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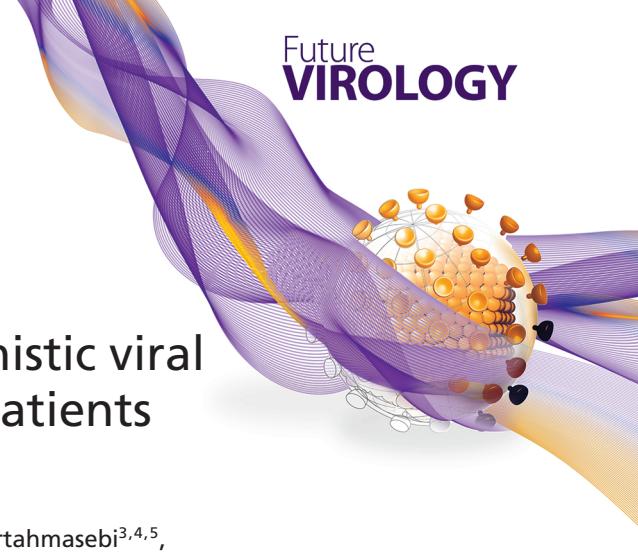

Simultaneous detection of opportunistic viral infections among renal transplant patients from Sina Hospital, Tehran

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Aim: A proportion of renal transplant (RT) recipients are at high risk for acquisition of opportunistic infections. **Methods:** A total of 101 plasma and urine specimens were collected from RT patients with raised plasma creatinine. These were tested for three common viral pathogens under suspicion of post-transplant nephropathy. **Results:** A total of 19 (18.8%), 15 (14.8%) and 10 (9.9%) tested positive for BK virus, cytomegalovirus and Epstein–Barr virus in their plasma and/or urine, respectively. Out of the 19 BK virus positive patients, 12 (63%) were in the tested plasma samples and 17 (89%) were in the urine samples. Four asymptomatic patients had high levels of Epstein–Barr virus shedding in their urine. No co-infected patients showed nephropathy. **Conclusion:** Relying on plasma creatinine rising levels alone may be an unreliable indicator for evaluating opportunistic viral infections in post-transplant RT subjects.

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Keywords: BK virus • cytomegalovirus (CMV) disease • diabetes • EBV-positive PTLD • Epstein–Barr virus (EBV) • multiplex real-time PCR • plasma creatinine • polyomavirus-associated nephropathy (PVAN) • quantitative PCR • renal transplantation

Aim

Despite great improvement in renal transplant (RT) outcomes and the introduction of new immunosuppressive agents that have reduced the rates of graft rejection, renal transplant recipients still suffer from opportunistic infections specifically due to the cytomegalovirus (CMV), Epstein–Barr virus (EBV) and BK virus (BKV). In RT patients, if these viruses remain uncontrolled, local reactivations are followed by wider dissemination into the bloodstream and the majority of cases are diagnosed during this hematogenous phase by the measurement of viral loads [1,2].

In seronegative individuals, CMV, BKV and EBV viruses establish nonreplicative life-long infections (latency) in $CD34^+$ myeloid progenitor cells [3]; renal, bladder and lymphoid cell [4]; and B cells [5] as major sites, respectively. Since latent infections by these viruses can spread in almost every organ, transmissions through organ transplantation are not surprising. In RT patients, in addition to the direct effects of these infections, indirect influences include acute and chronic rejections, poor long-term graft function and graft loss [5,6]. Moreover, the intensity of immunosuppression induction following transplantation alerts for viral reactivation [7] and any decrease in immunosuppression levels puts patients at higher risk of organ rejection and subsequently, the recipients are again at increased risk of infection [8].

Out of above viruses, polyomavirus-associated nephropathy is the major complication associated with high levels BKV replication in RT recipients. The incidence of BK virus-associated nephropathy has been correlated to specific immunosuppressive medications along with their exhaustive immunosuppression power; the most modifiable factor

for BKV infection and hence BK virus-associated nephropathy [9]. Therefore, laboratory measurement for viral DNA by quantitative PCR in urine and blood of patients has been advised by transplantation centers and authorities to avoid such life-threatening complications. Usually, BKV viruria is ahead of BK viremia [10] and for the diagnosis of BKVN, continuous BK viremia presents a more prognostic indicator compared with BKV viruria [11].

