DETECTION OF BK POLYOMAVIRUS USING REAL TIME PCR AND URINE CYTOLOGY IN 99 RENAL TRANSPLANT RECIPIENTS

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ABSTRACT

BK polyomavirus is one of the common post-transplant viral infections, affecting \(\sim 15\)% of renal transplantation recipients (RTR), leading to graft loss in more than half of cases. Plasma and urine samples were collected from 99 RTR patient, with 15 Living Donors (LD) and 15 patients with Chronic Kidney Disease (CKD) were taken as controls, BKV load in plasma was detected by real time PCR (RT-PCR), and urine cytology smears were Pap stained for detection of decoy cells (DCs). 12.12\% (12/99) cases were positive for BKV by RT-PCR, and 27.27\% (27/99) RTR patient were decoy positive, among which all of the 12 (RT-PCR) BKV positive patients were decoy positive, and 5 out of these 12 BK viremic patients had biopsy proven BKV nephropathy (BKVN). Our study suggests that BKV should be considered as a cause of nephropathy and allograft loss in RTRs in Iraq. Further research is required for better understanding of this entity.

KEYWORDS: Renal Transplant Recipients, BK Polyomavirus and Real Time PCR

INTRODUCTION

Opportunistic polyomaviruses infections mainly BK virus (BKV) and JC virus has become increasingly common problem in renal transplantation recipients (RTR). Polyomaviruses are circular, double-stranded DNA viruses (1). The most important and commonest among these viruses is BKV infection, which was reported in \(\sim 15\)% of renal transplant recipients (RTRs) in the first post-transplant year in the absence an effective prophylaxis strategy (2,3).

BKV presents with an asymptomatic gradual rise in serum creatinine with a tubulo-interstitial nephritis mimicking rejection, making a treatment dilemma. The decrease in immunosuppression that is needed to treat BKV infection is opposite to the increases in immunosuppressive drugs that are needed to treat rejection (4). 30 years ago, Gardner et al.
(5) warned: “The detection of polyomavirus infection is important, as increased
immunosuppression needs to be avoided, to prevent possible complications”.

After primary infection, BKV establishes its latency in the urinary epithelium and
renal tubular epithelial cells. When starting immunosuppression, the virus is reactivated and
begins to replicate, causing BK viremia and eventually invade the renal allograft, leading to
BK virus nephropathy (BKVN), the rates of BKVN varies, ranging from 1 to 10% (2,3,6,7).

BKV detection by quantitative real-time PCR (qRT-PCR) in plasma is very sensitive
and specific for predicting the development of BKVN. The focal nature of BKVN, and the
makeup of cellular infiltration in a renal allograft with BKVN that is very similar to those
with acute rejection, these major limitations for diagnosis by biopsy (8,9), make plasma BKV
qRT-PCR the preferred screening method at the majority of transplant centers (6,10,11)

In addition, urine cytology screening for viral inclusion-bearing, so called decoy cells
(DC) allows for the early identification of BKV infection, and it has a relatively high
sensitivity and a negative predictive value above 95%, besides being a cost-effective non-
invasive assay (12-14). Detection of decoy cells (DCs) in the urine is one of the earliest
assays, in this assay urine was Papanicolaou-stained and examined under light microscope to
look for virus infected cells "decoy cells", which are epithelial cells with enlarged nuclei, and
large basophilic ground-glass intra-nuclear viral inclusions (12-16).

In this study we aimed to investigate the prevalence of BKV in Iraqi RTRs using
quantitative real time PCR (qRT-PCR) and decoy cell screening.

Subjects and Methods

Subjects: This cross-sectional study was conducted from2013 to 2014. A total of 99
RTR patients who attend the (Center of Kidney Diseases and Transplantation) in the Medical
City of Baghdad, were enrolled in the study. A consent letter was signed by each patient, and
the study was approved by the ethical committee of Al-Nahrain University. Urine and plasma
samples were collected from the patients, 33 of them had normal renal function, and the
remaining 66 had impaired renal function, 78 were males. Two control groups were included
in the study, 15 Living Donors (LD) and 15 non-transplanted patients with Chronic Kidney
Disease (CKD).

Urine cytology: Urine (10-ml aliquots) was centrifuged in Falcon tubes at 1500 rpm
for 5 min for decoy cell screening. The supernatant was discarded and the sediment was re-
suspended in the remaining urine. For each patient; two slides were prepared; one was
immediately stained with the Papanicolaou method and examined under light microscope at
40 and 100 X; the other was stored unstained at –20 °C for confirmation of diagnosis if
required.

