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Development and validation with clinical samples of internally controlled multiplex real-time PCR for diagnosis of BKV and JCV infection in associated pathologies

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ABSTRACT

This article describes the development and validation with clinical samples of an internally controlled multiplex quantitative real-time PCR (QRT-PCR) for human polyomaviruses BK (BKV) and JC (JCV). Blood and urine samples from renal transplant recipients with suspected nephropathy, and cerebrospinal fluid (CSF) specimens from AIDS, natalizumab-treated and HIV-negative patients with suspected progressive multifocal leukoencephalopathy, previously checked for BKV and JCV by conventional PCR, were tested by QRT-PCR. All samples positive by conventional PCR were confirmed by QRT-PCR. Four cases of JCV-associated neurological infection, including all those detected in natalizumab-treated patients, and one case of BKV-related neurological infection were only identified by QRT-PCR. BKV was quantified in the CSF of neurological patients for the first time. Analyses of the Quality Control for Molecular Diagnostics 2010 panel were "highly satisfactory" for BKV and "satisfactory" for JCV. The QRT-PCR is specific and reproducible. It improves the sensitivity of conventional PCR for the diagnosis of BKV and JCV infection in various diseases.

1. Introduction

Polyomaviruses are small, nonenveloped DNA viruses that infect a wide variety of vertebrates including humans. Human polyomaviruses BK (BKV) and JC (JCV) are widespread among humans with seroprevalence rates of 82% for BKV and 39% for JCV in healthy adult blood donors [1]. Primary infection takes place during childhood and is usually asymptomatic. After primary infection, BKV and JCV remain latent in the urinary tract [2] but also in mononuclear cells [3,4] and the central nervous system (CNS) [5]. Asymptomatic reactivation and urinary shedding have been described, especially under immunosuppression [6–9]. Pathological reactivation is generally associated with severe immunosuppressive status. BKV is the aetiological agent of polyomavirus-associated nephropathy (PVAN) and urethral stenosis in renal transplant recipients and also of haemorrhagic cystitis (HC) in bone marrow transplant recipients [10]. The prevalence of PVAN, in renal transplant recipients, varies from 1% to 10% [11]. Viral DNA quantification is recommended for monitoring the clinical course of PVAN and for managing the immunosuppressive therapy [12]. JCV is associated with progressive

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Table 1
Sequences of the primers and probes used in the real-time PCR assay.

Primer or probe	Reference sequence (GenBank no.)	Sequence 5′–3′/alignment[a]	Position
PM2+		GCWGAMACTCTATGYCTRTGTGG	
	BKV Dunlop (V01108)	GCAGAAACTCTATGTCTATGTGG	4184–4206
	JCV Mad-1 (NC_001699.1)	GCTGACACTCTATGTCTATGTGG	4047–4069
PM2−		GGTAGAAGACCCYAARGACTTTCC	
	BKV Dunlop (V01108)	GGAAAGTCTTTAGGGTCTTCTACC[a]	4388–4411
	JCV Mad-1 (NC_001699.1)	GGAAAGTCTTTAGGGTCTTCTACC[a]	4251–4274
BKV probe	BKV Dunlop (V01108)	FAM-CCAGCACACATGTGTCTACT	4232–4251
JCV probe	JCV Mad-1 (NC_001699.1)	VIC-GCTTGACTGAGGAATGCATGCAG	4221–4243
IC probe[b]	–	NED-CGCATCACGCGCGACCTCTT	–

[a] Alignment of primer reverse PM2− with references sequences Dunlop (BKV) and Mad-1 (JCV).
[b] IC probe: internal control probe.

multifocal leukoencephalopathy (PML) in AIDS patients at a rate of approximately 3% of HIV-1 seropositive individuals [13]. Recently, JCV has been related to PML in multiple sclerosis (MS) patients treated with natalizumab with an incidence of 1 in 1000 [14]. However, BKV has been detected in cerebrospinal fluid (CSF) samples from patients with CNS disease [15–17], including PML [18,19], and JCV can be the aetiological agent of nephropathy [20,21] and HC [22–24]. Consequently, a differential diagnosis of JCV and BKV is necessary.

This study aimed to develop an internally controlled multiplex real-time PCR (QPCR) that can detect and accurately quantify both JCV and BKV in a single reaction tube. Moreover, its suitability for the diagnosis of human polyomaviruses infection in various associated pathologies has been validated with clinical samples.

2. Materials and methods

2.1. Clinical samples

Samples were selected from those tested for polyomavirus DNA by conventional multiplex PCR of BKV, JCV and SV40 [25] in the National Microbiology Centre between 1999 and 2010. One hundred and seven blood and 94 urine specimens from 12 renal transplant recipients with suspected PVAN were checked by QPCR. Thirty CSF specimens from 29 HIV-positive patients and 26 CSF specimens from 19 MS patients treated with natalizumab, all of them with suspected PML, were also tested. In addition, 11 CSF samples from 11 HIV-negative patients with suspected PML, including two cases of leukaemia, one renal transplant recipient, one case of breast cancer, one case of lymphoma, one patient treated with corticoids, one patient treated with gamma globulins, and four patients whose clinical condition was unknown, were analysed. Subtypes of some of the samples included in the present study had been determined in a previous study [26] and so samples containing BKV subtypes Ia (2 samples), Ib-1 (5), Ib-2 (3), II (5), III (2) and IV (2) were tested.

2.2. Control samples

Control samples were selected from those analysed in the National Microbiology Centre between 2006 and 2009. Seven CSF samples positive for Herpes Simplex virus (HSV) DNA, seven CSF specimens positive for human enterovirus RNA and ten CSF specimens negative for human enteroviruses and human herpesviruses by a previously described nested multiplex PCR [27] were included. Two cervical scraping samples positive for human papillomavirus DNA by conventional PCR [28] were also tested. Moreover, seven blood and seven serum specimens from immunocompetent individuals without suspected polyomavirus infection were analysed.

2.3. DNA extraction

Viral DNA was isolated from 200 μl of clinical samples with the QIAamp MinElute Virus Spin Kit (Qiagen, Izasa, Spain) and eluted in 50 μl of DNase-free water following the manufacturer's instructions.

2.4. Primers and probes

Previously described generic primers of both BKV and JCV, PM2+ and PM2−, were used to amplify a conserved fragment of the large T antigen gene [29] (Table 1).

Two-hundred and eighty JCV complete genome sequences belonging to subtypes 1–8 and 163 BKV complete genome sequences belonging to subtypes I–IV whose subtype had been determined in previous studies [30–33] were obtained from GenBank (http://www.ncbi.nlm.nih.gov/nucleotide) and aligned with BioEdit software (Ibis Theraupeutics, Carlsbad, CA, USA). Specific TaqMan-MGB probes (Applied Biosystems, Foster City, CA, USA) for BKV, JCV and the internal control were designed and labelled with FAM, VIC and NED dyes, respectively.

Dimers of primers and probes and self-formations of probes were tested with Primer Select software (Lasergene, DNASTAR Inc., Madison, WI, USA).

2.5. Internal control plasmid

A previously published internal control plasmid that includes a sequence of pseudorabies virus DNA flanked by the primers PM2+ and PM2− was used [29].

2.6. Construction of BKV and JCV plasmids for standard curves

The BKV and JCV PCR amplimers contained in the standard control plasmids were generated by amplification of