Iranian Red Crescent Medical Journal

ORIGINAL ARTICLE

Detection and Prevalence of Polyoma Virus BK among Iranian Kidney Transplant Patients by a Novel Nested-PCR

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Abstract

Background: BK virus is an increasingly recognized pathogen in transplant recipient patients associated with nephropathy and emerged as a cause of allograft failure linked to immunosuppressive regimens in renal transplant recipients. This study develops a sensitive PCR method to detect the viremia and viruria as well as the incidence of BK virus infection in renal transplant recipients.

Methods: A nested PCR method was developed and a total of 45 paired serum and urine samples from renal transplant recipient patients were collected and tested with the developed assay.

Results: The threshold of the developed detection assay was 10 copies/µl of BKV DNA in samples. Our results also indicated that about 40% of the urine and 26.7% of serum samples were positive for BKV in renal transplant patients in this study.

Conclusion: This Nested-PCR method was found a specific, sensitive and simple procedure for detection of viruria and viremia of BK virus in renal transplant recipients.

Keywords: BK virus; Renal transplant recipients; Prevalence; Iran

Introduction

BK virus (BKV) was described in 1971.[1] BKV is a non-enveloped icosahedral deoxyribonucleic acid (DNA) virus and represents a discrete species within the genus *Polyoma virus* of the family of *Polyomaviridae*.[2] Different studies have estimated that about 80% of the adult population worldwide are sero-positive for BKV with the exception of some segregated populations in South American and Southeast Asia.[3] Rates of BKV sero-positivity are significantly related to age, and sero-prevalence was shown to reach 91% at 5 to 9 years of age.[3,4] The BK virus is acquired during childhood, through the respiratory or gastrointestinal route.[5] The primary infections are trivial and resolve with the development of specific humoral and cellular immunities. After the primary infection, BKV has the tendency to remain latent in the reno-urinary epithelium.[4,6]

In the past decade, this virus has been recognized as an increasingly important cause of renal allograft dysfunction particularly in renal transplant recipients.[7-9] The relatively recent recognition of this condition probably reflects an increase in reactivation of latent BK virus infection as a consequence of the use of newer and more potent immunosuppressive agents such as tacrolimus or mycophenolate mofetil (MMF).[10,11] Most cases of BKV nephropathy "BKVN" have been diagnosed about 40 weeks post-transplantation.[8] Over the last few years there has been an increased recognition of the morbidity related to polyoma viruses, particularly BKV, in kidney transplant recipients.[10] Viruria directly correlates with

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Received: December 12, 2009 Accepted: April 12, 2010

the immune functions and is commonly present in renal transplant recipients with or without BKVN and approximately 30-40% of adult renal transplant recipients shed BK virus in urine.[8]

Studies by different investigators during the last few years have shown that BKV reactivation leads to allograft failure in 15 to 100% of the affected kidney transplant patients.[8,12,13] Shedding is typically detected by showing cytological abnormal cell in urine 'decoy cell', as well as culture, or through nucleic acid amplification tests on urine samples.[13-15] The aim of the current study was to develop a nested-PCR method to detect BKV viremia and viruria as well as determination the prevalence rate of BK infection among Iranian renal transplant recipients.

Materials and Methods

The study was a prospective study. Transplant laboratory centre teams were asked to submit paired urine and plasma samples from about 45 consecutive recipients of kidney who presented to the laboratory between 20 April and 28 July 2007. No preliminary screening was used to allow or prevent patient enrollment into the study, and all received samples during the study period were processed. Data regarding type and date of transplantation, received immunosuppressive agents, and renal function were collected. A group of 45 renal transplant patients from Tehran, Iran were enrolled.

DNA extraction was performed using two different methods for urine and serum samples. Specimens containing EDTA blood were centrifuged for 5 min at 2000 rpm. Subsequently 200 µl of the obtained plasma were used for DNA extraction using QIAamp DNA Blood Mini Kit (Qiagen, USA) as recommended by the manufacturer. DNA was extracted from urine samples as follows: 100 µl of urine incubated in 95°C for 20 min and then centrifuged at 14000 rpm for 10 min at 4°C. 100 µl of supernatant of urine were mixed with 100 µl of solution 1 (PEG 30%, 2 M NaCl), incubated at 4°C for 30 min and then centrifuged at 14000 rpm at 4°C for 15 min. The supernatant were discarded and 20 µl of solution 2 (10mM Tris-HCl, pH 7.6, 5%v/v Nonidet P40) were added to pellet and incubated at room temperature for 10 min before use.[16]

