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BK virus DNA detection by real-time polymerase chain reaction in clinical specimens

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SUMMARY

The BK polyomavirus (BKV) is widespread in the general population. In transplant recipients, the patients' weakened immune response may encourage reactivation of latent infection, leading to BKV-related diseases. Rapid and quantitative detection might help to delineate viral reactivation patterns and could thus play an important role in their clinical management.

In our study we developed an "in-house" quantitative real-time PCR to detect BKV DNA. The effectiveness of this assay was evaluated by a retrospective analysis of 118 plasma specimens from 22 bone marrow transplant (BMT) recipients and 107 samples from immunocompetent subjects.

Eight (36.3%) of the 22 bone marrow transplant recipients tested positive for BKV. The viral load varied from specimen to specimen (10 to 10^5 copies/ml). BKV related disease like hemorrhagic cystitis (HC) was diagnosed in three patients. Specimens from the control group all tested negative.

Our results showed the high sensitivity of the real-time PCR, allowing accurate and reproducible measuring of the viral load in order to identify patients at risk for BKV-related diseases. With due caution in interpreting threshold values, the real-time PCR could provide a rapid, sensitive and specific tool for detecting BKV and distinguishing latent and active infection.

KEY WORDS: Polyomavirus BK, real-time PCR, Bone marrow transplant recipients, hemorrhagic cystitis

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INTRODUCTION

The BK virus (BKV) is a human polyomavirus belonging to the *Papovaviridae* family. BKV is ubiquitous and infects a high proportion of the population: specific antibodies are detectable in 90% of 10-year-old children (Dolei *et al.,* 2000). Primary infection is thought to be acquired in childhood by oral or respiratory routes. It has been suggested that the virus can also be transmitted during organ transplants (Trofe *et al.,* 2004). The majority of primary infection is asymptomatic or minimally symptomatic; later BKV establishes latency in the urogenital tract and B lymphocytes (Ahsan and Shah, 2006; Bofill-Mas *et al.,* 2001; Degener *et al.,* 1997; Fedele *et al.,* 1999; Leung *et al.,* 2001; McNees *et al.,* 2005; Nickeleit *et al.,* 2000; Shah, 2000; Whiley *et al.,* 2001).

Weakened immune response plays an important role in the development of disease (ureteral stenosis, tubulointerstitial nephritis). In immunocompromised patients this may be associated with the reactivation of latent infection (Apperley *et al.,* 1987; Arthur *et al.,* 1989; Chatterjee *et al.,* 2000; Nickeleit *et al.,* 2000). Since 1995 there has been a 1-8% increase in the presence of polyomavirus nephropathy (PVN) in kidney trans-

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plant recipients. This pathology is often associated with significant graft dysfunction and the loss of the transplanted organ (Trofe *et al., 2004*). It has been suggested that BKV reactivation in bone marrow transplant (BMT) patients is associated with late onset of hemorrhagic cystitis (HC), a condition characterized by lower abdominal pain, dysuria, frequent micturition and hematuria (Arthur *et al.,* 1986; Fioriti *et al.,* 2005; Ilhan *et al.,* 1997; Leung *et al.,* 2001; Priftakis *et al.*, 2003; Seber *et al.,* 1999; Sehbani *et al.,* 2006; Vogeli *et al.,* 1999). HC affects between 7 and 68% of BMT patients. The effective clinical management of patients with compromised immune systems requires rapid detection and typing of BKV. But although the literature describes several viral assays, there is still no "gold standard" for the diagnosis and monitoring of BKV infections

Recently, many authors have focused on the possibility of using gene amplification (PCR) to monitor viral load in the blood and urine. Studies have shown that molecular techniques can be very effective in detecting BKV replication, even before the onset of viral pathology (Bressollette-Bodin *et al.,* 2005; Trofe *et al.,* 2004).

Is still unclear which is the most significant specimen for BKV DNA detection. Although some authors showed that BK viruria might be quantitatively related to the occurrence of HC, significant viral shedding can occur even in asymptomatic patients (Leung *et al.,* 2001). Moreover, BKV DNA detection by PCR in urine samples of renal allograft recipients, seems to be an inadequate tool for screening because of its high sensitivity: positive results were observed in patients without Decoy cells. It has been suggested that BK virus DNA detection in plasma samples may have higher positive predictive value (Nickeleit *et al.,* 2000b).

