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A globally applicable PCR-based detection and discrimination of BK and JC polyomaviruses

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ABSTRACT

BKV and JCV belong to the *Polyomaviridae* family and are opportunistic agents associated with complications in immunocompromised individuals. Although a single screening assay for both viruses would be convenient, the diversity of BKV and JCV serotypes and genotypes is a methodological challenge. In this paper, we developed a PCR method able to detect and segregate BKV and JCV, despite these genetic discrepancies. A duplex semi-nested PCR (duplex snPCR) was designed to target a conserved region (639nt-1516nt) within the VP2 gene. In the first PCR, a primer set common to all BKV and JCV serotypes/genotypes was used, followed by a semi-nested PCR with internal primers for BKV and JCV segregation. The limit of detection of the duplex snPCR was as low as 10 copies of BKV or JCV plasmids/μL. Specific products were observed when JCV and BKV plasmids were mixed in the same reaction. In field sample testing, the duplex snPCR detected and distinguished both viruses in different biological samples. Results were confirmed by Sanger's sequencing. The geographical complexity of BKV and JCV serotypes and genotypes imposes limits to a simple and universal method that could detect each virus. However, we describe here a sensitive and reliable PCR technique for BKV and JCV diagnosis that overcomes these limitations and could be universally applied.

KEYWORDS: BKV. JCV. PCR. Genotypes. Serotypes,

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BKV and JVC belong to the *Polyomaviridae* family and are small (40-45 nm), non-enveloped double-stranded DNA viruses (~5000 pb) with a high prevalence in the population, estimated as high as 80% and 70% for BKV and JCV, respectively[1].

The most common complications caused by BKV are the nephropathy (NPV) and hemorrhagic cystitis (HC) in renal transplant recipients, whereas the JCV is associated with progressive multifocal leukoencephalopathy (PML) in HIV-positive patients and in those with multiple sclerosis treated with natalizumab[2,3]. Recently, BKV and JCV have also been associated to some types of neoplasms[4-6] requiring further investigations.

In general, BKV and JCV can be detected in urine, whole blood, plasma, serum, tissues and rarely in cerebrospinal fluid (CSF)[2]. The diagnostic methods include cytological examination, immunofluorescent staining, enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR)[7-9]. The latter is the method of choice to detect active BKV replication in urine or blood since anti-BKV antibodies detection is not helpful and viral isolation is time-consuming[6]. For JCV, the immunohistochemistry of brain biopsies and PCR of CSF are the most accurate to diagnose PML[10].

Asmembers of the same viral family which are associated to disease in immunocompromised individuals, the development of a single discriminative test for both viruses is attractive. In addition, cases of JCV nephropathy have been reported in literature[8], supporting the need for an accurate identification of these agents. Although the use of quantitative real-time based assays became the standard diagnosis for both viruses, especially for BK in plasma, a cost-saving sensitive screening method would be helpful, especially in limited resourced centers that could not afford the high costs involved in the real-time PCR. However, due to genetic similarities (above 70%) and a diversity of serotypes and genotypes[11], the differentiation of BKV and JCV by molecular methods is challenging. Therefore, the goal of the present study was to design a PCR methodology to detect and differentiate BKV and JCV in clinical samples.

A duplex semi-nested PCR was designed targeting a conserved genomic region of the minor capsid protein VP2. To this end, VP2 sequences from all BKV viruses [V01108 (Ia), AB211371 (Ib1), AB211370 (Ib2), AB211375 (Ic), AB263916 (II), AB211386 (III), AB211387 (IV)] and JCV genotypes [J02226 (1A), AF015527 (1B), AF030085 (2A), AF015532 (2B), AF015534 (2C), AF015536 (2D), AF281606 (2E), U73500 (3A), U73501 (3B), AF015528 (4), AF015537 (6), U61771 (7)], available at GenBank database were aligned with the aid of the Molecular Evolutionary Genetics Analysis version 7.0 (MEGA 7.0)[12] software in search of potential regions for primer alignment. A region comprising ~870 pb (639 nt-1516 nt) was chosen due to its conservation along BKV and JCV genotypes, as well as its potential to discriminate between the viruses (Figure 1).

