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Molecular Characterization of Polyomaviruses (BKV, JCV) in a Symptomatic Kidney Transplant Recipients in Sudan

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Abstract **Background:** The present study was to investigate the polyomaviruses (BKV, JCV) in asymptomatic kidney transplant recipients and to characterize the polyomaviruses circulating in Sudan. **Methodology:** A total of 100 plasma and 100urine samples were collected randomly from renal transplant recipients, attending Sudanese Renal Transplantation Society, at the period between September 2012 to March 2013, and subjected to polymerase chain reaction assay to detect viral DNA, urine cytology also done to identify viral inclusion (decoy cell), DNA sequencing was done for some gene to confirm the result, phylogenetic analysis of BKV, JCV based on T large gene were done MEGA 6 software. **Results:** Among the 100 renal transplant patients the polyomaviruses were detected in 3 urine specimens 3/100 (3%) by using urine cytology technique, that showed nuclear inclusions (decoy cells). And also detected in 32 urine specimens 32/100 (32%) while only 6/100 (6%) of virus nucleic acid had been present in their patients plasma, by using conventional PCR method. Successful sequences of BK, JCV virus on T large gene were done for 6 specimens from the virus isolate PCR products. **Conclusions:** This is the first report on molecular characterization that describes the circulation of polyomaviruses (BKV, JCV) in Sudan, Human polyomavirus is related to different clinical manifestations among renal transplant patients. The routine use of urine cytology and PCR on urine and plasma is a useful tool for the rapid and sensitive detection of reactivated BKV in asymptomatic recipients.

Keywords: *BK polyomavirus, kidney transplant, PCR, Sudan*

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1. Introduction

Plyomaviruses are small size of virion (diameter 40-50nm), naked, icosahedral capsid, superhelical double strand circular DNA genome of molecular weight 3.2×10˙6, shared nucleotide sequence with other polyomaviruses and nuclear site of multiplication. The non-enveloped virion has icosahedral symmetry and 72 pentameric capsomeres. The virion is made up of 88% protein and 12% single cope of circular double strand DNA molecules which has about 5.200 bases [1].

There are three members in the family that infect humans; Simian Virus 40 (SV40), BK virus (BKV) and JC virus (JCV). SV40, JCV and BKV DNAs contain 5,243, 5,130, and 4,963 base pairs respectively [2].

BKV, JCV and SV40 share a high degree of nucleotide sequence homology. The JCV genome shares 75% sequence homology. The JCV genome shares 75% homology with the BKV genome and 69% with SV40 [3]. New human papovavirus (B.K.) were isolated from urine after renal transplantation in 1971 [4].

The BK, JC virus is ubiquitous in human populations worldwide. Infection typically occurs in childhood, with a seroprevalence up to 90% in adults. BKV and JCV are a urotheliotropic virus that becomes latent in the urinary tract after primary infection, reactivation mostly occurs in permissive cells, including renal tubular epithelial cells and the transitional cells of the lower urinary tract.

Active replication in the urinary tract typically correlates with a state of immunoincompetence in the context of immunosuppressive therapy. BKV can cause nephropathy in renal transplant recipients, resulting in tubulointestitial lesions known as polyomavirus associated nephropathy (PVAN) or, more specifically, BKV nephropathy (BKVN). JCV excretion in the urine is usually insignificant, although very rare cases of JCV-associated nephropathy are on record [5]. BKVN occurs in 1% to 10% of kidney transplant recipients, usually manifesting in the first year following transplantation and

leading to graft loss in 15% to 80% BKVN cases within 5 years [5].

Active replication of BKV in urothelial cells and subsequent tissue damage cause release of BKV, JCV into urine and blood that can be monitored by molecular assays, urine cytology. Monitoring of BK, JCV viruria and viremia can facilitate early diagnosis of BK replication, guide management of immunosuppressive therapy and monitor response to intervention [6].

Urinary viral replication precedes BK viremia by approximately four weeks. Histological changes in BK nephropathy become noticeable within 12 weeks after BK virus has been shaded in urine. Patients who have undergone renal transplant and have higher levels of BK viral DNA in their urine, will be more likely to have higher viral DNA levels in plasma. The presence of persistent viremia precedes the clinical signs of nephropathy [7].