Activation and replication of polyomaviruses was detected by identification of decoy
cells (DCs), which are viral inclusion-bearing epithelial cells characterized by a ground-glass
appearance with an enlarged nucleus, occupied by a basophilic inclusion surrounded by
chromatin (14,15). Some of the DCs appear resembling the tail of a comet (17). For decoy
cells quantification; a cut-off level of ≥ 10 decoy cells / ThinPrep slide, is defined as decoy
positive (11).

In addition to the quantification of common ground-glass decoy cells, the uncommon
(clumped) variants were also looked for; as their presence reflects the pathological stages of
BKVN, if the uncommon (clumped) variants are more than 25% of the total decoy cell count;
then BKVN can be predicted with more than 75% probability (18).

DNA Extraction: Viral DNA was extracted from 200μl of plasma using a Bosphore®
Viral DNA Extraction Spin Kit according to the manufacturer’s protocol which is based on
silica membrane column separation method (Anatolia Geneworks, Turkey), and then was
eluted from the column with 40μl elution buffer.

Real time PCR: Quantification of BKV plasma viral load was done Using Bosphore®
BKV Quantification Kit, which detects and quantitates the four main genotypes of BK Virus
DNA in human plasma. A region within the BKV large T-antigene (LT) encoding gene was
amplified. Fluorescence detection is accomplished using the FAM filter, and to check PCR
inhibition an internal control is incorporated into the system. The internal control was
detected with the Cy5 filter. Quantitation of BK viral load was performed using four
quantitated BKV DNA controls, ranging from 1x10^4 copies/ml to 1x10^7 copies/ml.

Statistical analysis was performed with the software SPSS version 21.0, and
Microsoft Excel 2013. Categorical data formulated as count and percentage. Fisher exact test
was used to describe the association of these data, in addition to relative risk study (RR).
Numerical data were described as mean, standard deviation of mean. Independent sample t-
test used for comparison between two groups while analysis of variance (ANOVA) was used
for comparison among more than two groups. p ≤0.05 was considered statistically significant.
Results

Out of these 99 RTR, 78 (78.79%) were males. The mean age was 37.01±1.31 ranging between 18 and 67 years, and the mean ages of the two control groups were (38.5±2.8) and (38.4±2.7) years for LD and CKD, respectively. The mean post-transplantation period in the RTR was 17.53±0.97 months ranging from 2-30 months, and their mean serum creatinine value was 2.33±1.7. Among these 99 RTRs, 19.19% had renal allograft rejection, five of them (5.05%) were receiving antithymocyte globulin (ATG) as anti-rejection therapy, 5.05% had biopsy proven BK virus nephropathy (BKVN), 4.04% had ureteric stenosis, and 43.44% had donor+/recipient+ CMV serostate.

Papanicolaou-stained urine cytology smears revealed 27 out of 99 RTR (27.27%) had positive decoy cells (DC) shedding, as compared with both control groups; LD and CKD who were all DC negative, p=0.001, in addition, uncommon DCs variants were present in 8 out of 99 RTR (8.08%). The most frequent variant of DCs was the amorphous, basophilic, ground-glass–like nuclear appearance. While in the other variants (uncommon type), the nucleus appeared eosinophilic and granular, and could be surrounded by a halo, or with a finely granular without a halo, figure (1).

In addition, the results of this study revealed that 19 out of these 27 cases (70.4%) were males, their mean age was 34±7 years with no significant correlation with decoy cell positivity, and their mean post-transplant period was 18.2±8 months which also not significantly correlated with decoy cell positivity. On the other hand, 21/27 (77.8%) of these DC positive patients had impaired renal function with a mean serum creatinine value 2.3±0.9 mg/dl, which is significantly correlated with DC positivity (p=0.01), and all of the 5 patients who had biopsy-proven BKVN had positive urine cytology for DCs, i.e. 18.5% of them.

Two main standard immunosuppressive regimes are mainly followed in our transplantation center in Baghdad; the old regimen which includes cyclosporine A (CSA), mycophenolate (MMF), and prednisolone, the second regimen includes tacrolimus (TAC) instead of CSA, in addition to MMF and prednisolone.

On comparing with the type of immunosuppression used, 55.6% of DC positive patients were on tacrolimus regimen, and 44.4% were on cyclosporine A regimen, which is not significantly correlated with DC positivity, while 4/5 (80%) of patients who were on ATG (anti-thymocyte globulin) were decoy positive, among the 26.3% (5/19) patients who had rejection. Finally, 3/4 (75%) of ureteric stenosis patients were DC positive.
Figure (1): Urine cytology: The activation and replication of polyomaviruses can be monitored by searching for viral inclusion-bearing epithelial cells, i.e., decoy cells (DC), in routine urine cytology specimens. (A,B) typical DC phenotype resembling the tail of a comet. And (C) uncommon (atypical) eosinophilic DC, (D) uncommon finely granular DC. Papanicolaou stain, (A&B) X400, (C&D) X1000.