In the first round of amplification, the forward primer BKJCf [5'-GCACTTTTGGGGGACCTAGT-3'; (nt 639-657 in BKV and nt 541-559 in JCV)] and the reverse primer BKJCr [5'-GGCAACATCCATTGAGGAG-3'; (nt 1,511-1,492 in BKV and nt 1,416-1,397 in JCV)] were used as they were common for both viruses. For the second round, the forward primers were designed to distinguish between BKV [BKf: 5'-GGACAATTGTAAATGCCCCYAT-3'; (nt 1228-1249)], and JCV [JCf: 5'-CCAAAGGGAGGGAACCTATATT-3'; (nt 1227-1248)], combined with the reverse primer BKJCr, already used in the first round. Due to a substitution of a T→C at the position 1,247 of BK genotype III, a degeneration (Y) was inserted at the 20th position of BKf primer. The inner primer set amplified a 284 bp fragment for BKV and 190 bp for JCV (Figure 1). The specificity of both pair primer pairs was confirmed with the aid of the BLASTn software, resulting in 100% similarity with BKV and/or JCV.

PCR mixture for both PCR steps contained 2.5 µL of 10X PCR buffer (200 mM Tris-HCl pH 8.4 and 500 mM KCl), 0.75 µL of 50 mM $MgCl_2$, 0.2 µL of 100 mM dNTP mixture, 0.2 µL of the 5 U/µL Taq polymerase enzyme (Invitrogen, Thermofischer Scientific, USA), 0.5 µL of each 25 µM forward and reverse primers in the first round, but in the second round 0.5 µL of each 25 µM forward primer and 1µL of the 25 µM reverse primer. The reaction mixture was adjusted to 25 µL of final volume with ultrapure water (Ambion, USA). In the first round 2 µL of DNA was added to the mixture, while in the second step 2 µL of the first PCR product was diluted 1:100 in ultrapure water and 2 µL of the dilution was added. The Thermal Cycling of both PCR rounds consisted of an initial denaturation step of 5 min at 9 ºC, following 40 cycles of 55 s at 94 ºC, 50 s at 55 ºC and 55 s at 72 ºC, and a final extension of 5 min at 72 ºC. The BKV and JCV VP2 amplicons were visualized on 1.5% agarose gels stained with 0.02% ethidium bromide.

As the internal reaction control, in-house designed primers (BAF1: 5'-AAGAGAGGCATCCTCACCCT-3'; and BAR2: 5'-TACATGGCTGGGGTGTTGAA-3'), amplifying a 220 bp fragment of the beta-actin gene[13] were used in the same cycling and reaction conditions, as described for the first round of PCR for BKV and JCV detections.

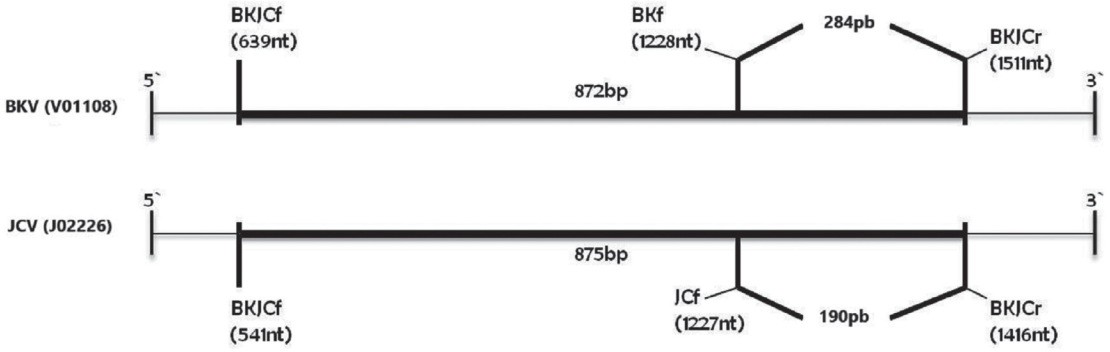

Figure 1 - Product sizes in the 1st and the 2nd (nested) PCR in BKV (V01108, Dunlop strain) and JCV (J02226, Mad1 strain) VP2 sequences

