Research Article

BK Polyomavirus (BKV) Infection and Nephropathy in Renal Transplant Recipients: A Prospective Study at Tertiary Care Center.

Sushma Singh[1], Narayan Prasad[2], Monisha Banerjee[3], Dharam Veer Singh[1], Swati Saxena[1], Jyoti Umrao[1], Tapan N.Dhole[1]*
[1]Department of Microbiology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India.
[2]Department of Nephrology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India.
[3]Department of Zoology, University of Lucknow, India.
***Corresponding author's E-mail:** tndhole@gmail.com

Accepted on: 10-01-2016; **Finalized on:** 29-02-2016.

ABSTRACT

BK virus (BKV) is a non enveloped, double stranded DNA virus of the polyomavirus family that primarily affects immuno compromised people. BK virus-allograft nephropathy (BKVAN) is an increasingly recognised complication after kidney transplantation and is a important cause of Kidney transplant morbidity and graft failure. Reported prevalence can vary significantly between centres ranging from 10% to 60%. Monitoring/screening of BK virus in urine and blood has been used as a surrogate marker of BKV nephropathy(BKVAN) post transplantation. So the purpose of our study was to assess the prevalence and severity of BK virus infection in renal transplant recipients in our center. We prospectively asseseed 150 kidney transplant recipients for the presence of BK viruria and BK viremia during the post transplant follow up period using quantitative Real-time PCR assay. We found that 30% of renal transplant recipients were having BK viruria whereas BK viremia was found in only 8.6% of renal transplant recipients. The median age of the patients was 35 years. Among 150 recipients, 18.6% recipients were treated with induction therapy. Living donor transplant consisted of 98% of the kidney donations. Maintenance immunosuppressive therapy included tacrolimus/cyclosporine and mycophenolate mofetil, plus tapering Prednisolone. The highest detected plasma viral load was 48000 copies per milliliter. Despite the occurrence of BK virus infection in 30% of our patients, BK nephropathy did not develop in only two of them. Routine screening of BK virus infection, particularly in centers with low prevalence of BK virus nephritis, may not be cost effective for predicting this disease.

Keywords: BK virus, BK virus associated nephropathy, Renal transplant recipients, viremia, viruria.

INTRODUCTION

BK virus nephropathy (BKN) is one of the most common viral complications in renal transplant recipients and has been recognized as an emerging cause of allograft dysfunction and graft loss in kidney transplant recipients.[1-3] Nearly 65-100% of the population revealed serological evidence of antibodies against the BK virus suggesting prior infection.[4] It has been proposed that after primary infection in childhood via oral and /or respiratory exposure, the virus maintains its latency in renourinary tract. After kidney transplantation, reactivation of the virus can lead to BKN with definite diagnosis based on demonstration of characteristic pathologic changes in renal biopsy samples.[5-6] BKN occurs in 1% to 10% of renal transplant recipients, during first year post transplantation, followed by graft loss in 15% to 80% of cases within 5 years if delayed diagnosis has been made.[7-9] Effective screening of BK virus in urine or plasma on regular intervals can lead to early detection of BKN, so that by decreasing or switching immunosuppression, the rate of graft loss may be reduced to 10% or less.[8-16] Several studies have demonstrated that most of the cases of BKN are preceded by an asymptomatic phase of persistent and significant viruria which is typically followed within few weeks by viremia. A significant and sustained viremia identifies patients with uncontrolled viral replication potentially leading to parenchymal injury which in turn leads to progression to BKVN and eventually deterioration of kidney graft function.

Although the reasons of recent increase in BK virus infection and nephritis remain mainly unknown.[17] Increased overall immunosuppression by antirejection therapy with methyl prednisolone pulses and lymphocyte-depleting agents and certain immunosuppressive agents like calcineurin inhibitors and mycophenolate mofetil are associated with an increased risk for BK virus infection.

Although the exact pathogenesis of BK virus infection that leads to BKN has still not been properly known, but the role of recipient humoral and cellular immunity, alloimmune activation, ischemic injury, and viral virulence, can not be ignored.[18]

In this prospective study of kidney transplant recipients, we intended to evaluate the prevalence and severity of BK virus infection, and related risk factors in our center.

MATERIALS AND METHODS

Participants

One fifty consecutive kidney transplant recipients at Sanjay Gandhi post graduate institute of Medical Sciences, Lucknow, (a Large tertiary care and transplant center) who signed the informed consent (between Feb 2013 and October 2013) were enrolled in the study.