Based on sequences of BKV and JCV in Genbank, an in-house nested PCR method was developed. The first PCR run was generic for detection of both BK and JC viruses, and the second PCR run was specific for detection of BKV. The external primer pair JCVF1 sense: 5'-CTGGGTTAAAGTCATGCT-3' (2185-2202 nt) and JCVR1 antisense: 5'-GGTAGAAGACCCTAAAGACT-3' (2589-2570 nt) was used to amplify a 385 bp fragment. For detection of BKV specific PCR product, a pair of internal primers BKVF2 sense: 5'-AAGTATTCCTTATTCACACC-3' (2252-2271 nt), BKVR2 antisense: 5'-CCCTCTGATCTACACCAG-3' (2566-2549)] that amplify a 278 bp segment was used for the second round amplification.

Based on homologies and data from multiple alignments, it has been shown that the first pair primers could amplify both BKV and JCV genome while the second pair of primers could only amplify BKV genome sequence.

For the first PCR run, 5 µl of purified DNA was used, and 4 µl of the first round product after tenfold dilution was used as a template for the second nested reaction. Final reaction volume was always 50 µl. Each assay included a negative control as well as confirmed BKV positive samples as a positive control. Briefly, 5 µl of extracted DNA was added to 40 µl of PCR solution containing 10 mM tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 200 µM of each dNTP and 4 pmol of each external primer. After an initial denaturation step of 2 min at 94°C, 5 µl (1U) of recombinant Taq DNA polymerase (cinnagen Co., I.R.I) were added and the first-round PCR amplification was carried out under the following conditions: 94°C for 30 sec, 48°C for 30 sec, 72°C for 30 sec for twenty cycles, then one cycle at 72°C for 5 min.

Four µl of 1/10 diluted first-round product was then transferred into a second 41 µl PCR solution mixture. The second-round reaction mix contained the same constituents as the first, but 8 pmol of each internal primer. After the initial denaturation step of 2 min at 94°C, 5 µl (1U) of recombinant Taq DNA polymerase (cinnagen Co., I.R.I) were added and the second-round PCR amplification was carried out under the following conditions: 94°C for 30 sec, 45°C for 30 sec, 72°C for 30 sec for thirty-five cycles, then one cycle at 72°C for 5 min.

The amplified PCR products were detected by direct gel analysis. A 20 µl sample of the second amplification product was run on a 2% agarose gel electrophoresis. Bands were visualized under UV light by ethidium bromide staining method.

To evaluate the threshold of detection of the developed nested PCR assay, briefly, the amplified

DNA fragment was extracted from the gel by gel extraction kit (Bioneer Co., Korea) and cloned in pTZ57R/T plasmid (Fermentas Co., Canada) and transformed into competent *DH5α* bacteria according to manufacture instruction. After cultivation of the bacteria, blue/white screening of the colonies was done, and colony PCR method has been developed. Four colonies of the bacteria have been selected for evaluation. The colonies have been cultured in separate flasks and after miniprep extraction, the concentration of the DNA has been calculated by optical density in 260 and 280 nm and the suitable formula. The serial dilution of the prepared DNA showed that the threshold of the developed detection assay was 10 copies/µl of BKV DNA in samples.

To access reproducibility, intra-assay and inter-assay analysis were performed. Five clinical samples were amplified in each assay. Positive results for clinical specimens were confirmed by testing a second fresh aliquot. To avoid false-positive PCR results due to carry-over contamination, aliquots of clinical specimens, preparation of reagents, DNA extraction, first round amplification, and nested PCR were performed in safety cabinets located in separate laboratories.

Results

Overall, 45 received kidney transplant patients who were admitted to the laboratory were included in this study. Patient ages were in the range of 12-59 years old; about 44% of the patients were female, and mycophenolate acid (MMF) was the most commonly used immunosuppressive agent. The samples were obtained a median of 24 months (1-108 months) after transplantation. Renal dysfunction (defined as a creatinine level of >1.5 mg/dl) was present in 51% of the patients, and the serum creatinine level was 2-2.5 mg/dl. Among our 45 patients, we found BKV viruria in 18 (40%) and viremia in 12 (26.7%) patients. In other words, 40% of patients had detectable BKV-DNA in urine samples while only 26.7% had detectable BKV-DNA both in urine and serum samples.