In Sudan there are very little information about detection and characterization of polyomaviruses (BKV, JCV) in kidney transplant recipients. The present study aimed to establish diagnostic techniques for rapid detection, monitoring and molecular characterization of polyomaviruses in renal transplant recipients in Sudan.

2. Materials and Methods

2.1. Study Design

This is a descriptive cross sectional hospital based study.

2.2. Study Area

This study was conducted in Khartoum State, among Sudanese renal transplantation patients, from September 2012 to March 2013.

2.3. Study Population

This study was conducted on volunteers of both males and females, adult and asymptomatic renal post transplanted recipients, visiting the Sudanese Renal Transplantation Society for routine checkup for renal function tests or immunosuppressive drugs monitoring, samples were collected.

2.4. Ethical Consideration

The study was approved by the Ethical Review Committee (ERC) of the Ministry of Health Khartoum State, Sudan. Prior to recruitment, the nature and objectives of the study were explained to potential participants and those who agreed to take part in the study signed a consent form.

2.5. Data Collection

Through a structured questionnaire, information were collected on age, gender, date of transplantation, date of sample collection, place of sample collection and the type of immunosuppressive drugs.

2.6. Specimen Collection

One hundred blood (plasma) and one hundred second morning urine samples were collected from asymptomatic renal transplanted recipients during 6 months of transplantation, and 50 urine samples were also collected from apparently healthy adult, randomly of both gender, whom did not undergo renal transplantation or any transplantation surgery and were considered as the control group. Blood specimens (5ml) in EDTA, were collected from the cubital vein and then centrifuged at 4000 rpm for 5 minutes to obtain the plasma. The clear plasma was taken immediately for DNA extraction or stored at -20 C˚. Urine specimens (20 ml) were collected from the patients using sterile wide mouth urine container, and then centrifuged at 3000 rpm for 15 minutes. The deposit was prepared for Papanicolaou stain immediately and the remained urine was preserved in -20 C˚ and for DNA extraction.

2.7. Urine Cytology

The supernatant of urine was decanted and the deposits were smeared in clean labeled microscopic slides. The slides were stained with Papanicolaou stain. Then slides were immersed in the fixative (95% ethanol) for 15 minutes, hydrated in 70% alcohol for two minutes, rinsed in water for one minutes. The nucleus was stained in Harris's Haematoxylin for five minutes, rinsed in water for two minutes, blued in tap water 14 minutes, rinsed in water for two minutes, dehydrated for two minutes as fellow's 70% alcohol, two changes of 95 % alcohol. The cytoplasm was stained in Orange G 6 for two minutes, dehydrated for two minutes in 70% alcohol, two changes of 95 % alcohol, stained in Eosin Azure 50 for three minutes, rinsed in 95 % alcohol for one minute, cleared in xylene and mounted in DPX. The smears were examined using light microscope (Olympus CH21) [9].

2.8. DNA Extraction from Urine and Plasma Samples

Seven ml of urine samples were washed by phosphate buffer saline (PBS), and then centrifuged at 3000 rpm for 10 minutes to collect the pellet. Two ml lyses buffer, 5 µl proteinase K, one ml guanidine chloride and 300 µl NH4 acetate were added and incubated at 37°C over. The samples were cooled to room temperature and then 2 ml pre chilled chloroform was added vortexed and then centrifuged for 5 minutes at 3000 rpm. The upper layer was collected to a new tube and then 10 ml cold absolute ethanol was added shacked and keep at -20°C for overnight, then centrifuged at 300 rpm for 15 minutes, carefully the supernatant was drained, and the tube was inverted on a tissue paper for 5 minutes. The pellet was washed with 4ml of 70% ethanol, centrifuged at 3000 rpm for 15 minutes. The supernatant was poured off and the pellet was allowed to dry for 2 hrs. The pellet was eluted in 200 µl distill water and store at -20°C until used [8].