In the study reported here, we investigated whether real-time PCR in plasma samples could be a useful tool for monitoring viral reactivation and identifying active infections in high-risk patients. To this end, we used a modified qualitative PCR and an "in-house" real-time PCR to compare levels of BKV DNA in plasma specimens from healthy immunocompetent subjects with those in specimens from bone marrow recipients with diseases of the urogenital tract (such as hemorrhagic cystitis).

MATERIALS AND METHODS

Patients and sampling

The study enrolled 22 bone marrow recipients attending the Haematology Department of the Sant'Eugenio Hospital, Rome, Italy. All subjects were receiving immunosuppressive therapy following standard protocols (Bolwell *et al.,* 2004; Loren *et al.,* 2005; Neumann *et al.,* 2005). For each patient, we analyzed plasma specimens collected within 2 months after transplant. Twelve of these patients displayed symptoms of viral infection of the low urogenital tract. During routine follow-up, we collected and analyzed 96 additional specimens, taking one specimen per patient once every three weeks for six months. As controls, we used plasma specimens from 107 healthy individuals undergoing routine check-ups at the Microbiology Outpatients Department of the Università Cattolica del Sacro Cuore.

All blood specimens from bone marrow transplant recipients and healthy individuals were collected in EDTA-tubes; plasma fraction was separated using low-speed centrifugation and stored at -80°C until analyzed.

DNA extraction and PCR qualitative assay

Nucleic acid was extracted from 200 µl of plasma using the spin column-based QIAamp DNA MiniKit (Qiagen S.p.A., Milan, Italy) and eluted in 100 µl of sterile water, according to the manufacturer's recommendations.

Qualitative PCR was used to test all samples for the presence of polyomavirus DNA. The primers used were PEP-1 and PEP-2 that bind to nucleotide sequences in the Large T antigen of BKV and JCV viruses as described by Arthur *et al.*, 1989. BKV produced a 176 bp amplification product; the length of the corresponding product for JCV was 173 bp.

This primer set was used in multiplex PCR, amplifying in the same reaction polyomavirus DNA and beta-globin gene fragments, to evaluate the DNA extraction process and the possible presence of amplification reaction's inhibitors. Beta-globin type specific primers amplify a 268 bp fragment (forward primer 5'-CAA CTT CAT CCA CGT TCA CC-3', reverse primer 5' -GAA GAG CCA AGG ACA GGT AC-3').

Each assay used 10 µl of DNA and 25 pmol each of the PEP-1 and PEP-2 and the Beta-globin

oligonucleotide primer sets. The reaction mixture contained 25 µl of HotStart Taq Master Mix (Qiagen S.p.a., Milan, Italy) and 5 µl of water RNasi-free in a final volume of 50 µl. The amplification profile consisted of 15 min at 95°C for activation of HotStar-Taq DNA polymerase followed by 45 cycles of denaturation (94°C for 30 s), annealing (55°C for 1 minute) and extension (72°C for 1 minute). Assays were performed on an iCycler Thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Amplicons were detected by electrophoresis of 20 µl of amplification products in a 2% agarose gel and visualized by ethidium bromide staining under UV light transilluminator Fluor-S^{TM}.

To discriminate between BKV and JCV sequences, we digested PCR products with *Bam*HI. The JCV sequence targeted for amplification contains a *Bam*HI site, which is absent in BKV. The molecular sizes of amplicons were determined by matching against commercial DNA molecular size markers (Molecular weight marker VIII, Roche Diagnostics, GmbH Mannheim, Germany). BKV produced a 176 bp amplification product, the digested JCV-DNA generated one 120 bp and one 53 bp fragment (Arthur *et al.,* 1989). The *Bam*HI site is not present in DNA fragments amplified by Beta-globin specific primers.

To assess sensitivity, specific PCR products were cloned into the pCR2.1 vector (Invitrogen, San Diego, CA, USA) and quantified by O.D. measurement. The construct was serially diluted and subsequently amplified using the PEP-1 and PEP-2 primers. PCR was shown to detect viral loads down to 5 copies per sample.

Procedures to prevent specimen contamination and PCR carryover were rigorously observed at all stages.

Real-time PCR

All plasma samples were tested by quantitative real-time PCR (Q-PCR) using the iCycler iQ Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA).