Sample Collection

Samples were taken from renal transplant patients who attended post transplant follow up clinic (OPD). The peripheral blood samples were collected in sterile EDTA tubes and the plasma fraction was separated out by low-speed centrifugation. Urine was collected as random midstream samples in sterile containers and was used without centrifugation.Both urine and plasma samples were stored at -80°C until assayed.

DNA Extraction

BK DNA was extracted from 200 µL of each patient's plasma, using a QIAamp DNA Blood mini kit (Qiagen), according to manufacturer's instruction. Briefly, 20 µL of protease was added to 200 µL of plasma in a 1.5-mL tube. Then, 200 µL of Al buffer was added to each tube, vortexed, and incubated for 10 minutes at 56°C. For DNA precipitation, 200 µL of ethanol was added to the mixture and centrifuged for 1minute. Components transferred to a collection tube contained filter tube. Trapped DNA was washed in two steps by AW1 and AW2 buffers to eliminate impurities together with centrifugation after each step. Finally, DNA was eluted using 50 µL of elution buffer was added after centrifugation and stored at -20°C. Similarly, DNA was extracted from 5mL of uncentrifuged urine using a QIAamp blood maxi kit (Qiagen).

Real-time Polymerase Chain Reaction Assay

The quantitative real-time PCR assay was performed using Geno–Sen's JC/BK real time PCR kit (Genome Diagnostics). The PCR amplifications were run in Rotor gene 2000/3000 (Corbett Research). The primers included in the Geno–Sen's real time PCR kit amplifies DNA fragment from large T antigen of BKV virus. The cycling profile includes: denaturation at 90°C for 50 sec, annealing at 50°C for 20 sec and extension at 72°C for 15 sec (45 cycles). Data analysis was performed with Rotor gene 3000 operator manual. The number of the BKV copies was calculated from the standard curve.

Statistical Analysis

Statistical analysis of data was done using the SPSS software (Statistical Package for the Social Sciences, version 17.0, SPSS Inc. Chicago, Ill, USA). Qualitative data were compared using chi-square test and the Fisher exact test, and quantitative data, by the Student *t* test test to identify associations of BKV infection with clinical variables, i.e., age, gender, donor type (related or unrelated), maintenance immunosuppression, rejection episodes and use of ATG. *P* values less than 0.05 was considered significant.

RESULTS AND DISCUSSION

150 renal transplant recipients were evaluated for the presence of BK virus at the OPD (out patient department) of Nephrology at SGPGIMS (Feb 2013 to Oct 2013). Median age of the patients was 35 years (range 10-63). There was a predominance of male gender with 83.3% males (n=125) and 16.6% (n=25) of females. Ninety eight percent (147/150) were recipients from living donors and three were recipients from deceased donors. Out of these 98% living donor transplants, 52 % (n=78) donors were parents, 15.3% (n=23) donors were siblings and 30.6% (n=46) were from spousal donors. Out of 150 recipients, 3 recipients had a second transplantation and the cause of their first allograft failure was chronic allograft nephropathy. Sixty two percent (n=93/150) patients received tacrolimus, mycophenolate mofetil (MMF), Prednisolone; 30% (n=45/150) received cyclosporine, mycophenolatemofetil ,Prednisolone and 8% (n=12/150) received cyclosporine, azathioprine, Prednisolone, as maintenance immunosuppression. Out of 150, 18.6% (n=28/150) were treated with induction therapy (ATG, Basiliximab and Daclizumab). Specifically, basiliximab was administered to 22 recipients, of whom 77.2% (17/22) developed viruria and 68.1% (15/22) developed viremia. Daclizumab was given to 6 renal graft recipients, of whom 33.3% (2/6) presented viruria and none of them were having viremia. Fourteen out of one fifty 9.3% (14/150) patients were induced by ATG. Fifty out of one fifty patients (33.3%) had a clinical history of acute rejection in the past and among these 12% (6/50) were treated with ATG and 88% (44/50) by methyl Prednisolone pulse for rejection episodes. Twenty nine percent patients had a history of CMV disease in the past and were treated with Valgancyclovir.

