV %) was between 0.15 – 4.46% for the intra-assay and 0.44 – 5.25% for inter-assay over three different days (Table 2).
**Tabel 2:** Intra- and inter-assay variation of different dilutions.

<table>
<thead>
<tr>
<th>BKV Subtype</th>
<th>Dilution</th>
<th>Intra-assay (Average Ct ± SD (%CV))</th>
<th>Inter-assay (Average Ct ± SD (%CV))</th>
</tr>
</thead>
<tbody>
<tr>
<td>BK GT1</td>
<td>Undiluted</td>
<td>19,74 ± 0,09 (0,44)</td>
<td>19,50 ± 0,18 (0,92)</td>
</tr>
<tr>
<td></td>
<td>Log 1</td>
<td>23,29 ± 0,06 (0,28)</td>
<td>23,70 ± 0,33 (1,38)</td>
</tr>
<tr>
<td></td>
<td>Log 2</td>
<td>26,97 ± 0,09 (0,32)</td>
<td>26,56 ± 0,41 (1,55)</td>
</tr>
<tr>
<td></td>
<td>Log 3</td>
<td>30,27 ± 0,16 (0,53)</td>
<td>29,99 ± 0,46 (1,63)</td>
</tr>
<tr>
<td></td>
<td>Log 4</td>
<td>34,01 ± 0,19 (0,57)</td>
<td>33,35 ± 0,65 (1,96)</td>
</tr>
<tr>
<td>BK GT2</td>
<td>Undiluted</td>
<td>20,58 ± 0,05 (0,23)</td>
<td>20,38 ± 0,22 (1,1)</td>
</tr>
<tr>
<td></td>
<td>Log 1</td>
<td>24,15 ± 0,05 (0,21)</td>
<td>23,85 ± 0,25 (1,07)</td>
</tr>
<tr>
<td></td>
<td>Log 2</td>
<td>28,00 ± 0,13 (0,46)</td>
<td>27,58 ± 0,41 (1,47)</td>
</tr>
<tr>
<td></td>
<td>Log 3</td>
<td>31,27 ± 0,08 (0,25)</td>
<td>31,07 ± 0,24 (0,77)</td>
</tr>
<tr>
<td></td>
<td>Log 4</td>
<td>35,15 ± 0,06 (0,18)</td>
<td>34,90 ± 0,38 (1,09)</td>
</tr>
<tr>
<td>BK GT3</td>
<td>Undiluted</td>
<td>18,92 ± 0,27 (1,41)</td>
<td>18,52 ± 0,5 (2,7)</td>
</tr>
<tr>
<td></td>
<td>Log 1</td>
<td>23,14 ± 0,09 (0,41)</td>
<td>23,05 ± 0,1 (0,41)</td>
</tr>
<tr>
<td></td>
<td>Log 2</td>
<td>26,37 ± 1,18 (4,46)</td>
<td>25,53 ± 1,34 (5,25)</td>
</tr>
<tr>
<td></td>
<td>Log 3</td>
<td>30,40 ± 0,27 (0,9)</td>
<td>29,74 ± 0,43 (1,45)</td>
</tr>
<tr>
<td></td>
<td>Log 4</td>
<td>34,62 ± 0,35 (1,01)</td>
<td>33,59 ± 1,21 (3,6)</td>
</tr>
<tr>
<td>BK GT4</td>
<td>Undiluted</td>
<td>21,54 ± 0,12 (0,57)</td>
<td>21,59 ± 0,31 (1,45)</td>
</tr>
<tr>
<td></td>
<td>Log 1</td>
<td>25,55 ± 0,34 (1,33)</td>
<td>24,98 ± 0,41 (1,66)</td>
</tr>
<tr>
<td></td>
<td>Log 2</td>
<td>28,67 ± 0,04 (0,15)</td>
<td>28,46 ± 0,35 (1,22)</td>
</tr>
<tr>
<td></td>
<td>Log 3</td>
<td>32,05 ± 0,32 (0,99)</td>
<td>31,94 ± 0,2 (0,63)</td>
</tr>
<tr>
<td></td>
<td>Log 4</td>
<td>36,51 ± 0,78 (2,15)</td>
<td>36,03 ± 0,38 (1,06)</td>
</tr>
</tbody>
</table>

The numbers in the table are absolute, calculations were done with not rounded numbers. BK GT = BK virus genotype.

**Limit of detection**

To determine the sensitivity 10-fold dilution series of BKGT I, II en IV were tested in the in-house BKV RT-PCR and the specific BKGT RT-PCRs. The in-house BKV RT-PCR is based on the VP2 gene, therefore the BKGT III serial dilutions could only be tested in the specific BKGT RT-PCR. Arbitrary log cp/ml were used for the comparison between the assays sensitivity.