2.9. Polymerase Chain Reaction (PCR)

Single set of primers was used to amplify both BKV and JCV T. large gene. The primer sequences used as follows: PEP-1 (5′-AGTCTTTAGGGTCTTCTACC-3′) and PEP-2 (5′-GGTGCCAACCTATGGAACAG-3′) [9]. 20µl of PCR mix was prepared in PCR tubes as follow: 10µl ready prepared master mix (Go Tag) was added to 4µl DNA then 1µl of both forward and reverse primers

were added to the mix. Finally the mix was completed to 20µl with distilled water. The amplification condition was as follows; initiated with a first denaturation step 94°C for 5 min; followed by 40 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 5min. Purified BKV genome as positive control and distilled water as negative control were included in all runs.

All samples were run in Thermocyler machine made in UK. All the amplified products were subjected to 1.5% agarose gel electrophoresis. Positive specimens for polyomaviruses resulted in PCR fragments of 176 bp.

2.10. Agarose Gel Electrophoresis

Clean electrophoresis tray was prepared, with sealed ends and a comb with the appropriate number of samples. The tank was filled with 1X of TBE buffer (54g of Trisbase, 27.5 g of boric acid, 20 ml of 0.5 M EDTA pH 8.0 dissolved in 800 ml distilled water and completed to 1L). 1.5 gram of low melting point agar was dissolved in 100 ml of 1X TBE buffer, boiled until clear. Then allowed to cool approximately to 60°C then 2µl of 10g/ml of ethidioum bromide was added before pouring into gel tank and the comb was inserted above the base of the tank. Agarose was added and allowed to seat for 30mins. Five µl of the PCR product was added carefully. 10 µl DNA marker (100 bp) was added to lane one. The lid of the tank was closed to generate an electric circuit, and run for 1hr at voltage of 35 volts/cm. When the migration of the marker is completed the gel was removed and visualized under ultraviolet light and photographed. The size of the separated bands was estimated in comparison to the DNA marker.

2.11. DNA Sequencing

DNA purification and standard sequencing was performed for both strands of PCR (175bp) by Macrogen Company (Seoul, Korea).

2.12. Bioinformatics Analysis

The nucleotides sequences of T.large gene achieved were firstly manually assembled by Finch TV software, then the cleaned sequences were searched for sequence similarity using nucleotide BLAST (http: //blast.ncbi.nlm.nih.gov/Blast.cgi). Highly similar sequences were retrieved from NCBI and subjected to multiple sequence alignment using the BioEdit [14]. Protein translation and modeling was done online by CPH model server-3.2 [16], and EXPASSY translate tools respectively. Chimera software [17] was used to predict protein 3D structure. Phylogenetic tree was generated by Maximum Likelihood method and Tamura-Nei model using the MEGA6 program from the aligned nucleotide sequences [18]. The statistical robustness and reliability of the branching order within each phylogenetic tree were confirmed by applying bootstrap resampling (n = 1000 replicates). Sequences from different countries were retrieved from Gene Bank and included in the generation of phylogenetic trees.

2.13. Data Analysis

Data generated were subject to statistical packages for social science (SPSS) VERSION 16. Descriptive statistics (frequency and percentages) were performed and then plotted into histograms. Chi-square was used to test significance between selected parameters of the study [10].

3. Results

Out of 32 PCR positive urine specimens, the BKV was identified in 11% of patients below 30 years, 7% were between 31-40 years, 6% were between 41-50 years and 8% were identified above 50 years (Table 1). Out of 6 PCR positive plasma specimens, the BKV were in 2% below 30 years and between 41-50 years, whereas only 1% was identified between 31-40 years and another one above 50 years. Out of 3 positive cytological changes in urine specimens the BKV were identified in 2% below 30 years, and only one 1% was between 31-40 years (Table 1). Out of 32 PCR positive urine specimens, the BKV were identified in 31% first 10 years of transplantation, whereas only 1% was in the second 10 years of transplantation. Out of 6 PCR positive plasma specimens, the BKV were identified in the first 10 years of transplantation (Table 2).

