Optimisation and analysis of polymerase chain reaction based DNA sequencing for genotyping polyoma virus in renal transplant patients: A report from South India

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Abstract

Purpose: To optimise a polymerase chain reaction (PCR) based DNA sequencing technique for genotyping polyoma virus in clinical specimens obtained from renal transplant patients. Materials and Methods: A hundred and thirty (106 peripheral blood and 24 urine) clinical specimens collected from renal transplant patients were included in the study for detecting the presence of DNA of BK virus (BKV), JC virus (JCV) by PCR targeting the viral protein 1 (VP1) gene. PCR based DNA sequencing was performed to determine the genotypes of polyoma virus and subjected to bioinformatics analysis to determine the amino acid sequences and screen for mutations in the VP1 gene. Results: Polyoma virus was detected in 23 (17.69%) specimens of which 19 (82.60%) were positive for BK virus, 3 (13.04%) for JC virus and 1 for both BK and JC virus. PCR based DNA sequencing detected BK virus genotype I in 12 (50%), genotype IV in 8 (33.3%) and JC virus in 4 (16.6%) clinical specimens. BKV genotype I was the predominant genotype (64.2% in peripheral blood and 33.33% in urine) prevalent in south India. Six novel mutations were found – at position 29, 30 to 47 of BKV genotype I; at position 11 and 15 of BKV genotype IV and at position 2 and 30 of JCV. Conclusion: BKV genotype I is the prominent genotype in India and novel mutations detected in the VP1 gene of BKV and JCV are being reported for the first time in literature.

Key words: BKV, DNA sequencing, genotypes, JCV, mutation, viral protein 1 gene

Introduction

Polyomavirus has emerged as an important pathogen in renal transplant patients.[1] Polyomavirus BK (BKV) has a worldwide seroprevalence of 60 to 80% in humans.[2] Primary infection occurs in childhood[3] and leads to viral latency in the urogenital tract and mononuclear cells. Following reactivation, which mostly occurs in immunocompromised patients, the virus is excreted in the urine.[4] BKV viruria in renal transplant recipients ranges between 10 to 60% and has been associated with ureteric stenosis[5]; BK viral nephropathy is characterised by tubular necrosis and interstitial nephritis.[6,7] BKV has also been linked to hemorrhagic cystitis in bone marrow transplant recipients.[6-10]

Characterisation of the genetic diversity of BK virus has biologic as well as clinical implications. This information is needed to seek potential relationships between viral genotype and clinical disease. The target genes for characterisation of BK virus are viral protein 1 (VP1) VP2, VP3 genes, large T and small T antigens, agnoprotein and non-coding control region. The most variable regions of the viral genome is the non-coding control region, which shows insertions, deletions, duplications, and complex rearrangements involving enhancer and/or promoter sequences.[11,12] Significant variation has also been recognised in the VP1 gene, which codes for VP1, a major structural protein that comprises approximately 80% of the total viral capsid protein. The present study focused on optimising a semi-nested polymerase chain reaction targeting the VP1 gene for rapid detection of BK virus (BKV) and JC virus (JCV) in renal transplant patients and subsequently determining its genotype.

Materials and Methods

Study population

A total of 130 specimens collected from renal transplant patients were included in the study for detecting the presence of BKV and JCV by PCR targeting the VP1 gene. Among the 130 clinical specimens, 26 were urine samples and the rest 104 were blood specimens. All 130 were non-repeat specimens collected from 125 patients including 5 patients from whom both urine and blood samples were obtained. The peripheral blood specimens were collected...
in a sterile EDTA vacutainer and the plasma was used for the quantitation of BK viral load. The urine specimens were collected in sterile universal containers and the deposit was subjected to DNA extraction. Extraction of DNA from clinical specimens were carried out with the QIAMP DNA extraction kit (QIAGEN, Hilden, Germany) following the manufacturers’ instructions.

**PCR for detection of BK virus and JC virus**

PCR for BKV was carried out as a 50 μl reaction with 200 μM of dNTPs, 5 μl of 10x PCR buffer, 1 unit of Taq polymerase and 50 picomoles of the primers (Bio Source Surgicals, India). The PCR amplification were carried out with the following primers:[13]

- **BKV-P1 (5’GAAATCTACTGTGGAGGCTG3’),**
- **BKV-P2 (5’GTACCATCTGGGTACTTTGTCCTG3’),**
- **BKV-P5 (5’ATGATTCATTACCTGGGACTGGGC3’),**
- **BKV-P1 (5’GAAATCTACTGAGGAGGCTG3’),**
- **BKV-P2 (5’GTACCATCTGGGTACTTTGTCCTG3’),**
- **JCV-P6 (5’CTTATCTAGGTACCGCTTGTGCTC3’).**