The BKGT I RT-PCR is more sensitive compared to the in-house RT-PCR, whereas the genotype II and IV BKGT RT-PCRs are less sensitive, with a difference within 1 log (Figure 1). Due to these differences and to be sure that all genotypes would be detected, the detection limit for the specific BKGT RT-PCRs is set on log 3 cp/ml instead of log 2 cp/ml, which is the detection limit of the in-house BKV RT-PCR. In clinical samples first the in-house RT-PCR is performed guaranteeing the sensitivity, followed by genotype specific PCRs.
A real time genotyping PCR

**Figure 1**: Sensitivity of the four BK type specific Real-Time PCRs. Values are means of 10-fold replications with SD error bars.

**Specificity**

The four specific BKGT RT-PCRs did not show non-specific amplification with the different BKGT in a range of log 2 to log 8 cp/ml. Furthermore, the assay didn’t show any cross-reactivity with the following viruses: B19, CMV, EBV, HHV6, HHV8, HSV 1, HSV 2, JCV and VZV.

**Accuracy**

In total, 212 patient samples ranging from log 2 to log 11 cp/ml in the BKV RT-PCR were tested with the specific internal controlled BKGT RT-PCRs. Of these 212 samples, the PCR results of 46 samples were confirmed by sequence analysis. In 9 out of the 55 samples, typing by sequence analysis was not possible due to a low viral load (below Log 3 cp/ml). However, an additional 4 of these 9 samples could successfully be detected by the specific internal controlled BKGT RT-PCR. There were no differences between the genotypes obtained with sequencing and RT-PCRs. In four samples, a double infection was seen; two of these four double infections were confirmed by sequencing (Figure 2).
Chapter 2

**Figure 2:** Confirmation double infection by sequencing the VP1 gene fragment (nucleotide 1630-1956). From 6 samples the PCR results, typed with the specific internally controlled BKGT RT-PCR, with either BKGT 1, BKGT IV or BKGT I + IV, were confirmed by sequencing the VP1 gene fragment. The circles show the double nucleotides in the double infected sample.

**The occurrence of the different BK genotypes in clinical samples**

Of the 212 clinical samples tested with the specific BKGT RT-PCRs, 70.8% was genotype I, 5.2% genotype II, 13.7% genotype IV, 1.9% genotype I and IV. No genotype III was found (Figure 3). Genotype 2 was only found in kidney Tx patients, whereby genotype I and IV was found in kidney Tx as well as in hematology patients. Genotype 4 was mainly found in kidney Tx and liver Tx patients.

**Figure 3:** Occurrence of the different BK genotypes in clinical samples. 212 samples are included from 191 different patients. (171 kidney Tx, 2 liver Tx, 16 hematology, 1 pancreas Tx and 1 other).
DISCUSSION

In this study, we developed 4 specific BKGT RT-PCRs on the VP1 gene for the detection of the 4 different BKGT. The specific BKGT RT-PCRs are internally controlled with PhHV and MGB probes are used for the discrimination between the 4 genotypes. All 4 BKGT RT-PCRs are 100% type specific. Due to differences in sensitivity, the detection limit was set on log 3 cp/ml, which has no effect on treatment as a viral load of log 2 cp/ml has no clinical implications. These differences could be explained by the ΔRn of the assays. With a lower ΔRn, the Ct value would also be lower. The ΔRn of the first sample from the 10-fold dilution series for the BKV RT-PCR is around 3 for genotype I, II and IV. In the specific BKGT RT-PCR the ΔRn for BKGT I is 3.2, BKGT II is around 2.5 and for BKGT IV it’s 1 (data not shown). The precision is within 1SD, with an exception by the Log 2 dilution of the BKGT III specific BKGT RT-PCR. The PCR results from 46 samples could be confirmed by sequence analysis of the VP1 gene. In 3 samples, a double infection with genotype I and IV was found with the specific BKGT RT-PCRs and the PCR result of 2 of these samples could also be confirmed by sequence analysis.

The advantage of using MGB probes for the discrimination is that they are more sequence specific for single base mismatches than standard DNA probes.\(^\text{17}\) However, when mutations arise in the probe region, genotypes can be missed due to this specificity. As a consequence, samples with a load above log 3 cp/ml that cannot be detected with our specific BKGT RT-PCRs, typing should be done by sequence analysis. Of the 212 clinical samples tested, all with viral loads above log 3 cp/ml could be detected with the specific BKGT RT-PCRs. If needed, sequence analysis could be performed in those cases if the specific assays did not give a detectable signal.

The specific BKGT RT-PCRs have at least two advantages over sequence analysis. Firstly, the specific BKGT RT-PCRs are more sensitive and able to determine the genotype of patient samples with lower viral loads compared to sequence analysis. Secondly, the specific BKGT RT-PCRs easily detect double infections. However, competition between the different genotypes in one sample may occur. As described before, the discrimination between the different BKGT RT-PCRs is based on the probe, so there will always be amplification with the primers. As a result if one genotype is present with a higher viral load the second genotype may fall under the detection limit, which means that double infections could be detected the best in samples with low viral loads. In this study the detected double infections were in samples with viral loads between log 3 cp/ml and log 5 cp/ml.