BKV infection was considered when BKV DNA was detected in plasma and/or urine of renal allograft recipients. BK viruria was detected in 45 patients (30 %), were as BK viremia was detected in only 13 patients (8.6 %). Statistical analysis of these 45 cases is shown in table no 1. Out of these 13 patients only two patients showed graft dysfunction, one had an unremarkable biopsy whereas one patient with highest plasma load displayed graft dysfunction along with histological evidence of nephritis in his biopsy. His baseline creatinine was 1.20 before the episode of graft dysfunction, which has risen to 2.3 at the time of diagnosis of BKN. The median urine viral load was 3415 copies/ml, whereas the plasma viral load was 1670 copies/ml. The highest detectable urine viral load was 4300000 copies, whereas the highest detected plasma viral load was 48000 copies. The median age of patients with BK virus infection was 37years (range, 17-58 years) and that of the overall transplant population was 35 years (range10-63 years; no significant difference). Of the patients 32% were male and 18% were female, which was not significantly different to the overall transplant population. The median time from transplantation to diagnosis of BK-virus infection was 23.30 months(range, 2-64months).

All the 45 BKV infected patients were on triple immunosuppression consisting of Tacrolimus/Cyclosporine, mycophenolate mofteil and prednisolone. 71.1%(n=32) of patients with viruria were on tacrolimus whereas 28.8% (n=13) of patients were on cyclosporine. On the other hand 84.6% (n=11) patients

with viremia were on tacrolimus and 15.3% (n=2) were on cyclosporine. (Fig.1)

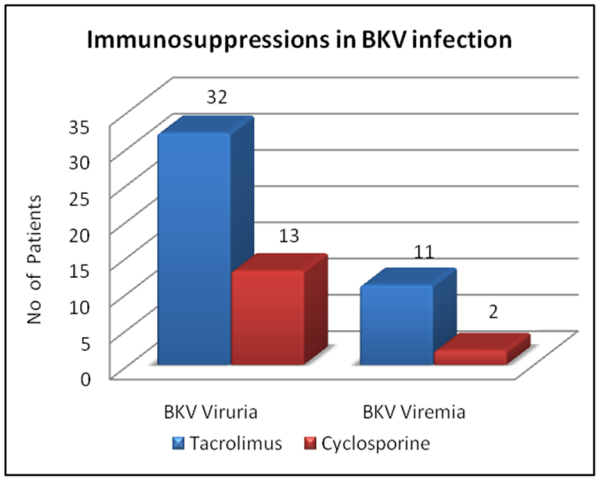

Figure 1: Immunosuppression in patients with BKV infection.

BK viral infection has emerged as a potential cause of progressive renal allograft dysfunction.[19] Early diagnosis of BKV and reduction of immunosuppressive therapy are the major strategies to improve graft survival and stabalize serum creatinine after kidney transplantation.[18,20,21] The prevalence of BK virus infection in post transplant patients with stable graft function has not been elucidated, especially in Indian scenario, so the purpose of our study was to determine the prevalence of BKV infection in post renal transplant patients with BKV infection and to explore the role of viral load in respect to stable graft function.

Viral replication begins early after transplantation and progresses through detectable stages-viruria, then viremia followed by nephropathy.[19] Progression to BKVAN occurs without clinical signs or symptoms except for increasing serum creatinine concentrations. The "gold standard" for diagnosis of BKVAN continues to be renal biopsy with demonstration of viral cytopathic tubulointerstitial changes. Sometimes, however, the biopsy result can be falsely negative due to the focal nature of the disease.[22] Urine cytology for decoy cells and BKV PCR assay for viruria and viremia are non-invasive methods for screening, monitoring of BKV infection. Viruria is sensitive for detecting active BKV infection but lacks specificity for nephropathy and hence can not predict tissue invasive disease, on the other hand BK viremia has been reported to have 100% sensitivity and 88% specificity[19]. It is speculated that viral DNA gains access to the blood stream once the cultivated virus spread to the renal tubules from the urothelium in the highly vascularised renal medulla.[23-24] It has been postulated that viremia only occurs under active replication and is not being observed during latent periods.[25,26] BKV-PCR can be clinically used as a non-invasive test in order to identify kidney transplant recipients under risk or suspicion of BKN.[27]