Table 1. Detection of polyomavirus in urine specimens of renal transplant recipients using PCR and cytology technique to age group

Age group (years)	PCR No (%)		Cytology No (%)		Total	
	+ve	-ve	+ve	-ve	No.	%
<30	11 (28.9)	27 (27)	2 (2%)	36 (36)	**38**	**38%**
31-40	7 (29.2)	17 (17)	1 (1%)	23 (23)	**24**	**24%**
41-50	6(27.3)	16 (16)	-	22 (22)	**22**	**22%**
>50	8(50)	8 (8)	-	16 (16)	**16**	**16%**
Total	**32(32)**	**68 (68)**	**3 (3%)**	**97 (97)**	**100**	**100%**

Table 2. Relationship between duration of transplantation and polyomavirus detection in urine and plasma of renal transplant recipients using PCR technique

Duration of transplantation	PCR (BKV) Frequency (%)				Total	
	urine		plasma			
	Negative	Positive	Negative	Positive	Frequency	%
<10 years	66 (66)	31 (31)	91 (91)	6 (6)	97	**97%**
>10 years	2 (2)	1 (1)	3 (3)	-	3	**3%**
Total	**68 (68)**	**32 (32)**	**94 (94)**	**6 (6)**	**100**	**100%**

DNA of BK virus was identified by PCR in 32 urine specimens and six plasma specimens (Table 3). All positive PCR samples revealed abundant amplicons of 176 bp indicative of large T-gene of the BK virus genomes (Figure 1). Decoy cells were identified by cytology technique in 3 urine specimens (Figure 2) (Table 1).

Table 3. Detection of polyomavirus in urine and plasma specimens of kidney transplant recipient using PCR in urine, plasma and cytology techniques

Technique	+ve		-ve		Total	
	No.	%	No.	%	No.	%
(urine) P.C.R	32	32%	68	68%	**100**	**100%**
(plasma) P.C.R	6	6%	94	94%	**100**	**100%**
Urine cytology	3	3%	97	97%	**100**	**100%**
Calculated x^2	43.1114					
P-value	= 0.0008**					

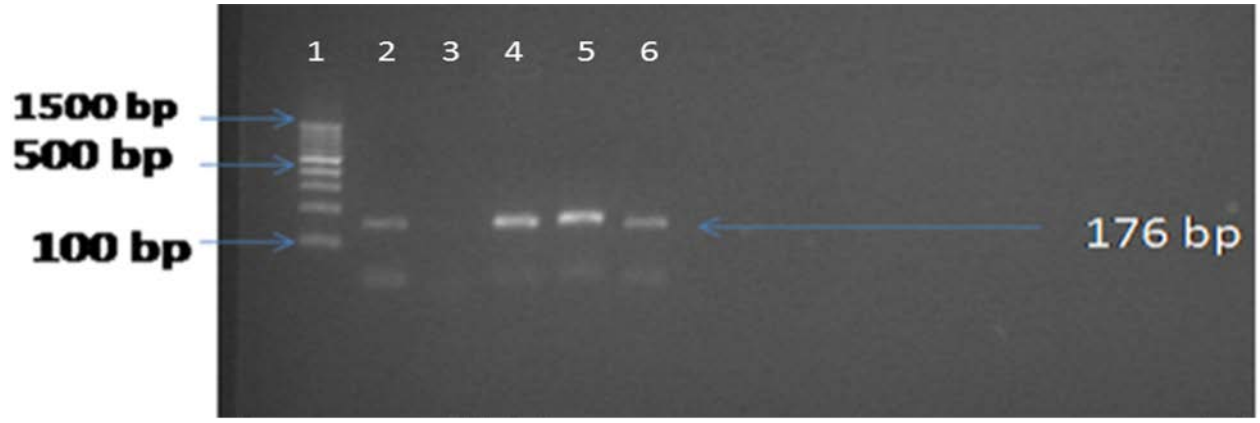

Figure 1. Agarose gel electrophoresis of PCR products generated by using BKV specific primers. (1) DNA size markers 50 bp, (2) positive control, (3) negative control, (4, 5, and 6) is positive samples, 176 bp

