Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 12 [5]April 2023 : 20-28
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Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD

ORIGINAL ARTICLE **OPEN ACCESS**

Prospective Monitoring of Bk Viral Load in Renal Transplant Recipients

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ABSTRACT
Polyomavirus BK (BKV) infection, which can result in BKV-associated nephropathy (BKVAN), is a growing issue for individuals who have undergone kidney transplantation. The gold standard of care for diagnosing infection and tracking treatment in kidney transplant patients infected with BKVN is viral load testing for the BK virus. This study's objective was to identify whether the BKV viral load in kidney recipients' urine and plasma might be utilised as a preliminary indicator of their risk for developing BK nephropathy.Urine and blood samples were collected from 89 kidney transplant recipients. DNA was extracted and stored at -80°C. Quantitative Real-Time PCR assay can be used to assess the BKV viral load or confirm the diagnosis of infection with BKV.89renal allograft recipients were evaluated. 33 (37%) had BKV viruria 17(19%) had BKV viremia and one had BKVN.The development of BK viremia/BKVN is early identified by the presence of viruria. Detection of higher viral load in urine should prompt early allograft biopsy and also a pre-emptive reduction in immunosuppression.After renal transplantation, routine screening of transplant recipients was recommended. This can improve transplant outcomes and prevent transplant rejection.
Keywords: *-BKV-BK Virus, BKVN-BK virus Nephropathy, PVAN-Polyoma Virus Associated Nephropathy, RT PCR-Real-Time Polymerase Chain Reaction,*

Received 12.02.2023 Revised 29.03.2023 Accepted 10.04.2023

INTRODUCTION

Most end-stage kidney disorders are treated preference by renal transplantation. Graft survival is the main adventure after transplantation. The development of newer immunosuppressive drugs is the key to successful kidney transplantation. But Organ toxicity can occur with long-term immunosuppressive drug usage, malignancy, and various opportunistic infections [1]. Opportunistic viral infections continue to be a critical factor in death and disability in recipients of kidney transplants. BKV associated with nephropathy is one of the serious causes of a newly transplanted kidney caused by BK Polyomavirus [2].
BK virus belongs to the human *Polyomaviridae* family, which may cause nephritis and urinary tract disorders in patients who have undergone Kidney transplantation [3]. A 39-year-old Sudanese man named BK, who had undergone kidney transplantation owing to chronic pyelonephritis and ureteric stenosis, provided the urine sample from which BKV was initially discovered in 1971[4]. The BKV capsid is a tiny, non-enveloped structure with a diameter of 40–45 nm and a circular, double-stranded DNA genome of 5 kilobases [5]. Its genomic structure consists of early non-structural genes encoding the large T and minor t antigens; the capsid proteins are encoded by late genes (VP1, VP2, and VP3) and noncoding controlling regions (NCCR), such as viral promoters and replication origins, and agnoprotein. BK virus is widely distributed in the adult population, but it is not associated with any illness in healthy individuals. The transmission mode is most likely through the mouth and throat, but Additionally, BKV can spread by seminal fluid, transfusions, or organ transplants, primarily renal allografts. The virus induces a latency phase following infection in urothelial and renal tubular epithelial cells. During renal transplantation or immunosuppressive therapy, the virus is reactivated, initiates replication, and triggers a series of actions beginning with the lysis of tubular cells that result in viruses in the urine. Subsequently,Allografts are invaded by the BK virus, which multiplies in the stroma and travels to the peripheral arteries. It causes various tubulointerstitial lesions and BK virus nephropathy, leading to graft loss. [6].