In our study, BK viral infection as quantified by Real-time PCR was evident in 30% of renal allograft recipients. Of the 150 collected urine and plasma samples, 30% of the urine and 8.6% of plasma specimens were positive for BK virus. The difference between urine and plasma samples was statistically significant. These figures are in accordance with other reports. In Switzerland, Hirsch[13] found that 30% of urine samples and 13% of plasma samples collected from kidney recipients were positive for BK virus. In France, 57% of urine and 29% of plasma specimens of this kind of patients have been positive for BK virus.[28] Almost similar result has been reported from Spain where 33% of urine and 7.5% of plasma specimens were positive for BK virus.[29] In a similar study in Kuwait, Nampoory[30] have found that 45% of urine and 26.1% of plasma samples were BK virus positive. Randhawa in their studies reported rates of 30% and 8% for viruria and viremia.[23,31] In our experience, we found the predominance of male gender and increased age of the transplant recipient as the risk factor for BKV infection. Other clinical parameters did not displayed any significant correlation with BKV infection. We also observed a strong correlation between immunosuppression (Tacrolimus, mycophenolate mofteil and Prednisolone) with BKV infection (P<0.0001). In our study, we found that out of 62% patients receiving tacrolimus, mycophenolate mofetil and prednisolone 34.4% (n=32) patients had BKV infection whereas out of 30% of patients receiving cyclosporine, mycophenolatemoeftil and prednisolone 28.8% (n=13) patients had BKV infection as compared to other maintenance immunosuppressive regimens, so we postulated that this tacrolimus and MMF may be the causative factor for the increased incidence of BKV infection, because most of the cases with BKV nephropathy have been associated with the use of these drugs[15] whereas fewer cases of BKV infection has also been reported to occur who received other immunosuppressive agents such as cyclosporine and azathioprine.[32]

According to several studies, in recent decade, the declining rates of rejection episodes after kidney transplantation has no longer been translated into similarly improved rates of graft survival due to better immunosuppressive drugs, including basiliximab as induction therapy which have led to the emergence of BKVAN, a major cause of renal failure early after kidney transplantation.[33] Our results are in accordance with the previously published studies by Anzivino and kaukulaki and colleagues,[32,34] we found a statistically significant correlation(P<0.0001) between BKV infection and patient receiving basiliximab as an induction therapy. Many studies have reported the association of BKV infection with the use of ATG for management of rejection,[18] but we did not find any such association in our study population.

Our study population with asymptomatic viruria showed urinary viral loads below proposed BKVAN cutoff of 1.0×10^7 copies/ml in 43 out of 45 samples. Only two

patients with 2.3×10^7 and 4.7×10^7copies/ml in urine and 22700 and 48000 copies/ml in plasma had graft dysfunction episode afterwards and only one patient's biopsy revealed viral cytopathic changes indicative of BKV associated nephropathy.

Another interesting finding is that, we observed BK viruria in 22 patients as late as in 21 months post transplantation, but did not lead to any kind of graft dysfunction, but it must be kept in mind that the viral load in such cases was below 1.0×10^4 in the urine and viremia was not seen in such cases, whereas we found that the two patients who showed graft dysfunction had urine viral loads above 2.0×10^7 and plasma load above 1.0×10^4 copies/ml and were in their third month of post transplantation. These findings indicate that transient viremia or viruria does not provoke irreversible damage to renal tissue. These results are comparable with other studies in this respect.[13,35]

Table1: Statistical analysis of forty five cases of BK viral infection.

Characteristics	Infected (%)	Non- infected (%)	Total no. of cases (%)	χ 2 DF P value
Total	45 (30%)	105 (70%)	150 (100%)	
Gender Male Female	 40 (32.5%) 5 (18.5%)	 83 (67.5%) 22 (81.5%)	 123 (82%) 27 (18%)	 2.067 p=0.150
Age(yr) 0-20 21-40 41-65	 2 (4.4%) 26 (57.8%) 17 (37.8%)	 7 (6.7%) 68 (64.8%) 30 (28.6%)	 9 (6%) 94 (62.7%) 47 (31.3%)	 1.36 p=0.507
Donor Parents Siblings Spouse	 21 (46.7%) 6 (13.3%) 18 (40%)	 59 (56.2%) 18 (17.1%) 28 (26.7%)	 80 (53.3%) 24 (16%) 46 (30.7%)	 2.64 p=0.266
Donor status Living Donor Cadever Donor	 43 (95.6%) 2 (4.4%)	 104 (99%) 1 (1%)	 147 (98%) 3 (2%)	1.96 p=0.161

CONCLUSION

Routine screening of BK virus infection, particularly in centers with low prevalence of BKN, may not be cost effective (especially in developing countries) for predicting this disease. However, monitoring of BKV viremia and viruria allows to identify renal transplant patients that could be at risk of BKVAN. Although limited by a relatively small sample size, our study suggests that even though BK virus reactivation and viruria are common and can occur at any time point and is more likely to commence in early post transplantation period but BK-associated renal dysfunction seems to be rare.