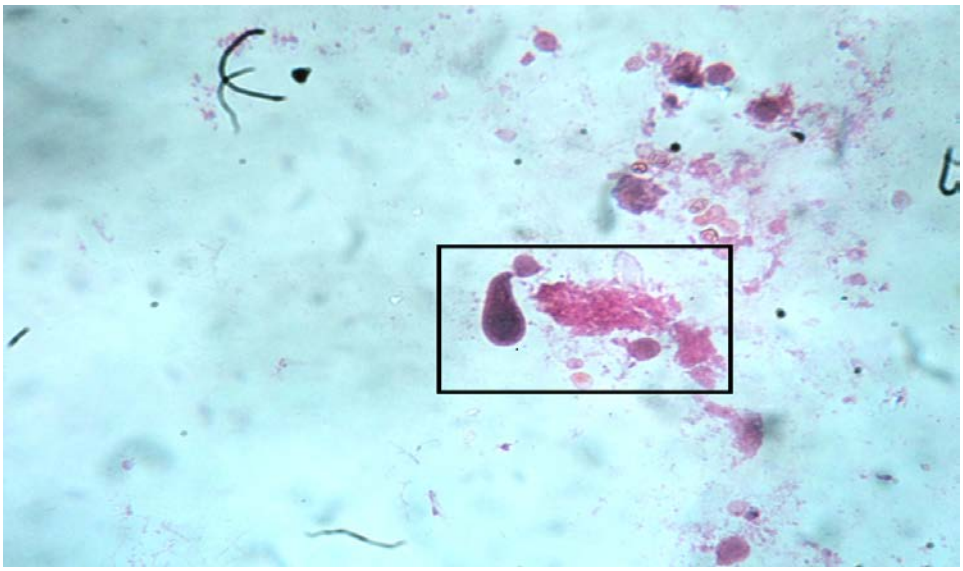

Figure 2. The nuclear inclusions of the decoy cell due to BK polyomavirus infection. Pap stain. X40

Fifty urine samples were collected from control group of apparently healthy adults who did not undergo renal transplantation, no decoy cells were found in cytological smear and 3 specimens were positive for BK viral DNA as shown in (Table 4).

Table 4. Relationship between duration of transplantation and Polyomavirus detection in urine of renal transplant recipients using Cytology technique

Duration of transplantation	BKV urine cytology				Total	
	Negative		Positive			
	Frequency	%	Frequency	%	Frequency	%
<10 years	94	94%	3/97	1%	97	**97%**
>10 years	3	94%	-	-	3	**3%**
Total	**97**	**97%**	**3**	**3%**	**100**	**100%**

Successful sequence of BKV based on T large gene was obtained for six positive PCR urine specimens to confirm the results. BLAST nucleotide search showed that three genes were 100% identity with BKV (ACY78383) from Iran and 99% with other isolated from South Africa (Gen Bank accession number BAF029), and Ethiopia (Gen Bank accession number BAF42908).

Multiple sequence alignment of nucleotides and translated protein showed that isolate 1 was 100% identical with isolates from Japan (BA143619), Kazakhstan (AG75434), Ireland (AE089605) and Iran (ACY78383) (Figure 3). And isolates 3 and 5 are 100% identical with isolates from Japan (BAE94733), Ethiopia (AAR89237) and Australia (CDJ78946) (Figure 4). Protein 3D structure of sequenced genes was showed in Figure 5 and Figure 6.

Phylogenetics analysis showed that the BKV isolated from Sudan is so closed to Iran isolate (Gen Bank accession number ACY78383) (Figure 7).