Decreased immunosuppression is the principal treatment but predisposes it to acute and chronic rejection. As the infection occurs in the early months after transplantation, reducing immunosuppression is risky and makes it vulnerable to denial [7]. The risk factors for BKPyV infection that have been examined may be divided into three groups: risk factors for donors, recipients, and transplant recipients. [8]. A strong immunosuppressive dose is a significant risk factor for the reactivation of BKV [9].A detectable antibody to BKV, which manifests early in childhood and remains raised throughout life, is present in around 80% of the general population. [10].
BKV infection is continuously tracked by quantitative real-time PCR measurements of BK virus DNA in urine and blood. The sensitivity and specificity of PCR for quantifying BKV DNA in plasma are 100 and 88% for BKVN, respectively. [11]. After 2–6 weeks, roughly half of people with extreme viruria acquire BK viremia, and another 2–6 weeks later, nearly half of those with BK viremia are identified with BKVAN after a biopsy. Consequently, by measuring the BKV load in urine, the early indicators of viral growth in the kidney may be assessed. The inability of urine cytology to quantify the severity of the disease due to its lack of specificity and sensitivity is addressed by determining the viral load using PCR [12]. Patients more likely to contract BKVAN should have their urine checked regularly for BKV to detect polyomavirus reactivation early [13]. BKV detection in urine peaked in the first six months before steadily declining after that[14]. In many ways, measuring urine virus count during checking BKVAN is preferable to measuring plasma viral load. When BKVAN develops,urine displays BKV initially, then plasma.; testing for urinary BKV DNA has a substantial negative predictive value long before the commencement of viremia; and BKVN. However, doctors should be cautious when interpreting a low BKV load in the urine. A low BKV load may indicate a decrease of immunosuppression in excess and the danger of eventual acute rejection due to poorer specificity anda failure to remove the virus from the urine after treatment. Real-time PCR urine and plasma viral load testing help detect initial viral proliferation, allowing for assistance, and stopping the development of viremia, BKVAN, or allograft loss[15].
Before considerable deterioration of the renal allograft's functionality develops, at least 90% of people at risk for BKVAN can be identified with BK viremia screening. Routinely checking plasma BKV levels every month until month 9, every three months until two years, and then decreasing annually until five years., even though the ideal frequency and screening methods remain uncertain. [16]. When the plasma BKV load exceeds three log10 copies/mL are discovered in two measurements in three weeks or if shipments grow to > four log10 copies/mL in two or more measures, but at least one multiple BKV replication is presumed, according to current guidelines (presumptive BKVAN). Doctors should taper immunosuppression when renal function is normal at baseline without requiring graft biopsies.[15].However, in India, most transplant centres still need to start using these screening protocols to recognize those most likely to develop BK viral infection[11].
This study explores the incidence of the BK virus infection in renal transplant recipients. BKV reactivation and replication in the urothelial cells are evident by the real-time quantitative PCR technique. It has been utilised to monitor plasma and urine BK virus load samples as a substitute indicator of BKV nephropathy. (BKVN). Quantitative BKV greater viral loads are positively correlated with a higher risk of acquiring BKVAN, according to real-time PCR data.

MATERIAL AND METHODS

The study was conducted from 2019 to 2022 at the Department of Medical Microbiology, Kannur University, Thalassery Campus. Samples were collected from a multispecialty hospital in Kozhikode, Kerala, India. The Institutional Ethics Committee approved the study. 89 kidney transplant recipients enrolled in this study. Each one of the participants provided their written, informed permission. The recipients were followed prospectively for six months after transplantation. The sample size estimation was done based on analytical stats of data from earlier research papers. The patients we have selected for our research have all undergone kidney transplantation, regardless of their sex, race, or nationality. The study excluded patients below 15 years of age and those who were 70 or older. Pregnant women were not allowed in the event.

Extraction of DNA From Urine and plasma Samples: -

After the kidney transplant, 60 days later, midstream urine samples (10-20ml) were taken from 89 kidney transplant recipients. Urine samples were processed as soon as possible.
If BKV was detected in urine (viruria), such patients' blood samples were collected
The following is how blood was drawn. First, Ethylene Diamine Tetra acetic Acid was added to a sterile collecting tube in which 3 to 5 ml of venous blood had been aseptically collected (EDTA). The blood was centrifuged for 5 minutes at 2000 rpm to separate the plasma. In a 1.5 ml sterile centrifuge tube, the plasma was transferred. Plasma samples were stored at -80 degrees

Using a MagGenome XpressRNA/DNA kit, (MagGenome[R]USA, Cat No: MG22Vrna-50 the DNA was extracted from 89 EDTA-anticoagulated plasma and urine samples of renal transplant recipients in accordance with the manufacturer's instructions.
After receiving, the kit should be stored at room temperature(2~25°C).
The extraction procedure of DNA extraction is suitable for using the Bioer NPA-32P nucleic acid purification instrument.
Extracted DNA was stored at -20^{0}C.