We used DNASIS version 2.1, for Windows (Hitachi Software Genetic System, San Francisco, CA, USA) to design PCR primers and probes specific for the BKV large T antigen. The sequences for primers and TaqMan probe were: forward primer, 5'-TTG GGT GGT GTT GAG TGT TGA G-3'; reverse primer, 5'-GGA GTC CTG GTG GAG TTC CTT-3'; probe, 5'(FAM)-CTG CTG TTG CTT CTT CAT CAC TGG CAA ACA-(TAMRA)3' (GenBank accession no. J02038). This pair of primers amplifies a 117 bp fragment. The probe was labeled dually at the 5'-end with 6-carboxyfluorescein (FAM) and the 3'-end with 6-carboxytetramethylrhodamine (TAMRA) (Applied Biosystems, Monza, MI, Italy). Primers and probes were stored at -20°C in small volumes to minimize multiple use and freezing and thawing of this reagents.

Quantitative PCR amplification was performed in a reaction volume of 50 µl containing 10 µl of DNA, 5 µl of primers (5 µM), 5 µl of probe (1.5 µM) and 25 µl of Platinum Quantitative PCR Super-mix-UDG (Invitrogen, San Diego, CA, USA). Thermal cycling was initiated with a first step at 95°C for 2 min, followed by 50 cycles at 94°C for 30 s and 58°C for 30 s.

The PCR product was cloned into pCR2.1 vector (Invitrogen, San Diego, CA, USA). The resulting plasmid was quantitated in triplicate by spectrophotometry at 260 nm, mixed with BKV-negative human DNA and serially diluted (from 10 to $1x10^7$ copies for reaction) to generate a standard curve. After confirming accurate preparation of the standard dilution series, the plasmid standards were stored at -20°C in small-volume aliquots to minimize multiple uses.

Amplification data measured as an increase in reporter fluorescence were collected in real-time and analyzed by Sequence Detection System software (Bio-Rad Laboratories Inc., Hercules, CA, USA). The fluorescence is generated as the reporter FAM on the 5' end of the probe is cleaved by 5'-3' exonuclease activity of the TaqMan polymerase during the extension phase of the cycle. The threshold cycle number, Ct, is the cycle number at which a significant increase in reporter fluorescence is detected above the mean background fluorescence. A standard curve constructed by plotting the log of starting plasmid concentration versus the Ct was used to determine BKV DNA concentrations in specimens. The dynamic range of real-time PCR assay (determined in preliminary studies) was 10 to 10^7 plasmid copies for reaction.

All samples were examined in triplicate. Viral load was expressed in terms of the copy number of viral DNA per milliliter of plasma.

The specificity of BKV real-time PCR assay was checked by testing DNA samples of the following viruses: JC polyomavirus, Cytomegalovirus, Herpes simplex virus type 1, Herpes simplex virus type 2, Adenovirus, Human Herpesvirus 8 and Epstein-Barr virus.
Negative controls containing distilled water instead of DNA were included in each run. Procedures to prevent specimen contamination and PCR carryover were rigorously observed at all stages.

RESULTS

Sensitivity of the "in-house" real-time PCR performed in this study was first evaluated by using serial dilutions of cloned BKV DNA amplification products. Each dilution was tested by real-time PCR in triplicate and the detection limit was estimated as 10 copies of BKV DNA per milliliter.
Specificity of the real-time PCR was evaluated by amplification of DNA samples from different viral pathogens. No positive results were observed testing DNA extracted from JC polyomavirus, Herpesviruses (Cytomegalovirus, Herpes simplex viruses, Human Herpesvirus 8, Epstein-Barr virus) and Adenovirus.
Plasma samples from 22 bone marrow transplant patients and from 107 immunocompetent subjects were then tested for polyomavirus DNA by qualitative multiplex PCR.
Beta-globin amplification products were observed in all DNA specimens demonstrating the suitability of the samples for PCR analysis and the absence of inhibitors in the reaction.
Amplification products from qualitative multiplex PCR (PEP 1-2) were digested with *Bam*HI endonuclease restriction enzyme to identify specimens that were positive for BK virus (Figure 1).
Of the 22 bone marrow transplant recipients 8 (36.3%) tested positive.
All plasma specimens from the control group tested negative for BKV.
A special effort was dedicated to 12 patients with infections of the lower urogenital tract: this group included all the patients who tested positive for BKV. The 96 additional specimens, collected during routine follow-up from all 12 patients, were also analyzed using both PCR and real-time PCR to evaluate viral load.