Figure 3. Amino acid multiple sequence alignment of isolate-1 (BKV) compared to other BKV from database. The alignment was performed using the Clustal W2 sequence alignment

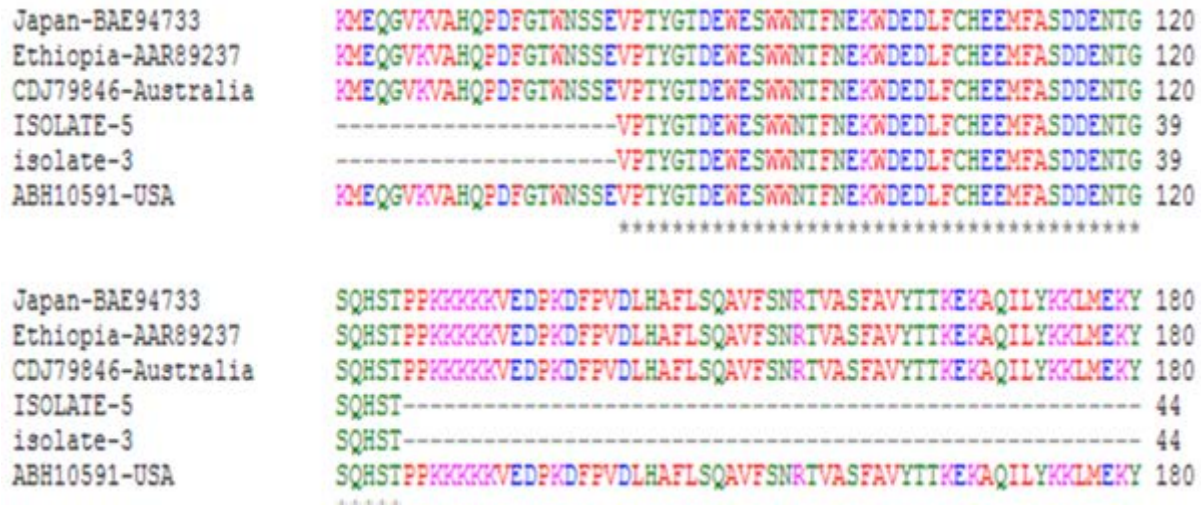

Figure 4. Amino acid multiple sequence alignment of isolate-1 (BKV) compared to other BKV from database. The alignment was performed using the Clustal W2 sequence alignment

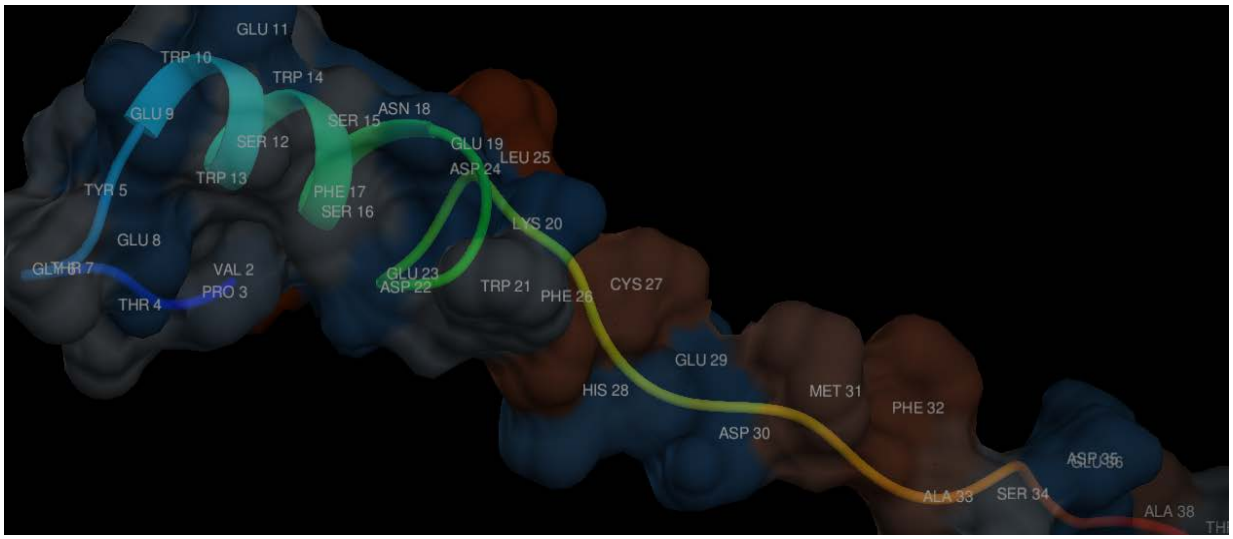

Figure 5. Tertiary protein structure of BKV of Isolate-1. Tertiary protein structure of BKV T.large gene that drawn by Chimera software version 1.9