On the other hand, CMV represents the most frequent viral opportunistic infection affecting RT recipients and continues to be a major complication in these patients. In these cases, CMV is a major cause of morbidity and mortality linked to aggressive CMV disease. Occurring in the first 6 months post-transplantation (during which CMV infection has generally been more likely to occur), universal prophylaxis antiviral regimen is the preferred strategy in many transplant center points in efforts toward eliminating or mitigating CMV replication before CMV disease development [12]. After prophylaxis treatment is withdrawn, CMV replication and disease still have been noted with significant rates and are more difficult to diagnose in outpatients [13–15].

Epstein–Barr virus (EBV) contaminates most populations worldwide and persists in hosts throughout their lifetime. The primary infection of EBV is predominantly asymptomatic, however, it sporadically causes infectious mononucleosis in young adults. EBV reactivation is a frequent possible concern for immunocompromised hosts, especially in organ transplantation recipients, as transformed cells become proliferating blasts which can lead to symptomatic disease, commonly presenting as associated post-transplant lymphoproliferative disorder (PTLD). As the immunosuppressive state must be sustained for the lifetime of the patient, the risk of PTLD development remains constant, although the possibility of early PTLD in the first year post-transplantation must not be ignored in the cases of primary EBV infection [11,12,16].

The aims of this study were: to investigate the rationale for increased plasma creatinine among RT recipients who were admitted to Sina Hospital; to find out the concomitant CMV, BKV and EBV coinfections using a multiplex real-time PCR and to explore the clinical significance of those coinfections between patients.

Materials & methods

Study designs

The study subjects comprised 101 plasma and urine specimens (202 samples in total) collected from renal transplant (RT) patients referred to Sina Hospital, Tehran, Iran between 2014 and 2017. The inclusion criteria included the hospitalized RT patients who showed any sign or symptoms regarding renal dysfunction and/or plasma creatinine rising post-transplantation. These patients were either symptomatic (fever, low glomerular filtration rate [GFR] and signs of urinary tract infection) or asymptomatic, diagnosed through rising levels of plasma creatinine during routine checkups. In this regard, all transplant patients who experienced raised plasma creatinine levels (0.3–0.5 mg/dl), underwent further clinical and laboratory evaluation (see below). According to available guidelines in our center, all RT individuals routinely receive prophylactic regimen post-transplantation comprised of Valcyte 450 mg (for 3 months according to their GFR) and co-trimoxasol (for 12 months, 400/80 mg according to GFR) for CMV and urinary tract infections, respectively. In addition, they all receive a methylprednisone pulse. For those who do not respond to treatment, renal biopsy is carried out for final diagnosis. The exclusion criteria were prerenal disorders, obstructive renal diseases and being under 18 years of age.

All our patients were negative for antibodies against hepatitis C, hepatitis D and human immunodeficiency virus. The study was evaluated and approved by the Ethical Committee of Tehran University of Medical Sciences. Before specimen collection, informed consent was obtained from all patients. From each participant, a urine specimen was taken as well as 5 ml aliquot of whole blood sample was withdrawn, and plasma was separated. Both specimens were then transported to hepatitis B laboratory at Tehran University of Medical Sciences until being tested. HBV serological markers including HBsAg and HBeAg/anti-HBe were evaluated by ELISA kits (Organon Teknika, Holland).

DNA extraction & polymerase chain reaction

Viral nucleic acids from plasma and urine samples were extracted using an automated extractor, Magnapure 96 (Roche, Basel, Switzerland), according to the manufacturer's recommendation. Before loading the trays, internal control was added to each specimen for exclusion of false negatives due to nonspecific inhibitors of the PCR reactions. DNA was then eluted using 100 µl of elution buffer, stored in -20°C.

A quantitative multiplex real-time PCR was carried out on 5 µl of extracted materials using BCE kit (for simultaneous detection of BKV, CMV and EBV, respectively; Fast Track Diagnostics, Luxembourg), according to manufacturer's instructions, as described previously [17]. Each reaction comprised a positive control as well as at

Table 1. Demographic, clinical and virological characteristic of patients.

