New Commercially Available PCR and Microplate Hybridization Assay for Detection and Differentiation of Human Polyomaviruses JC and BK in Cerebrospinal Fluid, Serum, and Urine Samples

Hélène Moret,1 Véronique Brodard,1 Côme Barranger,2 Nicolas Jovenin,3 Martine Joannes,2 and Laurent Andréoletti1*

Laboratoire de Virologie, CHU Reims et EA-3798, Faculté de Médecine, 51100 Reims,1 Argene Biosoft S.A., 09120 Varilhes;2 and Département d’Information Médicale, CHU de Reims et Faculté de Médecine, 51100 Reims,3 France

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JC and BK human polyomaviruses (family Polyomaviridae) may cause severe neurological or urinary tract pathologies in immunocompromised hosts. In the present study, we evaluated a new commercially available PCR and microplate colorimetric hybridization assay for the standardized differential detection of JC virus (JCV) and BK virus (BKV) genomes in clinical samples. This JC/BK Consensus test was first evaluated by testing serial dilutions of JCV or BKV plasmid DNA standards and was then compared with an in-house reference PCR assay for the detection of JCV and BKV virus genomes in 70 cerebrospinal fluid (CSF) samples of patients with neurological disorders and in 75 serum or plasma samples and 125 urine samples of renal graft recipients. This new test allowed a limit of detection of 10 copies and 1 copy of JCV and BKV virus genomes, respectively, and was able to differentiate various levels of JCV, BKV, and mixed JCV and BKV DNA genomes in a single reaction tube. Our results showed 100% specificity and sensitivity for the JC/BK Consensus test with CSF samples. With serum or plasma samples, this test had a specificity and a sensitivity of 100% for both JCV and mixed JCV and BKV DNA detection and a sensitivity and a specificity of 100 and 97.8% for BKV DNA detection, respectively. With urine samples, the sensitivity and specificity were 100 and 96.6%, respectively, for JCV DNA detection; 100 and 89.4%, respectively, for BKV DNA detection; and 44.4 and 100%, respectively, for mixed JCV and BKV DNA detection. In conclusion, our data indicate that this new test, the JC/BK Consensus test, is valuable for the sensitive and specific differential detection of single JCV and BKV infections in CSF, serum or plasma, and urine samples. The use of this reliable PCR assay would improve the routine virological diagnosis as well as the clinical care of immunocompromised patients with polyomavirus-related pathologies.

Human polyomaviruses JC and BK (family Polyomaviridae) are very similar viruses, based on their structures, DNA genome organizations, and nucleotide sequences. Both JC virus (JCV) and BK virus (BKV) share approximately 75% nucleotide homology with each other and 70% nucleotide homology with the simian polyomavirus simian virus 40 (21, 31). Human polyomaviruses JC and BK are endemic and infect a large proportion of healthy individuals worldwide (13, 29). Primary infections with these viruses usually occur during childhood, are largely asymptomatic, and may result in transient viremia (7). Following primary infections, JCV and BKV both establish latent infections in renal tissues and can also be detected in the B lymphocytes of healthy adults (1, 9, 19). Polyomavirus-related diseases usually develop under conditions of severe cellular immunosuppression, such as organ transplantation, AIDS, and leukemia (20). JCV is the causative agent of progressive multifocal leukoencephalopathy (PML), which primarily occurs in AIDS patients (4, 16, 30), and it has been also identified as a causal agent of hemorrhagic cystitis and graft nephropathy (5, 14, 25). Reactivation of BKV is related to urinary tract diseases, such as hemorrhagic cystitis, ureteric stenosis, glomerulonephritis, and graft nephropathy, which are most commonly found in transplant patients undergoing immunosuppressive therapy (10, 13, 15, 24, 28).