The detected BKGT in this study are in line with data published in the literature. The majority was typed as genotype I. Thus far, most studies determined the BKV genotypes and subgroups prevalence in the population by sequencing the typing region as described by Jin...
and colleagues.\textsuperscript{4,5,7,18,19} In contrast, Luo and his colleagues proposed that by using the large T-antigen as a genotyping scheme, a larger protein size containing more informative sites has an advantage over the 327bp of VP1 region.\textsuperscript{20}

However, in line with most studies, we also used the VP1 region, since primers and probes with a good discrimination between the genotypes was more straightforward than by selecting sequences from the large T-antigen. To our knowledge, only one other RT-PCR developed on the VP1 gene for genotyping BKV has been described.\textsuperscript{21} This RT-PCR shows cross-reactivity within the different genotypes. High concentrations, 10^6 copies/reaction, of BKGT I cross-react with genotype II and IV. Concentrations of 10^7 and 10^3 copies/reaction of BKGT II cross-react with genotype III and IV. BKGT III, 10^7 and 10 copies/reaction, cross-reacts with genotype IV and concentration of 10^7 copies/reaction of BKGT IV with BKGT II and III. In our validation procedure, the specificity of the different specific BKGT RT-PCR’s was tested using 10-fold serial dilutions (ranging from log 2 to log 8 cp/ml) of each genotype. In comparison to this described RT-PCR, the BKGT RT-PCRs developed in this study have a specificity of 100% resulting in no cross reactivity and therefore more suitable to detect double infections. In our study, two different genotypes were detected only in 4 samples from different patients (2 urine and 2 plasma). Matsuda and colleagues developed a genotyping tool for BKV by high-resolution melting analysis\textsuperscript{22}. The limitation with that assay is that it is difficult to differentiate among strains with the same amount of single nucleotide polymorphisms and the same extent of single nucleotide polymorphism variation.

In three other studies, co-infection with different BKV genotypes and subtypes have been described. Jin and colleagues reported double infections of BKGT I with other genotypes in HIV-infected patients, children and pregnant women, but not in BMT recipients.\textsuperscript{23} Luo and collaborators described co-infections of genotypes and subgroups in the urine of kidney transplant recipients and healthy individuals.\textsuperscript{24} Recently, Ledesma and colleagues reported co-infections of BKV genotypes as well as subgroups in paired urine and plasma samples from renal and bone marrow transplant recipients.\textsuperscript{19} Whether there is a difference among the various BKV genotypes/subgroups, and their causative role in development of clinical syndromes is not yet fully clear.

Tremoleda et al. demonstrated that in the urine and plasma of kidney transplant recipients with BKVAN, polymorphisms in the VP1 subtype region of the virus could be detected. Furthermore, they showed that these polymorphisms had a strong influence on the growth of the virus in cell culture and the capability to induce cytopathic effects.\textsuperscript{25} Pastrana and colleagues imply, with their in vivo study, that different BKV genotypes have different cellular tropisms and pathogenic potentials. These results are suggesting that not all genotypes/subgroups may
A real time genotyping PCR contribute equally in the pathogenesis towards disease and individuals who are infected with one BKV genotype/serotype may be vulnerable to other BKV serotypes after implementation of immunosuppression.

The debate whether the BK virus is of donor origin or the latent virus of the recipient causing the BKVAN is still ongoing. Recently, the study of Schmitt et al. 26 provides evidence of BKV donor origin in renal transplant recipients. In two cases the BKV sequence before Tx differed from the sequence detected in donor and recipient after Tx. Whereby in 20 cases the donor/recipient sequence after Tx was identical.

Also the recipients serostatus and the effect of mismatches in BK genotype/subgroups between recipient and donor on the development of BKVAN remain unsettled. In addition there are indications that pre-transplant viremia may add to the risk for development of BKVAN.

Thakur et al. 27 showed in a study amongst kidney transplant donors and recipients that dual positivity of donor-recipients pairs was related to a high risk of development of BKVAN. In 4 out of 6 donor-recipients pairs with viremia at the time of transplantation, a high outcome of BKV plasma loads was observed leading to the development of proven BKVAN in two cases.

Recently, the results of Mitterhofer and colleagues 28 also indicates that pre-transplant viremia is an additional risk factor for post-transplant BKV replication. They showed a significant difference in sustained viremia between patients with pre-transplant viremia versus patients with negative BK viral loads before transplant.

This raises the question whether polymorphisms occur more frequently if the donor has another genotype/subgroup then the recipient or if the donor and/or recipient have a BKV DNA load before transplantation. Therefore, research is needed to unravel the impact of the occurrence of double infections and BKV load before transplantation and to investigate the role of BKV infection in relation to the genotype and subgroup. The availability of a rapid, simple and cost-effective genotyping RT-PCR’s is useful for these studies to obtain more insight.

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