Real-Time PCR Assay

The quantitative BKV Real-Time PCR Kit was obtained from RTA Laboratories (RTA® BKV Real-Time PCR Kit, RTA Laboratories, Turkey. Cat. No: 09046100 is a nucleic acid amplification conducted in-vitro for quantifying BK Virus (BKV) DNA in human serum. The assay was carried out following the directions provided by the producer company. used the same procedure. The RTA BKV real-time PCR test includes both external standards for quantitative data gathering and internal control that governs target separation and amplification. To obtain exact quantification data for the Real-Time system, a standard curve should be created using four quantification standards. The Real-Time PCR apparatus should be accurately calibrated for the corresponding concentration of each standard before each run, and the standard curve will be generated in line with that after the reaction. 17µl of the Master Mix should be added to the capillaries or reaction tubes for real-time PCR for each sample. Add 3µl of DNA from each sample, a negative control, and quantification standards to the tubes. 20 µl was the total volume for all reactions. For the Quant Studio™ 5 Real-Time PCR machine, carry out the PCR technique. The target area is in several Glycoprotein G region sequences in the BKV genome. The goal for BKV is 169 bases long. Perform the following protocol for BIORAD CFX96: 95°C for 10 min, 1 cycle; 95°C for 30 sec, 54°C for 30 sec, 72°C for 30 sec, 45cycles. It is recommended to use the FAM and Texas Red channels for measuring fluorescence at 72°C. BKV DNA levels are shown in IU/ml.

RESULTS

The goal of the current study was to look into BK virus infections in renal transplant patients. Many risk factors were associated with this BKV-associated nephropathy. But the major cause of this illness was high dosages of immunosuppressive medications. Urine samples were collected from 89 renal transplant recipients two months after transplantation. Primarily, urine samples were processed. If BKV was detected in urine samples, such patients' blood samples were also collected.

DNA isolation from plasma and urine

DNA was extracted from the plasma and urine of renal transplant recipients (n=89) using a commercially available Mag Genome Xpress RNA/DNA kit (Mag Genome[R]USA, Cat No: MG22Vrna-50).

Real-Time PCR Assay for BK viral quantification

Quantitative real-time PCR was performed using a commercially available kit from RTA Laboratories (RTA® BKV Real-Time PCR Kit, RTA Laboratories, Turkey. Cat. No: 09046100) according to the manufacturer's protocol. The system was sensitive enough to detect as few as one copy of BKV DNA.