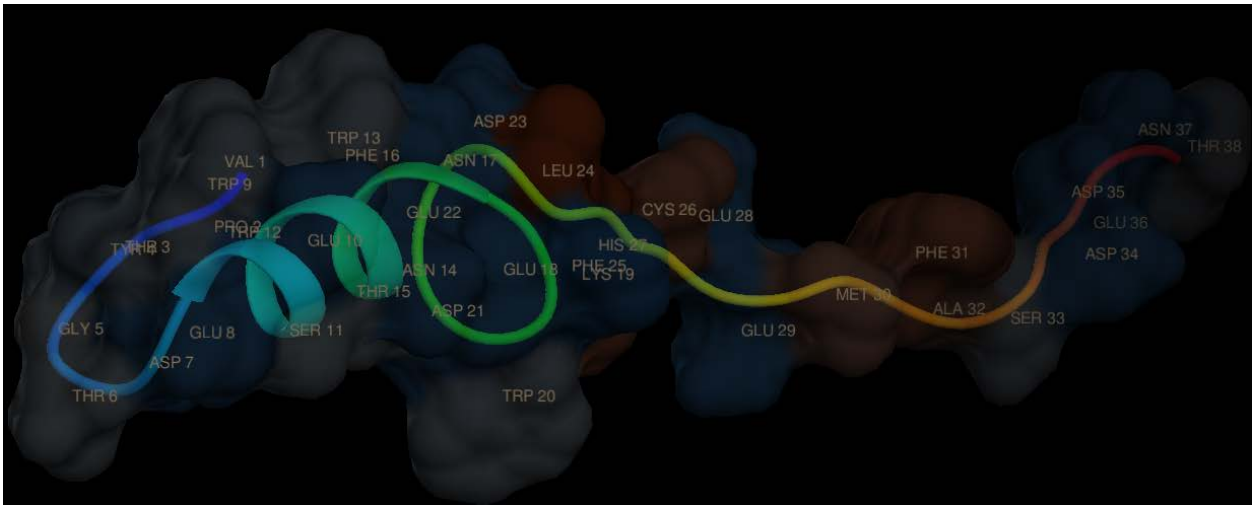

Figure 6. Tertiary protein structure of JCV of Isolate-3. Tertiary protein structure of JCV T.large gene that drawn by Chimera software version 1.9