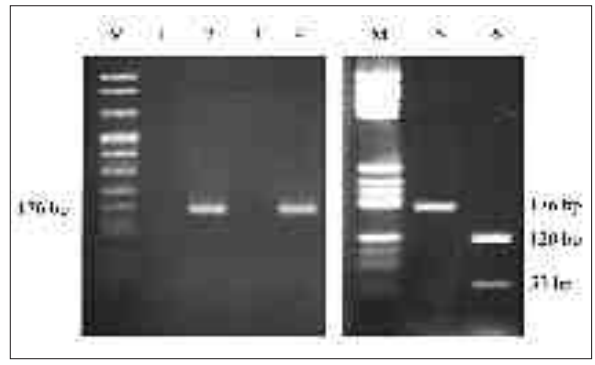

FIGURE 1 - *Detection of PCR-amplified BKV and JCV DNA in agarose gel. M: DNA Molecular Markers; lane 1: negative sample; lane 2: positive sample; lane 3: negative control; lane 4: positive control; lane 5: amplification product not digested by* Bam *HI; lane 6: amplification product digested by* Bam *HI (JCV).*

In this study, results from real-time PCR overlapped with those obtained by qualitative PCR. Real-time PCR confirmed all positive samples resulted for BKV DNA in qualitative test, displaying high sensitivity and specificity besides the advantage of the viral load evaluation.
The results, described in Table 1, show that viral load varies over time. In some patients (patients A, B, D, H, L, N) positive tests showing viral loads in the range 10^2 to 10^5 copies per milliliter of plasma alternated with negative test results. During follow-up, three of these patients (A, B, D) were diagnosed with HC. The HC occurred in patients A and B after 6 weeks, and in patients D after 4 weeks during the follow up.
One patient (patient M) initially tested positive (with a viral load of 2.9 x 10^3 copies/milliliter), and was negative on all subsequent tests. One patient (patient C) tested negative on all except the last test, which detected a viral load of 1.5 x 10^4 copies/milliliter. Four patients (patients E, F, G, I) were negative on all tests.

DISCUSSION

Given the increasing medical relevance of polyomavirus infection in humans, there is need for advancements in diagnosing and monitoring these infections.
In this study we developed a specific molecular assay to identify BKV infections. Human BK poly-

TABLE 1 - *BKV DNA determination by real-time PCR in plasma samples collected every three weeks from 12 bone marrow transplant patients.*

Patients	*Viral load (No. copies/ml)*	
	week 1-12	*week 13-24*
A	2.2×10^4	2.3×10^4
	Neg	3.8×10^3
	8.4×10^4	Neg
	Neg	7.6×10^3
B	6.4×10^2	4.9×10^4
	5.2×10^3	Neg
	Neg	9.1×10^2
	1.9×10^4	7.1×10^3
D	3.0×10^2	Neg
	4.5×10^4	8.5×10^4
	2.0×10^5	3.0×10^2
	3.6×10^3	2.1×10^3
H	Neg	3.1×10^3
	4.3×10^4	Neg
	Neg	Neg
	2.6×10^3	Neg
L	Neg	7.8×10^2
	7.3×10^4	Neg
	Neg	4.8×10^2
	1.5×10^4	Neg
N	1.2×10^5	9.2×10^2
	6.0×10^4	Neg
	Neg	Neg
	Neg	6.1×10^2
E, F, G, I	All negative	
M, C	Only one positive determination	

omavirus reactivation followed by overt disease is most frequent in patients with weakened immune systems such as organ or bone marrow transplant recipients (Dolei *et al.,* 2000). We report the development of an in-house real-time PCR for BKV DNA detection and a retrospective analysis of plasma specimens from BMT patients and healthy subjects by qualitative and quantitative PCR to evaluate the effectiveness of this assay.

There are a number of traditional methods for detecting and identifying polyomaviruses (Giraldo *et al.,* 1982; Knowles *et al.,* 1989; Nilsen *et al.,* 1991; Whiley *et al.,* 2001). These include serological assay, virus isolation by cell culturing, cytological examination and immunofluorescent staining of exfoliated urinary cells. Unfortunately, we have only a limited understanding of the specificity and sensitivity of these methods, which suffer from a high level of variability and poor interassay agreement. The usual way of obtaining a definite diagnosis is through graft biopsy.

Qualitative PCR (Arthur *et al.,* 1989), modified in this study to detect BKV DNA and beta-globin gene target, was very useful for its high sensitivity with respect to conventional virological methods with the further advantage of checking the goodness of DNA preparation.