Figure: - 1. Quantitation of BKV DNA by Real-time quantitative PCR

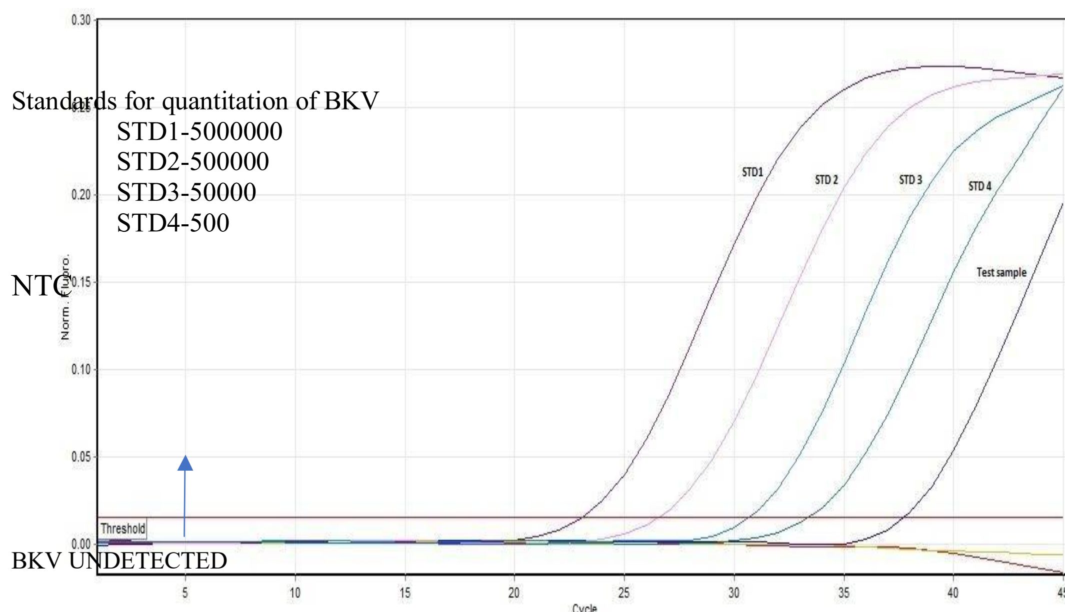

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Figure: -1. In order to construct a standard curve and collect correct quantification data for the Real-Time system, four quantification standards, such as STD1, STD2, STD3, and STD4, should be employed. Before each run, the corresponding concentration for each standard to the Real-Time PCR system should be properly established. At the conclusion of the reaction, the standard curve was formed appropriately. In addition to the four quantification standards, two test samples and one NTC (Negative Test Control) were utilised in this graph.
Figure 1.In order to construct a standard curve and collect correct quantification data for the Real-Time system, four quantification standards, such as STD1, STD2, STD3, and STD4, should be employed. Before each run, the corresponding concentration for each standard to the Real-Time PCR system should be properly established. At the conclusion of the reaction, the standard curve was formed appropriately. In addition to the four quantification standards, two test samples and one NTC (Negative Test Control) were utilised in this graph.
Real-Time PCR results showed that, out of 89 samples, 33 (37.07%) were BKV positive in urine (viruria). In the remaining 56 urine samples, BKV was not detected. Then BKV viruria patients' plasma samples were collected and DNA was extracted. From that, a total of 17 (51.5%) recipients showed the presence of BKV DNA in their plasma (viremia).

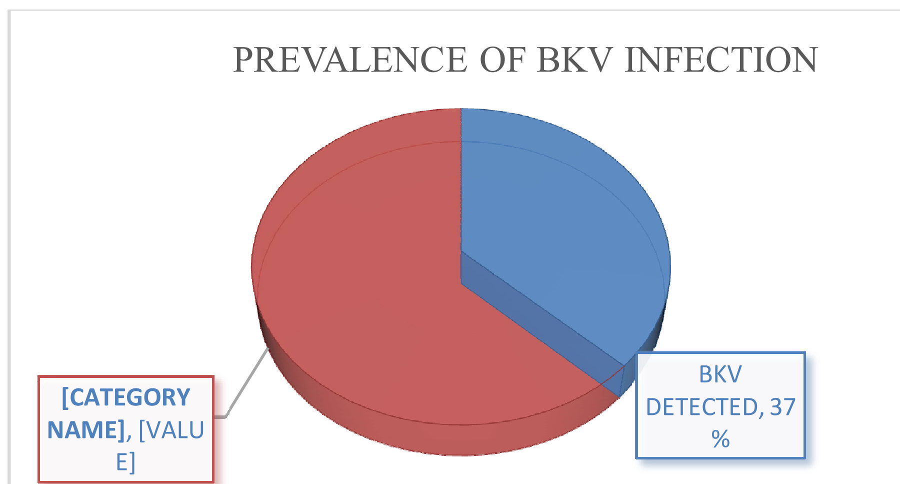

Figure: -2. Prevalence of BKV infection

In this study, 89 urine and plasma samples were collected from renal transplant recipients two months after transplantation. 33(37%) recipients detected BKV infection.63% of the recipient's samples showed BKV was not detected. Asymptomatic viruria was the major problem during the detection. Then we have to consider only the BKV viruria recipients only.