Conventional serological methods and traditional virus isolation by cell culture assays have previously been used to detect and identify human polyomavirus infections (20, 29). More recently, PCR assays for detection of JC and BK viruses have been developed, and several studies have shown PCR to be an effective tool for the detection of human polyomaviruses in a range of clinical samples (2, 8, 23, 32). PCR techniques have emerged as the “gold standard” for polyomavirus detection, particularly for the rapid detection and identification of the etiological agent in the clinical management of immunocompromised patients (3, 12, 17, 22, 32). However, the routine implementation of DNA PCR techniques followed by probe hybridization assays in clinical laboratories has some limitations, including amplification carryover contamination and the need for the use of technically cumbersome PCR product detection methods and standardized positive and negative controls, as well as an integrated coamplified internal DNA control to demonstrate the absence of PCR inhibitors for each sample tested (32). Several recent studies have demonstrated that real-time PCR technology could overcome many of these limitations and could be reliable tools for the rapid diagnosis of polyomavirus-related diseases in clinical practice. However, this PCR technology requires costly real-time PCR instrumentation and the development and the validation of specific fluorophore-labeled hybridization probes that allow the identifica-
tion of human polyomaviruses in clinical samples (6, 11, 18, 26, 27).

In the present study, we evaluated the sensitivity and the specificity of a new commercially available PCR and microplate colorimetric hybridization system that allows the qualitative differential detection of JC and BK virus genomes in clinical samples. This new PCR assay (the JC/BK Consensus test) was first evaluated by testing serial dilutions of JCV or BKV plasmid DNA standards. This test was then compared with an in-house reference PCR assay for the detection of JC and BK virus genomes in 70 cerebrospinal fluid (CSF) samples of patients with neurological disorders and in 75 serum or plasma samples and 125 urine samples of adult renal graft recipients.

MATERIALS AND METHODS

Clinical specimens. All of the clinical samples tested in the present study had originally been sent to the Virology Department of the University Hospital Center of Reims (Reims, France) from January 2000 to March 2004 for routine viral diagnostics by a human polyomavirus PCR DNA detection assay. Serum or plasma samples (n = 75) and urine samples (n = 125) had been taken from adult renal graft recipients. CSF samples (n = 70) had been taken from immunosuppressed patients, a majority of whom were human immunodeficiency virus-infected patients with neurological disorders potentially related to PML disease. Blood samples were collected in sterile vacuum tubes containing or not containing EDTA, and the serum or plasma fraction was separated by low-speed centrifugation. The samples were directly aliquoted and frozen at −80°C until they were used in the routine procedure for the detection of human polyomavirus DNA by PCR or in the present evaluation.

DNA extraction. DNA extraction was carried out with the spin column-based QIAamp DNA blood minikit (QIAGEN, Courtaboeuf, France), according to the manufacturer’s instructions. The final extraction volumes were 50 μl for CSF samples and plasma or serum samples and 200 μl for urine samples. A DNA extraction procedure was performed with a new aliquot of each sample tested at the time of the present study, and the purified DNA extracts were then stored at −20°C until they were used in the PCR procedures.

Routine in-house PCR for detection of polyomaviruses and hybridization proce- dures. An in-house PCR protocol for the detection of the two human polyomaviruses JCV and BKV was carried out with the primer pair PEP-1 and PEP-2, previously designed by Arthur et al. (2), and the PCR conditions described by Moret et al. (23). Following PCR amplification, 2 μl of each amplified product was analyzed by a differential microplate hybridization assay with, respectively, the two biotinylated probes (JEP-1 and BEP-1) specific for JCV and BKV strains (2). This differential hybridization protocol was performed according to the manufacturer’s instructions (Hybridowell kit; Argene Biosoft, Varti- hes, France).

JC/BK Consensus assay. In the present study, a new commercially available assay developed for the molecular detection and identification of human polyomaviruses JCV and BKV in clinical samples was evaluated. All the reagents for PCR amplification and microplate hybridization assay except the HotStarTaq DNA polymerase (QIAGEN) were included in the JC/BK Consensus kit (Argene Biosoft). For PCR amplification, 10 μl of extracted DNA and 2 μl of HotStarTaq DNA polymerase were incorporated in 40 μl of a specific PCR mixture containing primers specific for human JCV and BKV. For each sample, a second reaction was carried out to check for the absence of PCR inhibitors. In this second PCR amplification, a composite control plasmid was included at 2,500 copies per reaction tube. This plasmid contained an internal fragment of the same size and with the same thermodynamic properties as the template DNA and was amplified with the same primers, as described by the protocols of the manufacturer (Argene Biosoft). PCR was carried out in a Perkin-Elmer thermocycler (Applied Biosystems, Courtaboeuf, France) by using an amplification program containing the following steps: 10 min at 95°C for HotStarTaq DNA polymerase activation; 40 cycles consisting of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C; and a final extension phase of 10 min at 72°C. The amplified products were stored at −20°C for less than 1 week until detection.