REFERENCES

1. Hirsch HH. Polyomavirus BK Nephropathy: A (Re-) emerging Complication in Renal Transplantation. American journal of transplantation. 2(1), 2002, 25-30.
2. Purighalla R, Shapiro R, McCauley J, Randhawa P. BK virus infection in a kidney allograft diagnosed by needle biopsy. American journal of kidney diseases. 26(4), 1995, 671-3.
3. Randhawa PS, Demetris AJ. Nephropathy due to polyomavirus type BK. New England Journal of Medicine. 342(18), 2000, 1361-3.
4. Thakur R, Joshi K, Minz M, Singla A, Nada R, Arora S. Dual positivity of donor and recipient plasma for BK virus confers a high risk for development of BK nephropathy in renal allograft. Transplantation proceedings; 2012: Elsevier; 2012, 717-20.
5. Ramos E, Drachenberg CB, Papadimitriou JC, Hamze O, Fink JC, Klassen DK, Clinical course of polyoma virus nephropathy in 67 renal transplant patients. Journal of the American Society of Nephrology. 13(8), 2002, 2145-51.
6. Howell DN, Smith SR, Butterly DW, Klassen PS, Krigman HR, Burchette Jr JL, Diagnosis and management of BK polyomavirus interstitial nephritis in renal transplant recipients. Transplantation. 68(9), 1999, 1279-88.
7. Mengel M, Marwedel M, Radermacher J, Eden G, Schwarz A, Haller H, Incidence of polyomavirus-nephropathy in renal allografts: influence of modern immunosuppressive drugs. Nephrology Dialysis Transplantation. 18(6), 2003, 1190-6.
8. Mayr M, Nickeleit V, Hirsch HH, Dickenmann M, Mihatsch MJ, Steiger J. Polyomavirus BK nephropathy in a kidney transplant recipient: critical issues of diagnosis and management. American journal of kidney diseases. 38(3), 2001, e13. 1-e. 5.
9. Drachenberg C, Papadimitriou J, Wali R, Nogueira J, Mendley S, Hirsch H, Improved outcome of polyoma virus allograft nephropathy with early biopsy. Transplantation proceedings; 2004: Elsevier; 2004, 758-9.
10. Nickeleit V, Steiger J, Mihatsch MJ. BK virus infection after kidney transplantation. Graft. 5(suppl 1), 2002, S46.