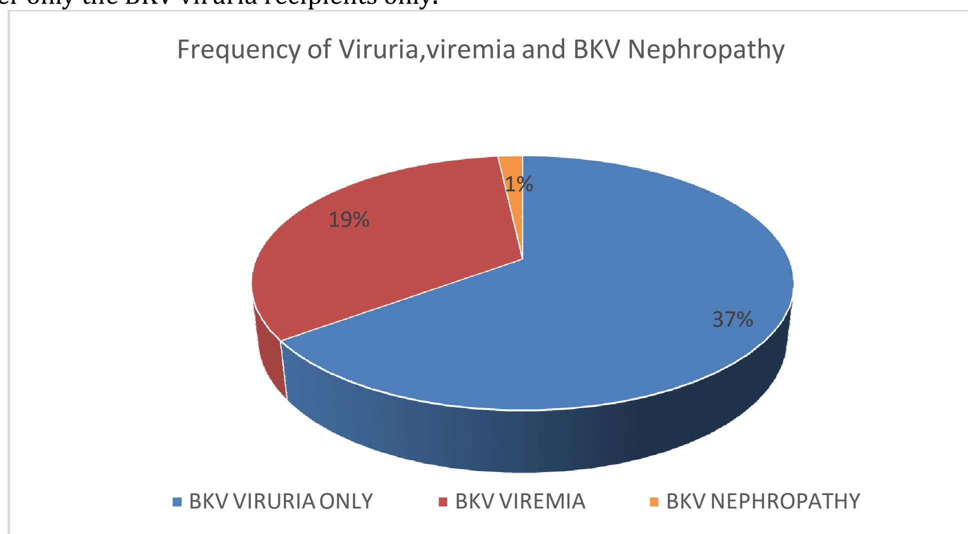

Figure 3. Frequency of viruria, viremia and BKVN.

From figure 2, we have to consider only the BKV-detected cases; out of 89 suspected patients, 33 (37%) had BKV infection, especially BKV viruria. From these 33 patients, 17 displayed both viruria andviremia.One exhibited BKVN. (Figure: -3) BKVN infection was considered when higher levels of BKV viral loads were detected in the plasma and urine of renal transplant recipients.
In the case of BKV viruria detected recipients were classified into three groups. In group I, BK viral load was a minimum of 1 copy to 10^5 copies /mL (median 400). Group II BK viral load in between 10^5 – 10^7 copies/mL (median of 1.02x10^6), and Group III Quantitation of BKV viral load was above 10^7 copies/mL (median of 6.71x10^8). In the first group, only seven recipients show viral load below the range of 5.58x10^4 copies/ml, and there is a slight variation in the number of recipients belonging to group II. Only nine positive samples were in the field of 8.58x10^6 copies/ml. But in Group III, 17 positive samples were detected in the range of viral load 1.49x10^10Copies/ml. Group III was the highly prevalent group in BKV viruria-detected cases. These 17 positive samples can cross the peritubular capillaries and cause BKV viremia. BKV viremia was never detected when viruria levels were less than 10^6 copies/ml. Also, a very high viral load in the urine may be at particular risk of developing BKVAN. From our study groups, we got 17 recipients who were BKV viremia positive. And also, we can say that these 17 recipients may suspect BKVN also. (Table: -1)

Table: - 1:Viral load in the urine of BKV patients

	Group-I	**Group-II**	**Group-III**
Viral load (Copies/ml)	1-10^5	10^5-10^7	>10^7
Positive Samples	7	9	17
Median (range)	400 (5.5868x10^4)	1.02x10^6 (8.58x10^6)	6.71x10^8 (1.49892x10^10)

Table: - 2. Seventeen renal transplant recipients show BKV viremia from 33 BKV viruria-positive samples. I have not encountered a single instance of viremia being detected in this study without detectable viruria.Ten positive samples had a low plasma viral load of 1- 10^2 copies/ml (median range of 488). The remaining seven recipients belong to the rest of the groups. But in group III, plasma viral load above 10,000 copies/ml. Only one recipient shows this higher viral load, such as 16,000 copies/ml. This patient suspects BKVN and has a chance of acute rejection (not shown in this table).This group III sample has the highest peak viral load in plasma.