For the microplate hybridization procedure, 5 μl of specific and inhibition control amplified products was chemically denatured. Then, the specific PCR product was used to coat two separate microwells, whereas the product amplified from the inhibition control was used to coat a third microwell for 15 min at 37°C. In each one of these coated microwells, a hybridization assay was carried out by using three 5′-biotinylated probes specific for JCV, BKV, and the inhibition control for 30 min at 37°C. After five washing phases, a reaction with a streptavidin-peroxidase conjugate was performed for 15 min at room temperature. The microwell was then washed five times, and the products were revealed by application of tetramethylbenzidine for 30 min at room temperature in the dark. Then, a stop solution was added to the microwells.

The optical density (OD) was measured at 450 nm versus 650 nm. The calculation of the cutoff (CO) value was obtained as followed: CO = OD (mean of the negative controls) + 0.075. Samples were considered negative if the OD was less than the CO value minus 10%, and samples were considered positive if the OD was greater than the CO value plus 10%. When the OD value was estimated to be within 10% of the CO value, the sample was tested once again after a new DNA extraction phase.

JCV and BKV plasmid DNA standards. The full genomic sequence of the JCV “Mad-1” and BKV “Dun” reference strains cloned in DNA plasmids. Plasmid DNA standards were diluted in sterile CSF. Cross-reactivity was evaluated by using a Qiagen spin miniprep kit (QIAGEN). Plasmid DNA concentrations were determined by measurement of the OD at 260 nm. Quantified JCV and BKV plasmid DNA standards were diluted to obtain a final concentration of 105 DNA copies per μl, aliquoted, and then stored at −20°C until they were used.

Statistical analyses. Student’s t test was carried out, when necessary, by using the software Epi-Info 6.04 (2001, Centers for Disease Control and Prevention-World Health Organization statistical analysis software, Geneva, Switzerland). The box plots were calculated and drawn by using the software Statview 4.51 (1995, Abacus Concepts, Berkeley, Calif.). P values less than 0.05 were consid- ered significant.

RESULTS

Analytical sensitivity and specificity of the new JC/BK Consensus test. The sensitivity and specificity of the JC/BK Consensus test were first evaluated by using 10-fold dilution series of JCV or BKV plasmid DNA standards that were diluted in sterile distilled water. Our results indicated that the new commercially available test was able to detect as few as 10 copies and 1 copy of the JCV and the BKV genomes, respectively, per reaction tube (Table 1). Identical sensitivity results have been obtained by using 10-fold dilution series of JCV or BKV plasmid DNA standards which were diluted in sterile CSF. Cross-reactivity was evaluated by the use of a protocol in which each one of the two probes specific for JCV and BKV was tested with standard dilution series of the two polyomavirus genomes (Table 1). Whatever the levels of human polyomavirus DNA templates tested, we observed a restricted detection of the
amplified JCV and BKV plasmid DNA standards by the respective JCV and BKV probes, indicating a highly specific differential molecular hybridization system (Table 1). In addition, the new JC/BK Consensus test was able to coamplify mixed copies of JCV and BKV plasmid DNA standards at ratios ranging from 1 to 10³ and to perform a qualitative differential detection of these two human polyomavirus genomes in a single reaction tube (data not shown). No positive amplification or hybridization assay results were observed with the DNA genomes of the human *Herpesviridae* family, simian virus 40, adenovirus, and proviral human immunodeficiency virus type 1 (data not shown).

**Evaluation of JCV genomic DNA detection in CSF samples of patients with neurological disorders by in-house reference PCR assay and the new JC/BK Consensus test.** We compared the rates of detection of JCV DNA in 70 CSF samples of patients with neurological disorders by the in-house reference PCR assay and the new JC/BK Consensus test. Table 2 shows that 28 and 42 of the CSF samples were positive and negative by the two assays, respectively, indicating 100% specificity and sensitivity for the JC/BK Consensus test with the CSF samples tested (Table 2). No CSF samples showed the presence of PCR inhibitors, as demonstrated by the amplification of an internal control included in the JC/BK Consensus test. No BKV DNA was detected in the 70 CSF samples tested by this new commercial assay.