Both viruria and viremia were significantly more common among recipients of kidney in Iran. The main demographic and clinical characteristics of patients who were positive for BK viremia and viruria are shown in Table 1.

Discussion

The aim of the study was to characterize the shedding of BKV in a group of Iranian renal transplant recipient patients. These viruses have been associated as the cause or as a cofactor in the development of certain diseases in immunosupressed individuals. However, the shedding of BKV and JCV in urine has also been described in healthy individuals and it varies from 20% up to nearly 50% .[17-19] Therefore, the study of their life cycle, as well as their epidemiology has earned special attention. Due to the role of BK virus in transplant recipients we used an in house Nested-PCR for detection and differentiation of BK virus for the first time in Iran in order to help prevention of allograft rejection after transplantation by BKVN. This Nested-PCR method was a specific, sensitive and simple procedure for detection of viruria and viremia of BK virus in renal transplant recipients. As confirmed previously by other investigators, serum samples were been positive only while the related urine samples had known as positive previously. Detection of viremia and viruria of BK virus depends on sensitivity of test. In this study regarding to high sensitivity of the test, the rate of recurrence was high in comparison to other reports. It seems that estimation

Table 1: The main demographic and clinical characteristics of patients who known as positive for BK viremia and viruria

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13
Age, mean years	48	34	56	22	12	34	54	30	29	52	39	41	59
Sex (F, M)	F	M	F	F	M	M	M	F	M	M	F	F	M
Immunosuppressive agent(s)													
• Mycophenolate	+	+	+	+	+	+	+	+	+	+	+	+	+
• Cyclosporine A	-	-	+	-	+	+	-	-	+	-	+	-	+
• Corticosteroids	+	+	+	+	+	+	-	+	+	+	+	+	-
Time of sample collection after Transplantation (Months)	23	11	14	9	108	28	13	38	12	80	1	42	23

Abbreviations: F, female; M, male; p, Patient

of recurrence of BK virus needs more investigation and a larger sample population. Altogether, monitoring of patients that their urine samples know as positive is requisite for prevention of graft failure post-transplantation. None of these patients developed permanent renal impairment, although this was present when plasma and urine samples were obtained. The role of mycophenolate as independent predictor of renal impairment in our study population is not clear. Limitations of our study include the absence of follow-up sampling of patients with BKV viruria (because this was a prevalence study), the unavailability of renal biopsy specimens, and the fact that quantitative PCR was not performed. In addition, the different schedule of operative revisions in each transplant program may have resulted in a selection bias in the submission of samples to the laboratory that could have caused us to overestimate the real prevalence and to prevent detection of earlier or later reactivation of infection.

Several centers (including ViraCor) now consider a titer above 10,000 copies per ml in plasma to be a significant marker of BKVAN.[11,20,21] The levels of circulating plasma BKV DNA correlating with BKVN remain controversial; Hirsch *et al.* reported copies greater than 7000 to be correlated with acute BKVN. However, BKVN can occur even with copies as low as 1000 copies (personal observation) and better correlation has been noted with persistent viremia.[22] BKVN is an important problem after renal transplantation that has limited improvements in graft survival. Increasing awareness of this condition, utility of BKV DNA estimation in blood and urine, careful renal histological evaluation, and avoiding further aggressive immunosuppression after the diagnosis of BKVN are changing the outcome of this disease. Approximately 40-60% of renal grafts with BKVN develop progressive graft loss.[23] Our results showed that the rate of reactivation of BK virus among renal transplant recipients in Iran approximately is high and seems to have more surveillance in screening programs in these patients for viremia and viruria of BK virus regarding its role in renal dysfunction.

Detection of viremia by PCR has high sensitivity, specificity and negative predictive value for nephropathy (100%, 88% and 100% respectively), although the positive predictive value is only 50%.[15] Urine BKV PCR is a very sensitive (100%) and specific (95%) marker for BKV infection and has a high negative predictive value (100%) and can be used as screening test.[24,25] More recent studies have shown that BKV associated nephropathy is seen in up to 8% of renal allograft patients.[14] Our Nested-PCR method was found a specific, sensitive and simple procedure for detection of viruria and viremia of BK virus in renal transplant recipients.

Acknowledgement

The authors acknowledge the Tarbiat Modares University for support of the study.

Conflict of interest: None declared.

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