11. Nickeleit V, Singh HK, Mihatsch MJ. Polyomavirus nephropathy: morphology, pathophysiology, and clinical management. Current opinion in nephrology and hypertension. 12(6), 2003, 599-605.
12. Medipalli R, Vasudev B, Zhu Y-r, Saad E, Cohen EP, Orentas R, Improved outcomes of BKVN: Impact on BK virus surveillance protocol. American journal of transplantation; 2007: BLACKWELL PUBLISHING 9600 GARSINGTON RD, OXFORD OX4 2DQ, OXON, ENGLAND; 2007, 150-.
13. Hirsch HH, Knowles W, Dickenmann M, Passweg J, Klimkait T, Mihatsch MJ, Prospective study of polyomavirus type BK replication and nephropathy in renal-transplant recipients. New England Journal of Medicine. 347(7), 2002, 488-96.
14. Hirsch HH, Brennan DC, Drachenberg CB, Ginevri F, Gordon J, Limaye AP, Polyomavirus-associated nephropathy in renal transplantation: interdisciplinary analyses and recommendations. Transplantation. 79(10), 2005, 1277-86.
15. Brennan DC, Agha I, Bohl DL, Schnitzler MA, Hardinger KL, Lockwood M, Incidence of BK with tacrolimus versus cyclosporine and impact of preemptive immunosuppression reduction. American journal of transplantation. 5(3), 2005, 582-94.
16. Hirsch HH, Snydman DR. BK virus: opportunity makes a pathogen. Clinical Infectious Diseases. 41(3), 2005, 354-60.
17. Hariharan S. BK virus nephritis after renal transplantation. Kidney international. 69(4), 2006, 655-62.
18. Soleymanian T, Keyvani H, Jazayeri SM, Fazeli Z, Ghamari S, Mahabadi M, Prospective Study of BK Virus Infection and Nephropathy During the First Year After Kidney Transplantation. Iranian journal of kidney diseases. 8(2), 2014.
19. Badwal S, Chopra G, Varma P, Hooda A. BK polyoma viral infection in renal allograft recipients. Medical Journal Armed Forces India. 67(2), 2011, 122-30.
20. Kuypers DR, Vandooren AK, Lerut E, Evenepoel P, Claes K, Snoeck R, Adjuvant low-dose cidofovir therapy for BK polyomavirus interstitial nephritis in renal transplant recipients. American journal of transplantation. 5(8), 2005, 1997-2004.
21. Zavos G, Gazouli M, Psimenou E, Papaconstantinou I, Bokos J, Boletis J, Polyomavirus BK infection in Greek renal transplant recipients. Transplantation proceedings; 2004: Elsevier; 2004, 1413-4.
22. Pang XL, Doucette K, LeBlanc B, Cockfield SM, Preiksaitis JK. Monitoring of polyomavirus BK virus viruria and viremia in renal allograft recipients by use of a quantitative real-time PCR assay: one-year prospective study. Journal of clinical microbiology. 45(11), 2007, 3568-73.
23. Randhawa PS, Vats A, Zygmunt D, Swalsky P, Scantlebury V, Shapiro R, Quantitation of viral DNA in renal allograft tissue from patients with BK virus nephropathy1. Transplantation. 74(4), 2002, 485-8.
24. Drachenberg RC, Drachenberg CB, Papadimitriou JC, Ramos E, Fink JC, Wali R, Morphological spectrum of polyoma virus disease in renal allografts: diagnostic accuracy of urine cytology. American journal of transplantation. 1(4), 2001, 373-81.
25. Drachenberg CB, Hirsch HH, Ramos E, Papadimitriou JC. Polyomavirus disease in renal transplantation: review of pathological findings and diagnostic methods. Human pathology. 36(12), 2005, 1245-55.
26. Reploeg MD, Storch GA, Clifford DB. BK virus: a clinical review. Clinical Infectious Diseases. 33(2), 2001, 191-202.
27. Montagner J, Michelon T, Fontanelle B, Oliveira A, Silveira J, Schroeder R, BKV-infection in kidney graft dysfunction. Brazilian Journal of Infectious Diseases. 14(2), 2010, 170-4.
28. Bressollette-Bodin C, Coste-Burel M, Hourmant M, Sebille V, Andre-Garnier E, Imbert-Marcille B. A prospective longitudinal study of BK virus infection in 104 renal transplant recipients. American journal of transplantation. 5(8), 2005, 1926-33.
29. Vera-Sempere F, Rubio L, Moreno-Baylach M, García A, Prieto M, Camañas A, Polymerase chain reaction detection of BK virus and monitoring of BK nephropathy in renal transplant recipients at the University Hospital La Fe. Transplantation proceedings; 2005: Elsevier; 2005, 3770-3.
30. Nampoory M, Johny K, Pacsa A, Nair P, Said T, Halim M, BK virus nephropathy in renal transplant recipients in Kuwait: a preliminary report. Transplantation proceedings; 2005: Elsevier; 2005, 3048-50.
31. Vats A, Shapiro R, Randhawa PS, Scantlebury V, Tuzuner A, Saxena M, Quantitative viral load monitoring and cidofovir therapy for the management of BK virus-associated nephropathy in children and adults1. Transplantation. 75(1), 2003, 105-12.
32. Anzivino E, Bellizzi A, Mitterhofer AP, Tinti F, Barile M, Colosimo MT, Early monitoring of the human polyomavirus BK replication and sequencing analysis in a cohort of adult kidney transplant patients treated with basiliximab. Virol J. 8(1), 2011, 407.
33. Harza M, Tacu D, Mitroi G, BUCŞA C, Gherghiceanu M, Preda A, Polyomavirus BK-associated nephropathy after kidney transplantation: a single-center retrospective analysis. Romanian journal of morphology and embryology= Revue roumaine de morphologie et embryologie. 55(1), 2014, 123.
34. Koukoulaki M, Grispou E, Pistolas D, Balaska K, Apostolou T, Anagnostopoulou M, Prospective monitoring of BK virus replication in renal transplant recipients. Transplant Infectious Disease. 11(1), 2009, 1-10.
35. Nickeleit V, Klimkait T, Binet IF, Dalquen P, Del Zenero V, Thiel G, Testing for polyomavirus type BK DNA in plasma to identify renal-allograft recipients with viral nephropathy. New England Journal of Medicine. 342(18), 2000, 1309-15.

Source of Support: Nil, **Conflict of Interest:** None.