**Evaluation of JCV, BKV, or mixed JCV and BKV genomic DNA detection in serum or plasma samples of adult renal graft recipients by in-house reference PCR assay and the new JC/BK Consensus test.** We compared the rates of detection of JCV, BKV, and mixed JCV and BKV DNA genomes in 75 serum or plasma samples of adult renal graft recipients by the two assays. Table 3 shows that 5 samples were positive for JCV DNA, 29 were positive for BKV DNA, and 1 was positive for mixed JCV and BKV DNA, whereas 39 were negative for JCV and BKV DNA by the two assays. Only one sample appeared to be positive by the new JC/BK Consensus test and negative by the in-house PCR assay for BKV DNA detection. None of these serum or plasma samples displayed the presence of PCR inhibitors. Taken together, these data show that the new consensus test has a sensitivity and a specificity of 100% for both JCV and mixed JCV-BKV detection and a sensitivity of 100% and a specificity of 97.8% for BKV detection in serum or plasma samples.

**Evaluation of the JCV, BKV, or mixed JCV and BKV genomic DNA detection in urine samples of adult renal graft recipients by in-house reference PCR assay and the new JC/BK Consensus test.** In the last part of the retrospective evaluation of the new JC/BK Consensus test, we compared the rates of detection of JCV, BKV or mixed JCV and BKV in 125 urine samples of adult renal graft recipients by both the in-house reference PCR assay and the JC/BK Consensus test. Table 4 shows that 35 samples were positive for JCV DNA, 40 were positive for BKV DNA, and 8 were positive for mixed JCV and BKV DNA, whereas 30 appeared to be negative for JCV and BKV DNA by the two assays. Ten samples appeared to be positive by the in-house test for mixed JCV and BKV DNA detection. Of these 10 samples, 2 were positive only for JCV DNA and 8 were positive only for BKV DNA by the new JC/BK Consensus test. One sample was positive for BKV DNA by the new test, whereas it was negative for polyomavirus by the in-house test. Moreover, another sample appeared to be positive for JCV DNA by the new test and negative by the in-house test (Table 4). None of these urine samples displayed the presence of PCR inhibitors. For urine samples, we assessed a sensitivity and a specificity of 100 and 96.6%, respectively, for JCV DNA detection; a sensitivity and a specificity of 100 and 89.4%, respectively, for BKV DNA detection; and a sensitivity and a specificity of 44.4 and 100%, respectively, for mixed JCV and BKV DNA detection. Figure 1 shows the distributions of positive OD values obtained by detection of JCV or BKV DNA in 95 urine samples by the in-house reference PCR assay and the new JC/BK Consensus test. The mean optical density values obtained by the JC/BK Consensus test appeared to be significantly higher than those obtained by the in-house PCR assay for JCV- and BKV-positive urine samples (*P < 0.0001* and *P = 0.0001*, respectively) (Fig. 1). Taken together, these data indicate the reliability and the suitability of this new JC/BK Consensus test for the sensitive and specific differential detection of single JCV and BKV infections in urine samples.

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<tr>
<th>Table 2. Comparison of rates of detection of JCV DNA in CSF samples of patients with neurological disorders by in-house reference PCR assay and the new JC/BK Consensus test.</th>
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<tr>
<td>JC/BK Consensus test result for JCV</td>
</tr>
<tr>
<td>Positive</td>
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<td>Positive</td>
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<td>Negative</td>
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<th>Table 3. Comparison of rates of detection of JCV, BKV, or mixed JCV and BKV DNA in serum or plasma samples of adult renal graft recipients by in-house reference PCR assay and the new JC/BK Consensus test.</th>
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<tr>
<td>JC/BK Consensus test result</td>
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<tr>
<td>JCV + BKV−</td>
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<td>JCV− BKV+</td>
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*+, positive result for the indicated virus; −, negative result for the indicated virus.*
FIG. 1. Distribution of positive optical density values obtained by detection of JCV or BKV DNA sequences in 95 urine samples by the in-house reference PCR assay and the new JC/BK Consensus test. The length of each box corresponds to the interquartile range, with the upper boundary of the box representing the 75th percentile and lower boundary of the box representing the 25th percentile. The horizontal line in the box indicates the median value. The lines extending upward and downward from the box mark the 10th and the 90th percentile ranges, respectively. Outlier values are shown as circles.