Table2: Viral load in plasma of BKV patients

	Group-1	**Group-2**	**Group-3**
Viral load (Copies/ml)	1-10^2	10^2-10^3	>10^3
Positive samples	10	6	1
Median (range)	91 (488)	2215 (4033)	16000 (16000)

Sustainable BKV viruria and viremia were found in these seven patients. (Both group 2 and group 3). With more than 10^2 - 10^3 copies /ml viral load inpatients of renal transplants, plasma is thought to be the most trustworthy surrogate marker for suspected BKVAN. (Table: -2.). But BKV-associated nephropathy is made by demonstrating typical viral cytopathic findings at histopathology.

HISTOPATHOLOGICAL STUDIES

Expert clinicians evaluated renal biopsies of all seven suspected patients. Clinically only one patient presented with acute graft dysfunction. The remaining 06 patients had stable graft function and became slowly negative for the BKvirus in subsequent biopsies and urine/plasma PCR. The formation of donor-specific antibodies (DSA) following transplantation is hazardous, according to kidney biopsy reports, and immunofluorescence staining C4d. Only this can lead to antibody-mediated rejection (ABMR). These antibodies may develop primarily as a result of class II HLA molecules. C4d-negative ABMR often develops >12 months after transplantation, however, it might happen suddenly in very sensitive individuals with long-lasting DSAs (even after desensitisation). Sampling for light microscopy included thirty glomeruli identified; one is globally sclerotic. Epithelial cells were the only cells to exhibit BK

nephropathy light microscopic findings, such as intranuclear viral inclusions, and a biopsy revealed the nephropathy-related characteristics of the BK virus.

Immunohistochemistry

After careful histological examination, immunohistochemistry was also performed. Immunohistochemistry revealed nuclear viral staining for Polyoma virus SV40 inthis case. BKVN showed focal viral cytopathic changes in the tubular epithelial cells associated with less than 1% of tubules. There is mild tubulitis. There is no peritubular capillaritis or vasculitis. No lesions were seen in the glomeruli or blood vessels. But this patient had no documented acute rejection after the diagnosis of BKVN.

Demographic and clinical characteristics of patients with BKVN

Only one patient was suffering from BKVN. Positive for BKV viruria and BKV viremia through Real-time PCR. The age of patient was 58 years old and male gender. The leading cause of kidney failure was glomerulonephritis. He started dialysis 12 months ago and had no previous history of Diabetic Mellitus. According to the Tacrolimus Genotyping, it belongs to the fast metabolizer category. He received a graft from his 54-year-old wife. This was his first allograft. It is an ABO-incompatible transplantation. Tacrolimus, Mycophenolate mofetil sodium and Prednisolone were used for maintenance immunosuppression. On the sixth day after transplantation, his creatine level rises to 2.22 mg/dL. A graft biopsy was done, suggesting cellular rejection with vascular involvement. He was treated with Thymoglobulin (150 mg) to reduce the rejection episodes. His graft function started improving and creatinine was 1.6 mg/dl, and his Tacrolimus level was 5.5 ng/ml after the transplantation surgery.

After two months, BKV was detected in urine and plasma with a higher viral load. His creatine level increased from 1.7 mg/dl to 2.5 mg/dl. At the time of renal biopsy, rabbit anti-thymocyte globulin was used to induce immunosuppression.

BKVN patient, maintenance immunosuppression was arbitrarily reduced. Consisted of a change in the target level of Tacrolimus from 8.8 ng/ml to 6.6 ng/ml, and the Mycophenolatemofetil sodium (360mg) dose was decreased to half twice a day for one week and after one week, again reduced by half (once a day). But there is no change in the dosage of prednisone after the reduction of immunosuppression and progressive but slow deterioration of graft function at the end of the observation period.