The VP1 gene of BKV was amplified by a semi nested PCR, with BKV-P1 and BKV-P5 primers yielding a 259 base pair (bp) DNA fragment for the first round followed by BKV-P1 and BKV-P2 primers, yielding a 214 bp DNA fragment for the second round. The VP1 gene of JCV was amplified by uniplex PCR, with BKV-P1 and JCV-P6 primers, generating a 321 bp fragment. The thermal profile consisted of initial denaturation at 96°C for 6 minutes, followed by 40 cycles at 96°C for 30 seconds, annealing at 64°C for 1 minute, with extension at 72°C for 1 minute followed by a final extension at 72°C for 7 minutes.

**Real Time PCR for quantitation of BK virus**

The Real time PCR assay for detection of BK virus was performed using BK Virus R-gene™ - Quantification complete kit (BioMérieux Corporate, SA). The peripheral blood specimens were collected in sterile EDTA vacutainers and the plasma was used for the quantitation of BK viral load. The urine specimens were collected in sterile universal containers and the deposit was subjected to DNA extraction. The BKV DNA detected was expressed in DNA copies/ml of plasma.

**PCR based DNA sequencing for the amplified products of BKV and JCV**

The amplified PCR products of BKV and JCV VP1 DNA fragments were eluted from the gel with the Qiagen elution kit (Hilden, Germany) according to the recommendations of the manufacturer. Cycle sequencing was performed with Big Dye cycle sequencing kit with BKV and JCV forward and reverse primers, thus allowing both strands to be sequenced.

Sequenceing of the amplified products was performed in a 10 μl reaction volume, containing 1.0 μl of Ready Reaction (RR) mix, 3.0 μl of sequencing buffer, 2.0 μl of each primer (1:100 diluted), 3.0 μl MilliQ water and 2.0 μl of the PCR eluted product.

Amplification was carried out in Perkin-Elmer thermocycler with initial denaturation at 96°C for 1 minute followed by 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. The cycle-sequenced products were then purified using 125 mM EDTA and 3M sodium acetate. The purified products were then denatured with 10 μl formamide and loaded onto ABI Prism 3130 AVANT (Applied Biosystems, USA) genetic analyser that works on the principle of Sangers’ dideoxy termination method.

The sequences were then analysed with the help of BioEdit sequence alignment software. BLAST analysis [www.ncbi.nlm.nih.gov/BLAST] was done to compare the sequenced data with the standard strains to determine the percentage of homology.

**Sequence analysis using bioinformatics tools**

Multalin analysis is a freely available online tool to align DNA sequences to detect homology or variations. The FASTA formatted (single line sequence data) nucleotide bases of the amplified BKV and JCV VP1 DNA fragments of the clinical specimens were first multaligned (http://multalin.toulouse.inra.fr/multalin/) along with forward and reverse primers with the GenBank sequence. The nucleotide sequences were then converted to amino acid sequences and again multaligned with the GenBank Sequence to check for mutations which may cause structural alterations in the protein.

**Results**

**PCR targeting VP1 gene for detection of polyoma virus**

Polyoma virus was detected in 23 (17.69%) out of 130 clinical specimens by PCR targeting the VP1 region. Among the 23 clinical specimens, 19 (82.60%) were positive for BK virus, 3 (17.39%) for JC virus and 1 for both BK and JC virus. The results of PCR targeting the VP1 gene of polyoma BK virus after first and second round of amplification and for JC virus are shown in Figure 1a-c respectively. Polyoma BK virus was detected in both blood and urine specimens obtained from two patients. The JC virus was not detected in any of the dual specimens.

**Real Time PCR for quantitation of BK virus**

The polyoma BK virus positive specimens were subjected to Real time PCR to determine the copy number of BK virus. Among the 23 clinical specimens, Real Time PCR for BK virus revealed the presence of BK virus in 21 clinical specimens with copy numbers ranging from $1.56 \times 10^2$ to $9.94 \times 10^6$ copies/ml of BK virus DNA. One urine and one blood specimen which were negative for
BK virus by Real Time PCR were positive for JC virus by qualitative PCR.

The mean viral load of BK virus was $7.48 \times 10^7$ copies/ml. The analysis of Real time PCR results of BK virus revealed a high viral load in urine specimens ($1.9 \times 10^8$ copies /ml) as compared to the peripheral blood specimen ($2.57 \times 10^4$ copies /ml). The distribution of BK viral load in peripheral blood and urine specimens is shown in Figure 2a and b.