Variable	All patients, n = 101 (%)	BK positive, n = 19 (%)	BK negative, n = 82 (%)	p-value
Sex, n (%)				
Males	69 (68.3)	12 (63)	57 (70)	0.594
Females	32 (31.7)	7 (37)	25 (30)	
Age	46.59 ± 15.08	48.7 ± 15.58	46.15 ± 15.2	0.562
Types of kidney donors, n (%)				
Living donor	31 (30.7)	4 (21)	27 (33)	0.461
Deceased donor	68 (67.3)	14 (74)	54 (66)	
Unknown	2 (2)	1 (5)	1 (1)	
The time elapsed from transplantation, year	3.9 ± 3.11	2.10 ± 2.30	5.21 ± 5.31	0.003
High creatinine, n (%)				0.608
Yes	94 (93)	18 (95)	76 (93)	
No	7 (7)	1 (5)	6 (7)	
Urinary tract infection, n (%)				0.342
Yes	7 (7)	1 (5)	6 (7)	
No	94 (93)	18 (95)	76 (93)	
Reduce urinary volume, n (%)				
Yes	2 (2)	1 (5)	1 (1)	0.608
No	109 (98)	18 (95)	81 (99)	
Fever, n (%)				
Yes	17 (17)	1 (5)	16 (20)	0.119
No	84 (83)	18 (95)	66 (80)	
Diabetes, n (%)				
Yes	18 (17.8)	4 (21)	15 (18)	0.781
No	83 (82.2)	15 (79)	67 (78)	
Number of kidney transplants, n (%)				
First transplant	95 (94)	16 (84)	79 (96)	0.116
Second transplant	5 (5)	2 (11)	3 (4)	
Third transplant	1 (1)	1 (5)	0 (0)	
Coinfection with CMV, n (%)				
Yes	15 (15)	7 (37)	8 (10)	0.001
No	86 (85)	12 (63)	74 (90)	
Coinfection with EBV, n (%)				
Yes	10 (10)	6 (32)	4 (5)	0.001
No	91 (90)	13 (68)	78 (95)	

least three standards for analysis of quantification curves in each sample per run. For BK virus, values of $>10^4$ and $>10^7$ copies per ml were considered clinically significant in blood and urine, respectively. For CMV, levels above 1000 IU/ml were deemed clinically significant; however, in the presence of clinical symptoms, levels below 1000 IU/ml were also considered significant as being possibly clinically correlated. With regard to EBV quantitative results, initially the clinical symptoms indicating the occurrence of PTLD were considered; otherwise, the mere detection of EBV was considered as being clinically meaningless.

Statistical analysis

Data were analyzed with the Statistical Program for Social Sciences (SPSS-24, SPSS Inc., IL, USA). Continuous variables were demonstrated as the mean ± standard deviation (SD) and were compared using independent *t*-test. Categorical variables were expressed as numbers and percentages and compared with the chi-squared or Fisher's exact test. For all comparisons, p-value <0.05 was considered as statistically significant.

Results

Table 1 shows demographic, clinical and virological characteristics of transplant patients. Periods of hospitalization ranged between 5 and 30 days. The total number of patients were 101; of whom 69 (68.3%) and 32 (31.7%) were males and females, respectively without any significant differences (p-value 0.594; Table 1), and 44 (43.4%) of total participants were positive for at least one of the three viruses in either their plasma or urine. A total of 19 (18.8%), 15 (14.8%) and 10 (9.9%) out of 101 patients were positive for BKV, CMV and EBV in their plasma and/or urine, respectively (Table 1). Patients were classified into two groups according to the presence of BK virus in their plasma: BK-positive 19 (18.8%, group I) and BK-negative 82 (81.2%, group II). The mean age of all patients was 46.59 ± 15.08. There was no significant difference between the mean age of BKV-positive and BKV-negative

Table 2. Demographic, clinical and virological details of cases with Epstein–Barr virus shedding in the urine.

Characteristics	Case 1	Case 2	Case 3	Case 4
Age	44	47	54	52
Gender	F	F	F	M
Reasons for Tx	Diabetes/hypertension	Hypertension	Diabetes/hypertension	Unknown
Post-transplant month†	14	10	9	20
Creatinine†	1.8	1.4	1.5	5.6
CMV plasma/urine	CMV 1.8×10^3/neg	3.9×10^2/ 4×10^3	7×10^3/neg	Neg/3×10^5
BKV plasma/urine	Neg/neg	Neg/neg	Neg/neg	Neg/neg
EBV plasma/urine	3.5×10^3/ 4.2×10^3	Neg/3.2×10^3	Neg/8×10^8	Neg/2.5×10^3
Follow-up CMV plasma/urine	Neg/5×10^3	Neg/neg	Neg/neg	Neg/7×10^6
Follow-up BKV plasma/urine	Neg/neg	Neg/neg	Neg/neg	Neg/neg
Follow-Up EBV plasma/urine	Neg/neg	Neg/neg	Neg/neg	Neg/neg

All viral loads are reported as international unit per milliliter.
†At the time of admission.
F: Female; M: Male; Neg: Negative.