BK viremia was detected in (12.12%) 12 out of 99 RTR with a viral load (VL) ranging from $1 \times 10^2$ to $1 \times 10^9$ copies/ml, and none of the control groups (LD and CKD) was BKV positive. Among the 12 BK positive cases, 7 patients were above 40 years old and 6 of these 7 patients had BK VL more than $10^3$ copies/ml, 10 out of 12 were males and 7 out of these 10 males had BK VL more than $10^3$ copies/ml, 9 out of 12 had renal transplantation within less than 12 months and 6 of these 9 patients had BK VL more than $10^3$ copies/ml,

According to our standard curve, 58.3% (7/12) had BK viral load more than $1 \times 10^3$ copies/ml, with a mean of $1.99 \times 10^8$ copies/ml. In addition, 41.7 % (5/12) had biopsy proven BKVN, i.e. 5.05% (5/99 RTR) had BKVN, 4 of these 5 cases (80%) had BK viral load of more than $1 \times 10^4$ copies/ml, and three of these 5 cases (60%) have lost their graft.

Regarding donor/recipient CMV serostate, there was no significant difference in donor/recipient CMV serostate among the 12 BK viremic patients. However, the study revealed a significant positive correlation between serum creatinine value and BK VL; ($r=+0.576$, $p=0.05$), figure (2). But, only 3 out of the 19 RTR patients who had rejection, had BK viremia; which shows no significant correlation ($p=0.581$), and only one of the 5 patients who were receiving ATG (anti-thymocyteglobuline) had BK viremia. There was a significant difference in BKV viremia rates between samples from the biopsy proven BKVN and remaining BK positive cases ($p=0.03$). Furthermore, the study showed a highly significant association between BKV positivity and ureteric stenosis cases ($p=0.007$).
Despite the none significant correlation between the type of immunosuppressive regimen used and BK viremia (p=0.42), this study showed that 50% (4 out of 8) BKV positive patients who were receiving TAC regimen had BK VL more than $1 \times 10^4$ copies/ml, and all of the 5 patients who had biopsy proven BKV nephropathy (BKVN) were on TAC regimen.

Finally, the results of this study demonstrated a highly significant association between BKV positivity and decoy cells positivity in Pap-stained urine cytology smears (p=0.001). Moreover, all cases who had uncommon decoy cells in urine cytology smears were positive for BKV (p=0.001), table (1) A and B respectively. However, there was no significant correlation between the number of decoy cells per urine cytology smear and BK VL ($r=0.111$, p=730).

**Table (1): The prevalence of: A; Decoy cells shedding, and B; Uncommon decoy cells shedding in cases positive for BK viremia among the 99 RTR.**

<table>
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<th>A</th>
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Discussion

By using more potent immunosuppressive regimens to decrease acute rejection rates in RTRs, viral infections had emerged as an important cause of allograft loss. BKV is one of the common post-transplant viral infections (2,6), in this study, the prevalence of BK viremia was 12.12%, which lies within the range of overall incidence of BK viremia that range from 11% to 29% (19,20). Following the detection of viruria, some patients develop viremia. BK viremia is believed to result from a more extensive infection leading to severe tubular injury with rupture of tubular basement membranes and entry of the virus into the blood stream via peritubular capillaries. Ultimately, sustained viremia is associated with BKVN in 1% to 10% of RTRs (11,21,22), in which also the prevalence of BKVN (5.05%) in our study lies within this range.

Studies found that BK viremia and BKVN are most common in the first year after transplantation, when immunosuppression is most intense (23,24), which are in line with our results which showed that (75%) 9 out of 12 had renal transplantation within less than 12 months, and 66.7%, 6 of these 9 patients had BK VL more than $10^3$ copies/ml. However, some workers found that early, during the first month following transplantation, BKVN can occur only exceptionally (7). This explains our reason behind collecting RTR patients with a post-transplant period ranging between 2-30 months and not less than 2 months.

In addition, these studies showed that BKV reactivation is usually associated with ureteric stenosis and bacterial urinary tract infections (23,24), which also support the results of this study in which 2 out of the 12 viremic patients had bacterial urinary tract infection, and another 2 out of the 12 viremic patients had ureteric stenosis (among the only 4 out of 99 RTR who had ureteric stenosis) (p=0.007).