**PCR based DNA sequencing for detection of genotypes of polyoma virus**

**Table 1: Distribution of polyoma virus genotypes determined by PCR based DNA sequencing in clinical specimens**

<table>
<thead>
<tr>
<th>Clinical specimens</th>
<th>PCR for polyoma virus positive</th>
<th>Results of PCR based DNA sequencing for detection of polyoma virus genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BK virus</td>
<td>JCV, BK virus and JC virus</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N=14</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>Urine</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>N=9</td>
<td>1*</td>
<td></td>
</tr>
<tr>
<td>Total N=23</td>
<td>19</td>
<td>3</td>
</tr>
<tr>
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</tbody>
</table>

Note: 24 genotypes of polyoma virus were detected from 23 clinical specimens. *Mixed genotype of BK virus genotype I2 and JC virus was detected in one urine specimen. PCR: Polymerase chain reaction, DNA, BK: BK virus, JC: JC virus

![Figure 1](https://www.ijmm.org)

**Figure 1:** (a) Results of PCR (Round-I) targeting VP1 gene of BK Virus, 100 bp: Molecular weight marker 100 bp ladder, NC: Negative Control, VRF 4911/12: Urine – Negative, VRF 4960/12: Urine – Negative, VRF 4960/12: Blood – Negative, VRF 6719/12: Urine – Positive, VRF 6178/12: Blood – Negative (b) Results of PCR (Round-II) targeting VP1 gene of BK Virus, NC 2 - Negative Control II round, NC 1 – Negative control I round, VRF 4960/12 - Urine – Positive, VRF 0143/13 - Blood – Negative, VRF 6196/12/Blood – Negative, VRF 0517/13 - Blood – Positive, VRF 0719/13 - Blood – Positive, VRF 0423/13 - Blood – Positive, VRF 5794/12 - Urine – Positive, VRF 6249/12 - Urine – Negative, VRF 1354/13 - Blood – Positive, VRF 1655/13 - Urine – Negative, VRF 1654/13 - Urine – Negative, VRF 1690/13 - Urine – Negative, 100 bp: 100 bp ladder molecular weight marker (c) Results of PCR targeting VP1 gene of JC Virus, NC - Negative Control, VRF 4354/12 - Blood – Negative, VRF 4945/12 - Blood – Negative, VRF 4945/12 - Blood – Negative, VRF 5794/12 - Urine – Negative, 5795/12 - Urine – Positve, 5794/12 - Urine – Negative, 100 bp: Molecular weight marker 100 bp ladder

![Figure 2](https://www.ijmm.org)

**Figure 2:** (a) Scatter plot representing the BK virus copy numbers in peripheral blood (b) Scatter plot representing the BK virus copy numbers in urine
virus genotyping was not performed with this specimen due to the low viral load.

Among the 4 dual specimens subjected to PCR based DNA sequencing, BK virus genotype IV was detected in 4 (2 blood and 2 urine) clinical specimens and BK virus genotype I detected in 4 clinical specimens (2 blood and 2 urine). BK virus genotype I and genotype IV were detected in the dual specimens of the same patients.

Bioinformatics analysis of polyoma virus

BK virus

The multalin analysis (http://multalin.toulouse.inra.fr/multalin/) of BLASTX of VP1 gene of BKV genotype I revealed two amino acid mutations at position 11 (glutamine to lysine) and at position 29 (lysine to glutamic acid) of BKV strain detected in peripheral blood (VRF 4354/12) and deletion of amino acids from position 30 to 47. All other BKV strains belonging to genotype I were similar. The multalin analysis of BLASTX of VP1 gene of BKV genotype IV revealed two amino acid mutations at position 11 (histidine to arginine) and at position 15 (lysine to glutamine) of BKV strain detected in urine (VRF 5795/12). All other BKV strains belonging to genotype IV were similar. The multalin analysis of representative amino acid codes of BKV genotype I and IV are provided in Figure 3a and b respectively.

JC virus

The multalin analysis of BlastX results of JCV of VPI gene revealed two mutations at position 2 (isoleucine to leucine) and 30 (threonine to alanine) when compared to the standard AB262401.1. The four JCV strains exhibited similar mutations at position 2 while only one JCV strain (JCV 5795/12) exhibited the mutation at position 30. The other 3 JCV strains were similar. The results of multalin analysis performed on the VP 1 gene using BlastX are provided in Figure 3c.

Multalin analysis showed change in the amino acid code L to I at position 2 of JCV strains 714, 812, 4911, 5975 and T to A at position 3 of JCV strain 5975.