patients (p-value 0.562; Table 1). In group I, 12 (63%) and 7 (37%) were males and females, respectively. No strong association was found between the genders of both groups (p-value 0.594; Table 1). 31 (30.7%) and 68 (67.3%) patients received kidney transplant from living and deceased donors, respectively. However, no substantial difference was found between the type of donation and the status of plasma BKV (p-value 0.461; Table 1). The mean time elapsed from transplantation was 3.9 ± 3.11 years among all patients. These mean values for groups I and II were 2.10 ± 2.30 and 5.21 ± 5.31 years, respectively. These values showed significant relationship with BKV status (p-value 0.003; Table 1) signifying the more recent elapsed time from transplantation, the more chance for being positive for BKV. There was no strong association between the number of transplantation episodes and the BKV laboratory results (p-value 0.116; Table 1).

Out of the 19 patients in group I, 12 (63%) and 17 (89%) were positive for BKV in plasma and urine, respectively (results not shown). A total of 10 patients (53%) had BKV in both body compartments. The mean values for BK viremia and BK viruria were 2.6×10^5 and 3.1×10^7 IU/ml, respectively (results not shown). However, a majority did not have any chief complaints due to the obligation for checking out their urinary status for following up. Although the only initial finding for all patients was rising in their plasma creatinine (94, 93%), we were unable to find any substantial association between high plasma creatinine levels between BKV-positive and BKV-negative patients (p-value 0.608; Table 1). Moreover, we did not discover urinary tract infection or reduced urine volume in many patients and between BKV negative and positive subjects indeed (see below) (p-values 0.342 and 0.608, respectively; Table 1). 16 out of 17 patients had fever and were negative for BKV; however, when comparing all patients, this finding did not have a statistically significant relationship (p-value 0.119; Table 1). Despite this, diabetes was one of the main reasons for kidney transplantation in 18 subjects (17.8%); however, no significant association was found between BKV test results and the diabetic status of the patients (p-value 0.781; Table 1). Applying a multiplex approach, we did find a considerable link between coinfection of BKV and CMV; 7 (37%) of 19 BKV positive patients were also positive for CMV (p-value 0.001; Table 1). The mean levels of CMV in blood and urine of patients were 3.7×10^5 and 3.3×10^5 IU/ml, respectively (results not shown). In addition, out of the ten total patients who were found to be EBV-positive in either their plasma and/or urine, 6 (32%) belonged to BKV-positive group and 4 (5%) were BKV negative, which showed a significant correlational difference (p-value 0.001; Table 1). During the multiplex approach, we came across four patients with unusual laboratory findings. The urines of these patients were positive for EBV at levels between 2500 and 8×10^8 IU/ml (Table 2). All were cadaver kidney transplant recipients. However, nobody had any symptoms concerning these laboratory results. Three patients had abnormal sonography findings in their urinary tracts and subsequently they were positive for bacteria in their urinary cultures. At 3 months follow-up after being discharged from the hospital, these patients were completely negative for EBV in their plasma and their urines (Table 2).

Discussion

In our center, for all kidney post-transplantation recipients, routine laboratory tests are carried out during patient follow-up. In the case of plasma creatinine rising and for BKVN, usually a multiplex quantitative real-time PCR is

performed for simultaneous detection of BKV, EBV and CMV in the urine and plasma specimens. The ability to identify the presence of two of herpesviruses plus BKV within the same sample simultaneously is one advantage of this PCR method.

In kidney transplantation patients, the first signs of renal disease often include high-level BKV viruria and subsequent BKV viremia, however, usually in the absence of any apparent deterioration in the graft function [18,19]. Generally, BK viruria, BK viremia and BKVN have been reported in renal transplant recipients at prevalence of 30, 14 and 8%, respectively [20–22]. Moreover, positive BKV viruria and BKV viremia occur early after transplantation even within the first hours of post-transplantation and usually peak at approximately 1–3 months post-transplantation [21–23]. It is believed that BK replication status may be an indicator of immunosuppression level [10]. Urine and blood BKV DNA PCR greater than 10^7 and 10^4 copies/ml, respectively may specify BKVN [23,24]. Guidelines for BKV screening recommended by Kidney Disease Improving Global Outcomes (KDIGO) included monthly screening using nucleic acid testing for the first 3–6 months, then every 3 months until the end of the first year [25]. On the other hand, BKVN has been illustrated in the absence of plasma creatinine elevation, BK viremia and BK viruria or other signs of disease activity [10,26]. In addition, elevated plasma creatinine in the presence of BK viremia or BK viruria signifies BK-induced interstitial nephritis [10].