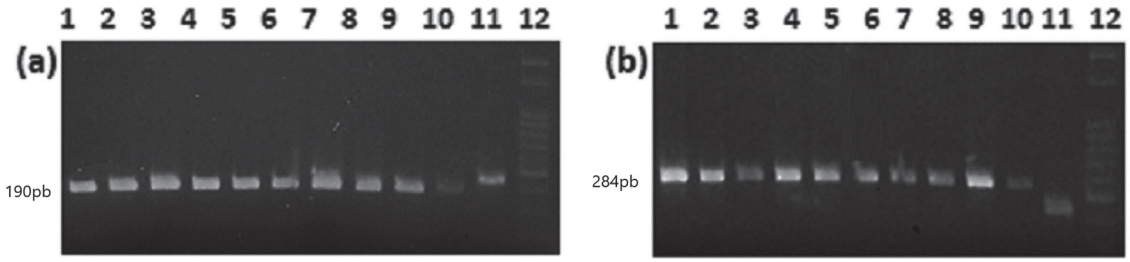

Figure 2 - Agarose gel showing the limit of detection for JCV (190 pb): A) and BKV (284 pb); B) plasmids. Lines 1 to 10: Plasmids ranging from 10^{10} to 10^{1} copies; line 11: the human beta-actin used as an endogenous control (220 pb); line 12: 100 bp DNA ladder (New England Biolabs, USA)

The sensitivity of the reaction was evaluated with plasmids containing complete genomes of BKV and JCV. The theoretical plasmid copy number was calculated using the ENDMEMO software available at www.endmemo.com/bio/dnacopynum.php, which is based on the size (in base-pairs) of each plasmid, the average nucleotide molecular weight and DNA quantity. The plasmids were quantified by the Nanodrop Spectophotometer (ThermoFisher Scientific®, USA) at 260 nm wave length 10-fold diluted in TE (Tris-EDTA) buffer (ThermoFisher Scientific®, USA). The 10^{10} to 10 copies per microliter dilutions were subsequently submitted to the duplex-snPCR. A mixture of BKV and JCV plasmids were also tested to check out the capacity of PCR to co-detect both viruses in the same sample. Finally, 87 clinical samples (60 whole blood; 21 urines; 5 plasma samples; 1 renal biopsy) from renal transplant patients, one per individual, were tested. Samples were stored at -80 °C until DNA was extracted. Biopsies and 200 µL of whole blood samples DNA were extracted by the RTP® DNA/RNA Virus Mini Kit (Stratec Molecular Biomedical - Berlin, Germany) according to the manufacturer's instructions, while 200 µL of urine and plasma samples DNA were extracted by the PureLink™ Viral RNA/DNA Mini Kit (ThermoFisher Scientific®, USA), also according to the manufacturer's instructionsSamples were all eluated in 50 µL of TE buffer provided by the extraction kits. This study was approved by the Ethic Committee of the Hospital Universitario Clementino Fraga Filho (CAAE Nº 43624814.2.0000.5262).

The duplex snPCR could detect as few as 10 copies of template plasmidic DNA of each virus per microliter (Figure 2). It was also able to co-detect JCV and BKV in the same mixture (Figure 3). Moreover, sn-PCR was conducted to test the cross-reactivity between second round BKV and JCV primers and JCV BKV plasmids, respectively, revealing that the designed primers do not amplify non-specific plasmids. Of the clinical samples, 34 (39%) were positive for any of the two viruses, of which 31(35.6%) were positive for BKV and 3 (3.4%) for JCV. Regarding the sample type, JCV was detected only in urine, while BKV was detected in 11 urine samples, one kidney tissue fragment and 19 whole blood samples. None of the viruses were detected in plasma samples. No dual detection was observed in the tested samples and all of them were positive for the beta-actin reaction.