Discussion

Monitoring of BK viral load in urine and blood has been used as a surrogate marker of BKV nephropathy (BKVN). To facilitate clinical management of renal transplant recipients, it becomes essential to identify relevant threshold levels of BK viruria and BK viremia as well as the genotype of the virus. The present study was undertaken to optimise nucleic acid amplification assays for rapid detection and quantitation of polyoma virus and subsequent determination of its genotype. Polyoma virus, BKV was detected in 10.76% peripheral blood specimens and 6.92% of urine specimens. Among the 23 clinical specimens positive for polyoma virus, BK virus was detected in 68.42% of plasma specimens and 31.57% of urine specimens. In India, a study by Thakur et al.,[14] revealed BKV in 44.7% plasma specimens after amplification by PCR. In another study, Mitterhofer et al.,[15] from Italy reported that 33.3% (12/36) of plasma samples and 58.3% (21/36) of corresponding urine samples had detectable BKV DNA. Our findings correlate with the findings of the other research group regarding the increased detection of BKV as compared with JCV. The incidence of JC virus was 17.39% (peripheral blood and plasma) in the present study. Although Kannanganai et al.,[16] have reported the presence of JCV in 62% cerebrospinal fluid specimens in HIV infected individuals with neurotropic viral disease, there are no studies in Indian literature associated with detection of JCV in renal transplant patients.

Qualitative PCRs are sensitive enough for detecting active BKV infections but lack specificity for nephropathy because the detected virus could originate anywhere along the urinary tract. Quantitation of BKV DNA in the plasma or urine is a superior indicator of nephropathy. In general, renal transplant recipients with BK virus nephropathy have a higher BK viral load than transplant recipients without BK virus nephropathy. However, there are no clear threshold levels for urinary viral loads that could predict viremia, and no universal viruria and viremia cutoff values exist for BK virus nephropathy.[17] Hirsch et al.,[18] have reported the threshold values of plasma BK viral load as greater than 1,000 copies or more per millilitre; and greater than 100,000 copies or more per millilitre in urine. In the present study, BKV copy number ranged from 662 to 1.5×10^5 copies/ml in peripheral blood specimens and 1.1×10^5 to 9.9×10^10 copies/ml in urine. All the 6 BKV positive urine specimens revealed a high copy number greater than the threshold value of BKV in urine (Hirsch et al. criteria >1×10^5 copies/ml significant viruria). In the present study, 4 (28.5%) peripheral blood specimens and 7 (77.77%) urine specimens revealed significant viraemia and viruria. In India, a study by Thakur et al.,[19] revealed
BKV positivity, in 15.7% and 25% urine and plasma specimens of renal transplant recipients with a peak viremia and viruria occurring at 1-3 months post transplantation. Our findings of incidence of BKV significant viraemia correlates well with the findings of Thakur et al.[19]

The regional distribution of the BKV subtypes has been established in several studies[3,20-26] Genotype I is most prevalent throughout the world, followed by genotype IV, with genotypes II and III occurring less frequently. BKV prevalent in East Asia except for Japan,[23] although there is evidence that the prevalence of this subtype was underestimated in other regions.[27] In the present study, PCR based DNA sequencing targeting the VP1 gene of BKV revealed genotype I as the predominant genotype (64.2% in peripheral blood and 33.33% in urine) in south India.

BKV genotype IV was detected in 28.5% peripheral blood specimens and 44.4% urine specimens in this study whereas BKV genotypes II and III were not detected. In published literature, the incidence of BKV genotype I varied from 64 to 90% and BKV genotype IV varied from 5 to 36%.[22,23,26,28-31] There are no reports in literature on the BKV genotypes prevalent in India. This is the first report on the prevalence of BKV genotypes in renal transplant patients in India.

The major capsid protein of BKV VP1 is critical for recognition of the host cell receptors and for virion assembly. The mutations of VP1 gene reported in the present study at position 29 and from 30 to 47 of BKV1 and mutations at position 11 and 15 are novel mutations identified in the VP1 gene which has not been reported earlier in literature. The altered viral protein hinders the viability of the virus since the mutations in VP1 gene can alter the binding of VP1 with sialic acid as reported by Dugan et al.[32] Two novel mutations at position 2 and 30 were detected in VP1 gene of JC virus. The VP1 gene of BK virus is essential for the attachment of the virus to the host cells. There are reports which state that the mutations in VP1 gene might lead to the alteration in the structure of VP1 hindering the binding properties of the virus. Further structural bioinformatics analysis can determine whether these mutations alter the conformation of VP1 of BKV.

PCR based DNA sequencing is a rapid and reliable method to genotype BKV and JCV. To the best of our knowledge, the mutations detected in the VP1 gene of BKV and JCV are novel mutations reported for the first time in literature.

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References


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