DISCUSSION

Screening for BKV viremia and viruria is now the standard procedure for all kidney transplant recipients.BKV is released into urine and blood by active replication in urothelial cells, which leads to tissue damage and is detectable by molecular testing. The diagnosis and follow-up of BKVAN in patients who have had renal transplantation can be aided by quantitative PCR for BKV. According to studies, serial quantitative PCR testing for BKV in the urine and blood can be used to determine whether individuals are at risk for developing BKVAN and to track treatment response. According to studies, patients who have >10^4 copies/mL of BKV in their plasma or >10^7 copies/mL of BKV in their urine are at risk of developing BKVAN, the fact that serial monitoring could be more beneficial than single-time point detection. [17].

In our investigation, urine samples from 89 renal transplant patients were taken two months following the transplant. DNA was isolated from the urine samples and refrigerated at -80°C. Using the RTPCR method, BKV infection incidence may be identified. 33 (37%) of the 89 urine samples were positive for BKV (BKV viruria), while BKV was not found in 56 (63%) of the samples (no viruria). We were more concerned when BKV was found in the urine (viruria). Plasma samples from individuals with BKV-detected cases were taken to determine the virus load in their blood. Plasma was used to extract DNA. DNA viral load can find out by Real-time PCR assay. In this assay, we can find a viral load of at least 1copies/ml. So, it was a highly sensitive test as compared with normal conventional PCR. A total of 17 plasma samples show BKV viral loads. From the BKV-positive samples 17 recipients have both BKV viruria and viremia the remaining 16 patients' population have viruria only. However, a similar study from India reported a higher incidence of BKV in plasma (viremia) and BKV in urine (viruria) in their patient group. [18] Viremia often follows viruria and comes before BKVN symptoms appear. Compared to viruria, the positive predictive value of viremia for BKVN is greater, hovering around 60%. Viruria is more sensitive than viremia, for this reason. Other studies from across the world include a study from Greece, where 9% (3/32) of renal transplant recipients were BKV positive in their plasma after transplantation [19]. In Switzerland, Hirsch et al (2002a) found that 30% of urine samples and 13% of plasma samples collected from kidney recipients were positive for BKV [20]. In France, 57% of urine and 29% of plasma specimens [21] and in Spain, 33% of urine and 7.5% of plasma specimens were positive for the BK virus [22].

A positive sample denotes the beginning of viral activation, which requires quantification and ongoing viral load monitoring to determine the likelihood of developing renal illness. Patients had the highest

chance of developing viremia if their urine viral load remained consistently higher. [23][24]. Initially, viral reactivation in susceptible patients remains usually asymptomatic followed by elevation of serum creatinine presented in the form of allograft dysfunction which ultimately leads to BKV-Nephropathy [19]. A preventive decrease in immunosuppression is required in cases where persistently high levels of viremia are a sign of continuous renal injury that does not heal on its own [25]. The 89 kidney transplant recipients in the current study were tested for the BK virus, and the results showed a wide range of viral loads in positive samples, from a few to millions of copies per ml. BKV DNA was found in 17% of plasma samples and 37% of urine samples when renal transplant patients' BK viremia and viruria were monitored over the course of a 6-month follow-up period. (Figure no: - 2) as mentioned earlier. Among them, only one patient with the highest viral load in urine and plasma had BKV nephropathy, whereas 16 recipients with viral loads below 10,000 copies/ml presented with good graft function (Table 1). These findings indicated that graft function is directly correlated with viral load i.e., high viral loads ≥10,000 copies/ml are predictive of a greater risk of BKV nephropathy [26]

Likewise, in other reports, the highest BK virus replication occurred within 2 to 6 months post-transplantation during the strongest immunosuppressive period [19]. In order to identify other risk factors, serum creatinine (levels) were analysed and correlated with various demographic, virological and histologic indices. None of the other factors displayed a significant association with serum creatinine except the viral load of BKV in plasma. It was found that patients with high plasma viral loads >10,000 copies/ml and with histological evidence of viral nephropathy displayed a significantly high serum creatinine. Most of the patients had cleared BKV infection. Therefore, it can be concluded that plasma viral loads >10,000 copies/ml and histological evidence of inflammation in biopsies are predictive of the subsequent decline in graft functional outcome.