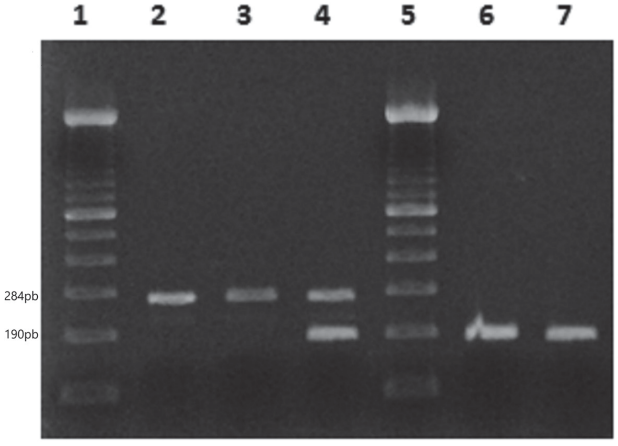

Figure 3 - Agarose gel showing the duplex snPCR products. Lines 2 and 3: 284 bp BKV fragment; lines 6 and 7: JCV 190 bp fragment; line 4: JCV and BKV dual detection; lines 1 and 5: 50 DNA bp ladder (New England Biolabs, USA)

BKV and JCV share many genetic and biological properties, but are associated with different illnesses[4]. Meanwhile, other pathogenic properties of both viruses need further investigation, especially concerning their association with different neoplasias[14].

In this study, a duplex semi-nested PCR was developed to detect and discriminate BKV and JCV in diverse types of clinical samples. The technique was proven highly sensitive, as observed by the limit of detection of 10 copies per µL. In addition, the primer sets chosen after extensive alignment of the different BKV and JCV genomes, and confirmed by the BLAST analysis, confirmed their inclusiveness. In addition, the results obtained with acellular (urine supernatant, plasma) and cellular (tissue, whole blood) clinical samples, indicate a wider application.

The development of a reliable multiplex tool for the identification of polyomaviruses is not trivial, which may

explain the small number of existing protocols. BKV and JCV are classified into major subtypes (4 BKV and 7 JCV) that are further subdivided into geographically dispersed genotypes[15,16]. Therefore, the inclusion of these variants should be considered under the penalty of a misdiagnosis. For instance, using the protocol established by Fedele *et al.*[17], which is the most referenced method for simultaneous detections of BKV and JCV, 7 patients of our study were co-infected with JCV and BKV. However, the sequencing analysis confirmed only BKV, which was further confirmed by our primer sets. Therefore, the conclusion was that the JCV primers described by these authors were not specific, probably due to the extensive degeneration of the primer sequences. In 2000, Bofil-Mas *et al.*[18] developed an interesting multiplex tool for BKV and JCV diagnosis, although the nested reaction required a separated BKV and JCV amplification. More recent publications on multiplex PCR for both viruses are based on the methodology described by Fedele *et al.*[19] which can, as shown, generate non-specific results, and also the methodology proposed by Bofil-Mas *et al.*[20].

Although real-time PCR is the standard diagnosis for polyomaviruses, the use of qualitative methods may be of interest in centers that lack the infrastructure or receive a small number of samples to compensate for the use of more expensive diagnostic methods. In addition, qualitative tests have shown to be compatible with quantitative tests for detecting BKV in blood samples (unpublished data).

Based on our results, we conclude that the original duplex snPCR presented here is a sensitive, specific and reliable molecular methodology to detect and differentiate BKV and JCV in clinical samples. Although we established the technique using samples from renal recipients only, we could extend it to other types of biological materials from patients presenting different clinical manifestations.

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