In this work, viral DNA was never detected in plasma samples from healthy subjects where the latent infection is probably controlled by the immune system.

PCR easily detects BKV DNA in specimens from BMT recipients, patients at risk for BKV-related diseases.

However, to identify active infections, we need a quantitative assay. Given that the presence of BKV in plasma is associated with viral replication (Hirsch *et al.,* 2002; Nickeleit *et al.,* 2000; Splendiani *et al.,* 2004), levels of BKV DNA in plasma could be used as quantitative markers for polyomavirus replication. The lymphocytes B are considered the principal cell carrier of BKV during viral reactivation and so blood appears a significant biological sample to evaluate the presence of an active BKV infection.

These findings are in accordance with recent studies suggesting that BKV reactivation is a risk factor for the HC tin BMT patients and persistent high levels of viral DNA in plasma may be associated with the disease. (Apperley *et al.,* 1987; Arthur *et al.,* 1986; McNees *et al.,* 2005; Schatzl *et al.,* 1994; Sencer *et al.,* 1993; Whiley *et al.,* 2001). In fact, the mean viral load in plasma was significantly higher in patients with biopsy-proven BKV nephropathy than in patients without histological evidence of nephropathy (Hirsch *et al.,* 2002).

Our results support the hypothesis that quantitative PCR may discriminate between controlled viral reactivation with low levels of viral load and trends of viremia significantly associated with lower urogenital tract diseases.

In this study 36.3% of the BMT patients followed-up were positive for BKV DNA in more than one specimen and a diagnosis of HC has been report-

ed in the three patients with persisting detectable viral load (patients A, B, D).
Although similar viral load was found in samples of other patients without related pathology during the follow-up period, we cannot exclude the HC developing in the future.
Other authors have reported that reductions in immunosuppressive therapy eliminate viral DNA from plasma (Nickeleit *et al.,* 2000). There is currently no specific antiviral treatment for BKV infection. Therapy thus consists of adjustments to immunosuppressive therapy, making it possible to control the replication of the virus (Nickeleit *et al.,* 2000; Randhawa *et al.,* 2004). Taken together these results suggest that viral load in plasma reflects disease dynamics.
It is possible that the oscillating levels of viral DNA we measured in some of our own patients reflect precisely this kind of adjustment to immunosuppressive therapy (though it could also be due to variations in leukocyte number).
Early detection of viral reactivation is essential to make immunosuppressive therapy modulation effective. Real-time PCR provides a fast and sensitive way of measuring the viral load, overcoming the limitations of alternative molecular techniques and the risk of false positive results (Arthur *et al.,* 1989; Chang *et al.,* 1996; De Santis and Azzi, 1996; Leung *et al.,* 2002; Mackay *et al.,* 2002, Si-Mohamed *et al.,* 2006**)**.
In our study, results from BKV-specific real-time PCR assay overlapped with those obtained by qualitative PCR, displaying high sensitivity and specificity. The real time PCR eliminates post-amplification processing of PCR products, resulting less time-consuming and allowing the implementation of nucleic acid amplification techniques in clinical laboratories.
Our findings also show that viral loads can vary greatly between patients and in different specimens from the same patient. It follows that to identify at risk patients, it is important to evaluate trends in viral load using a series of specimens. It should also be noted that the sensitivity of quantitative PCR varies from laboratory to laboratory. In our own study, persistent viral loads in excess of 10^3-10^5 copies/milliliter reliably identified BKV reactivation and the risk for developing severe pathology. Values of viremia ranging between 10^3-10^4 copies/milliliter of plasma could identify at risk patients and the necessity of monitoring viral load in the follow-up. This range is supported by other study carried out in renal transplant recipients (Hirsch *et al.,* 2002). However, we believe that to determine a viremia cut-off is extremely difficult because of the lack of standardized assays, so the threshold values should be treated with caution (Randhawa *et al.,* 2004; Trofe *et al.,* 2004). Other studies including larger number of patients may improve the predictive value of this test**.**

CONCLUSIONS

According to a number of reports our observations emphasize that real-time PCR can be effective in distinguishing active from latent BKV infection in monitoring disease progression and in guiding therapy.
Our own work suggests that if real-time PCR is used with due precautions it become a "gold standard" for clinical laboratories engaged in the detection and monitoring of active BKV infection.

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