The BKVN patient had varied worsening of graft function that was seen in elevated serum creatinine. Only the analysis of renal biopsies was able to provide a correct diagnosis and separation from other causes of graft malfunction due to the lack of specificity in clinical presentation. BKVN was identified in our research three months after transplantation. According to Ramos et al. (2002), histologically verified BKVN might be seen as early as 4 months following transplantation, with a mean diagnostic time of 13.5 months [27].

In our investigation, we discovered that Tacrolimus, MMF, and prednisone were given to all patients. 37% of people had BKV viruria, and 17% had both. As opposed to other maintenance immunosuppressive regimens like Tacrolimus, Azoran, prednisone, and cyclosporine, 16.6% of patients receiving Cyclosporine, Mycophenolate Mofetil, and Prednisolone in Anzivino et al.,2011 study developed BKV infections [28]. Based on our research, we hypothesised that the higher prevalence of BKV infection may be caused by Tacrolimus and MMF. The usage of these medications has been linked to the majority of occurrences of BKV nephropathy. In our patients, the immunosuppression was reduced in a random and inconsistent manner, and each patient's diagnosis was made at a different period. Patients with and without reduced immunosuppression experienced a similar chronic loss of renal function, which may be related to relatively late intervention, an insufficient reduction in immunosuppression, or the failure to completely eliminate the viral pathogen once nephritis developed.

CONCLUSION

Viral load testing for BK Virus (BKV) in urine, and plasma, has become the standard of care for the diagnosis of infection, monitoring BKV reactivation and monitoring of therapy of kidney transplant patients' infection.The initial indicator of an active viral replication is BK viruria. Urine BKV monitoring is required for early diagnosis and prevention because early discovery increases the likelihood that immunologic control of BK replication will be successful. Following effective BKVN therapy, BKV remains detectable in urine for a longer period of time than in blood, and BK viruria measurements can be used to track changes in BKV replication. In order to predict the functional stability of the graft, measurement of viral load, particularly in plasma, may be used to monitor the clinical course of viral nephropathy which is a non-invasive approach.

ACKNOWLEDGEMENT

I am extremely thankful to the Department of Medical Microbiology, Kannur University for supporting me in times of need.

CONFLICTS OF INTEREST

All authors have no conflicts of interest to declare.

C0- AUTHOR'S CONTRIBUTION

Dr Arun B provides me with valuable suggestions for this research and excellent guidance. Dr Firoz Aziz, (MBBS, MD). His meticulous guidance, generous moral support, patient understanding and critical review of work done. Dr Vipin Vishwanath,for the guidance and support during the entire work. He spent a lot of time spent during the evaluation and analysis of the results.Mis Anagha KHer helpduring the course of my research work.

FUNDING

This work was financially supported DEPARTMENT OF SCIENCE AND TECHNOLOGY-WOMEN SCIENTIST SCHEME-A. (DST-WOS-A)

ETHICS STATEMENT

The Institutional Ethics Committee approved the study. (Ref No- CAU/SHS/2400/IEC/2014 DATED 28/11/2018). (Appendix 1)

INFORMED CONSENT

89 kidney transplant recipients enrolled in this study. Each one of the participants provided their written, informed permission. (Appendix 2)

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CITATION OF THIS ARTICLE

Aswathi M Nair, Arun B, Feroz Aziz, Vipin Vishwanath, and Anagha K.. Prospective Monitoring Of Bk Viral Load In Renal Transplant Recipients..Bull. Env. Pharmacol. Life Sci., Vol 12[5] April 2023: 20-28.