None of the subjects in the present investigation with raised plasma creatinine suffered from BK-associated nephropathy ultimately. Also, this was the case for those who were co-infected or even triple-infected individuals. Despite the enormous impact of these opportunistic viral infections on the outcome of solid organ transplantations, this intermittent viremia and/or viruria either by BKV and CMV in the absence of clinical symptoms have been reported globally, without any known clinical outcome or consequence. Although substantial CMV infections are being diagnosed during the viremic phase by viral load or antigen testing [27,28], the mere detection of CMV does not essentially exclude the presence of co-pathogens in the blood. On the other hand, the lack of CMV does not completely rule out CMV disease in these patients, as very low or transient periods of CMV viremia could be found in some cases due to the compartmentalized or localized CMV diseases [29,30]. In the present study, all subjects had received CMV antivirals including ganciclovir (1.25 mg/kg IV daily as induction for 1 month, which then was switched to oral valgancyclovir) or valcyte (450 mg, according to their plasma creatinine levels) for the first 3 months post transplantation. In the cases of CMV DNAemia, and upon clinical evaluation, they did not show any manifestation typical of CMV disease. Therefore, these patients should be checked in defined interval periods using multiplex testing approaches and also laboratory data should be interpreted carefully, because late-onset CMV disease still develops in approximately 18% of patients even in the presence of either prophylactic or pre-emptive strategies [15,31].

Despite strong correlation between the presence of EBV and BKV positivity, we did not find any sign and symptoms among patients regarding this finding. However, four patients had different levels of EBV in their urine. In spite of this, they did not show any symptoms regarding the kidney or any systemic involvements. These patients were positive for EBV IgG and were negative for EBV IgM. They had already presented as the first report of asymptomatic urinary EBV shedding in immunocompromised cases elsewhere [17]. All patients were followed up for 3 months post discharge by testing on their urine and plasma. All were negative for EBV. We hypothesized that in transplant recipients, asymptomatic shedding of EBV in urine in the absence of signals for aggressive disease (presence of virus in blood and/or tissues) should be observed with suspension. Lacking EBV DNAemia together with alternative periods of detectable EBV in urine might mirror the presence of functionally effectual central/effector memory T cells against EBV. The implication of this finding among immunocompromised patients requires prospective longitudinal studies.

Conclusion

In conclusion, most patients in the current investigation had asymptomatic shedding of three opportunistic viruses in different body compartments without any clinical significance outcomes. No further medical interventions were carried out on these individuals. Relying on plasma creatinine rising might not be a reliable indicator for evaluation of opportunistic infections in these subjects. Therefore, these patients should be routinely checked in defined interval periods using multiplex testing approaches and laboratory data should be interpreted carefully to avoid unnecessary medical interference. The implication of this finding among immunocompromised patients requires prospective longitudinal studies.

Summary points

- This is the first study from Iran, using a multiplex real-time PCR approach for simultaneous detection of three opportunistic pathogens among kidney transplant patients.
- Fourty four (43.4%) of total participants were positive for at least for one of three viruses in either their plasma or urine. Only 19 (18.8%) of the patients were BK positive.
- More than a third of BK virus (BKV)-positive patients had simultaneous high levels of cytomegalovirus (CMV) in their specimens.
- There was no significant difference between the mean age, genders, type of donation and the number of transplantation episodes between BKV-positive and BKV-negative patients.
- The mean values for BK viremia and BK viruria were 2.6×10^5 and 3.1×10^7 IU/ml, respectively. However, a majority did not have any chief complaint due to the obligation for checking out their urinary status in follow-up.
- There was not any substantial association between high plasma creatinine levels between BKV-positive and BKV-negative patients, nor urinary tract infection or reduced urine volume in a majority of patients and between BKV-negative and -positive subjects.
- We did find a considerable virological link between coinfection of BKV and CMV; however, no clinical relevance was found between these co-infections.
- Four patients showed high levels of Epstein–Barr virus shedding in their urines; however; nobody had any signs and symptoms during following up survey 3 months post discharge from hospital.

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Financial and competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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•• **Valganciclovir prophylaxis in the pediatric renal transplant population is safe and effective in preventing CMV infection. Prolonging valganciclovir prophylaxis to 6 months may decrease the incidence of late-onset CMV disease without a significant increase in toxicity and resistance.**