In the majority of patients, BK viremia and progression of BKVN are asymptomatic except only for steadily increasing serum creatinine concentrations (25-27). A presentation that is very obvious in our viremic patients is that there was a significant positive correlation between serum creatinine value and BK VL; ($r=+0.576$, $p=0.05$), figure (2). And this increasing serum creatinine often makes most of the physicians to misdiagnose BKVN as an acute rejection or drug toxicity (28).

Results of our study showed a significant difference between the median BK VL in plasma from the biopsy proven BKVN and the remaining BK viremic patients ($1.82 \times 10^5$ versus $1.82 \times 10^2$) copies/ml ($p=0.03$), in which 4 out of 5 BKVN cases had BK VL levels more than $1 \times 10^4$ copies/ml. The American Society of Transplantation (AST) defines a BK
VL of $\geq 4 \log_{10}/mL (1 \times 10^4$ copies) as presumptive BKVN, and recommends reduction in immunosuppression (25), which is also supported by other studies (11), which is in agreement with our results.

However, a recent study showed that these current AST guidelines have underestimated the diagnosis of BKVN, and a cut off BK VL of $\geq (1 \times 10^4$ copies) might not necessarily indicate a presumptive BKVN, and even lower values of plasma viral load could sometimes associated with BKVN (29). This finding explains why one of the 5 BKVN cases in our study had BK VL less than $(1 \times 10^4$ copies).

The suggested risk factors for BKVN and BK viremia, included mainly the recipient factors (older age, male gender, low or absent BKV-specific T-cell activity) (25,30), in the present study, although it was not significant by relative risk study, 7 patients out of the 12 BK positive cases were above 40 years old and (85.7%) i.e. 6 of these 7 patients had BK VL more than $10^3$ copies/ml. Also (83.3%) 10 out of 12 were males and (70%) 7 out of these 10 males had BK VL more than $10^3$ copies/ml.

Although RR study showed none significant correlation between the type of immunosuppressive regimen (whether TAC or CSA) and BK viremia, 66.7% (8/12) patients were on TAC regimen, 50% (4/8) had BK VL more than $1 \times 10^4$ copies/ml, and all of the 5 patients who had biopsy proven BKV nephropathy (BKVN) were on TAC regimen, a finding that is in agreement with the study of Hirsch et al (3), which showed that high-titer BK VL ($>4 \log_{10}$) $10^4$ copies/ml, and the overall median BK VLs were higher in the TAC group.

In 2009, Egli et al. (31) demonstrated that TAC therapy inhibits BKV-specific T cell immune response, and the reduction of immunosuppressive therapies has led to an increase in the BKV-specific cellular immune response. However, both TAC and CSA were shown to cause dose-dependent inhibition of IFN-$\gamma$ expression by BKV-specific T cell response (4,32).

BKV shedding into the urine occurs in 10-30% of renal transplant recipients, and prospective monitoring of RTRs may identify patients with active infection before deterioration of the renal function. BKV cytopathic effect is a well-recognized entity in urine cytology specimens. Virus-infected cells termed (decoy cells) can be found in urine samples, and may mimic the nuclear changes that occur in urothelial cancer (33).

Results of this study showed that the rate of occurrence of DCs as a marker of viral replication was 27.27%, and all of the 12 BK viremic patients were decoy positive that is similar to the majority of studies on the detection of DCs in RTRs (34-37), which make it a cost-effective and powerful screening method. On the other hand, this study showed no
significant correlation between BKV load and the number of DCs which is supported by the study of Nickeleit et al., which showed that higher numbers of DCs do not indicate a higher risk level (37).

Based on the morphologic features alone, one cannot always distinguish between BKV excretion and other viral infections. DCs might result from infection with BKV, JCV, and less commonly, adenoviruses (34,38). However JCV and adenoviruses rarely cause nephropathy in RTRs (39,40).

BKV PCR does not face the obstacles that limit urine cytology and can ultimately prove superior in screening for BKVN. A study compared BKV PCR with urine cytology, by evaluating 114 patients for evidence of BK viremia and DCs, along with concurrent renal biopsy in order to correlate with an actual BKVN. Results indicated that, identification of DCs as a marker of BKV viruria had a sensitivity of 25%, and a specificity of 84%. While the sensitivity and specificity of BKV PCR on plasma for BK viremia was 100% and 91% respectively. Also, BKV PCR on plasma and urine had superior positive and negative predictive values for biopsy-proven, concurrent BKVN (10).

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Conflict of Interest: Authors declare no conflict of interest.
List of abbreviations:
BKV= BK virus
BKVN= BK virusnephropathy
RTR= renal transplant recipient
LD= living donor
CKD= chronic kidney disease
RT-PCR= real time PCR
DC= decoy cell

References