DISCUSSION

Evaluation of commercially available polyomavirus-specific PCR assays is a prerequisite for the further standardization of JCV and BKV genome detection in CSF samples of immunocompromised patients with neurological syndromes, as well as in urine and serum or plasma samples of organ graft recipients undergoing immunosuppressive therapy. In this study, we evaluated for the first time a new commercially available PCR assay which uses several pairs of primers to amplify JCV and BKV genomes, differentiates the JCV and BKV amplicons in a microwell colorimetric hybridization assay, and demonstrates the absence of PCR inhibitors through the use of a positive control tested with a duplicate sample after DNA extraction within 6 h. We evaluated this new JC/BK Consensus PCR method for the virological diagnosis of JCV and BKV infections in CSF, serum or plasma, and urine samples.

In the first part of the present study, the levels of sensitivity of the new JC/BK Consensus PCR test appeared to be equivalent to those previously obtained by classical or real-time PCR assays for the detection of polyomavirus genomes (3, 6, 27). Evaluation of the specificity demonstrated the capacity of this new assay to perform the specific differential detection of JC or BK virus genomes whatever the levels of human polyomavirus DNA templates per reaction tube were (Table 1). This absence of cross-reactivity for the detection of the two human polyomaviruses suggested that this new test might allow the reliable differential detection of these viruses in various anatomical compartments. Moreover, our analytical results indicated that the new test was able to detect various levels of mixed JCV and BKV genomes in a single reaction tube, suggesting that this assay may be also valuable for the detection of JCV and BKV coinfection in samples of graft transplant recipients or immunocompromised hosts. Altogether, these analytical data suggest that the JC/BK Consensus PCR test might be of major use for the management of immunocompromised patients in which the presence or the absence of JCV or BKV DNA detection does not imply similar clinical and therapeutic consequences according to the clinical sample tested (20).

In the second part of the present study, the new JC/BK Consensus test was compared with an improved in-house reference PCR assay for the detection of JCV and BKV virus genomes in 70 CSF samples from patients with neurological disorders (2, 23). Our data demonstrated similar rates of sensitivity and specificity for JCV detection in CSF samples by the two assays (Table 2). Moreover, the use of this new assay allowed us to exclude the presence of PCR inhibitors. Altogether, these findings demonstrate the reliability of this new assay for the clinical diagnosis of JCV-induced neurological disorders, particularly PML (23).

In the present investigation, 75 serum or plasma samples and 125 urine samples of adult renal graft recipients were tested by the two PCR and hybridization techniques. The rates of sensitivity and specificity appeared to be very similar between the two assays (Tables 3 and 4). Of the 75 serum or plasma samples tested, only 1 sample appeared to be BKV positive by the JC/BK Consensus test and negative by the in-house PCR. This discrepant result was verified twice, demonstrating low OD values (<0.5) with the JC/BK Consensus kit and OD values lower than the cutoff values (<0.04) by the in-house technique (data not shown). This discrepant result obtained with one serum sample was also observed with two urine samples (Table 4); these discrepancies could be explained only by a higher sensitivity of BKV DNA detection with the new JC/BK Consensus test. Of the 125 urine samples tested, 10 samples appeared to be positive by the in-house PCR test for mixed JCV and BKV DNA detection, whereas they were positive for only one of the two polyomaviruses by the JC/BK Consensus test (Table 4). In all of these cases of mixed polyomavirus infections detected by the in-house PCR assay, we observed that one of the OD values ranged from 0.150 to 0.400, whereas the other was greater than 3.000 (data not shown). By contrast, by using the Consensus JC/BK technique, the same samples appeared to be positive for either the JCV or BKV genome, with OD values greater than 3.500 (data not shown). Because, we have observed the highly specific differential detection of the two human polyomaviruses by the new JC/BK Consensus test (Table 1), these findings suggest the presence of false-positive mixed JCV and BKV DNA detection in the urine samples by the in-house PCR assay. However, further prospective evaluations are needed to confirm the high levels of sensitivity and specificity of the JC/BK Consensus test for the detection of mixed JCV and BKV genomes in urine and other clinical samples.

In conclusion, this report describes the first evaluation of a new commercially available test that allows the sensitive and specific differential detection of single JCV and BKV infections in CSF, serum or plasma, and urine samples. This standardized test should be very useful for the discrimination of JCV and BK viruses in clinical samples. The use of this reliable polyomavirus detection assay would improve the virological diagnosis as well as the clinical care and the therapeutic man-
agement of immunosuppressed patients with JCV-related neurological disorders or BKV-related nephropathy.

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REFERENCES