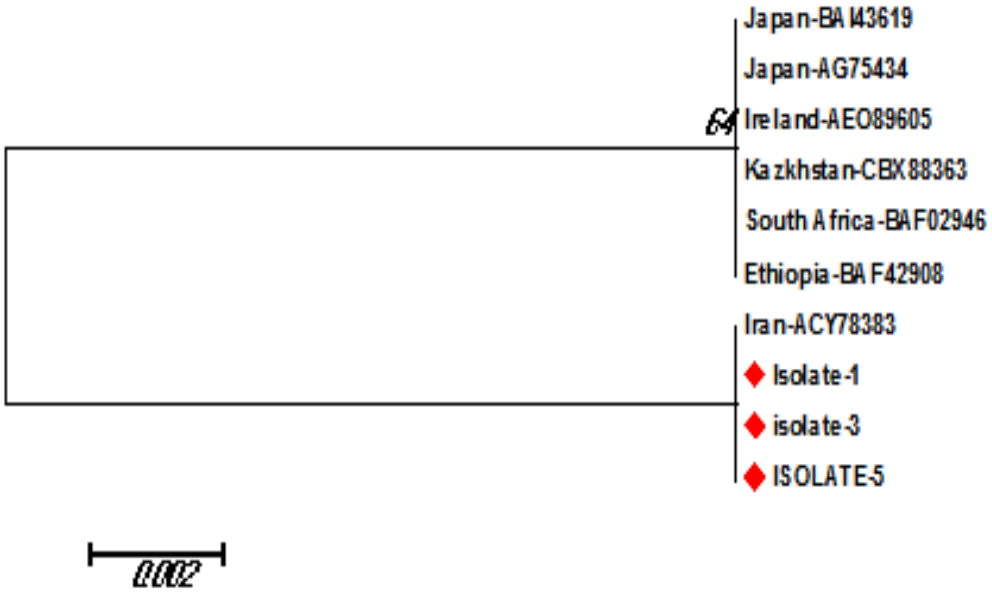

Figure 7. Phylogenic tree of T large gene of BK and JC viruses (strains from Sudan indicated by red square

4. Discussion

There has been limited investigation of BK polyomavirus reactivation in renal transplant recipients with asymptomatic nephritic syndrome. Given the association of BK polyomavirus reactivation with the use of immune suppressive therapies, we sought to investigate whether polyomavirus BK virus reactivation occurred in renal transplant recipients with asymptomatic nephritic syndrome.

Early detection of BKV reactivation in the urine and plasma is a powerful clinical tool for identifying patients at risk for developing BKVN and for monitoring response to therapy.

BK viral infections progress through detectable stages. Urinary viral replication precedes BK viremia by approximately four weeks. Histological changes in BK nephropathy start to be noticeable 12 weeks after BK virus has been shaded in urine. Patients who have undergone renal transplant and have high levels of BK viral DNA in their urine, will be more likely to have high viral plasma DNA levels. The presence of persistent viremia precedes the clinical signs of nephropathy [7].

This study sought to establish the screening diagnostic techniques for early detection of polyomavirus BK virus viruria and viremia and presence of intranuclear viral inclusion bodies (decoy cells) in tubular epithelial cells in urine of renal transplant recipients. The hypothesis was that polyomavirus BK virus viruria is more commonly detected among asymptomatic renal transplant recipients than viremia and urine cytology.

In this study 100 renal transplant recipients were investigated for the presence of BK virus. The BKV were identified in 32/100 urine specimens, 6/100 plasma specimens using PCR. This finding agrees with the study done by Koukoulaki., *et al* (2008), whom diagnosed BKV in 43/50 of urine specimens, and 4/50 in plasma specimens using PCR. The augmented immunosuppressive can lead to continuous viral replication and shedding of the virus in urine, It is important to state that immediately after renal transplantation, there is also clinically silent BKV viruria, preceding the development of BKV nephropathy.

The presence of circulating virus in plasma appeared at late stages, this explain that only 6 specimens were BKV

positive, that associated with active nephropathy, this may be due to the virions enter the circulation through per tubular capillaries following tubular damage. Thus demonstrating BKV DNA in blood could be surrogate diagnostic marker to urine PCR for diagnosis confirmation and further patient's monitoring. The small percentage of plasma PCR positivity, as compared to urine PCR results, shows that limited number of patients could be at potential risk at developing BKV nephritis. It seems that in most of the cases viruria is not linked to distinct pathologic entity, and testing for BKV DNA in blood combined with urine PCR has much higher clinical significance.

Urine cytology is inexpensive and displays a high negative predictive value, but polyomavirus shedding in urine is very common and occurs in 20% to 45% of renal transplant recipients. In our data, urinary decoy cell shedding was positive in 26.2% of allograft recipients. Thus, the predictive characteristics of urinary decoy cell shedding are limited, and distinguishing between the BK and JCV cannot be made based on urine cytology but requires performance of urine PCR. A combination of significant viremia and viruria leads to a diagnosis of presumptive BKVN [12].

The cytology technique applied to 100 urine specimens Decoy cell were identified in 3 urine patients. This is consistent with a previous study done on renal transplant recipient by Elhoweris., *et al* (2011) whom reported five (2.7%) BK polyomavirus infection [13]. Applying the decoy cell screening test requires a trained cytologist and otherwise it will be less sensitive. These explain the low percentage of patients with positive urine cytology when compared to most sensitive and specific PCR.

In this study 100 renal transplant recipients were investigated, the mean age of study group was 40 years. This age group is in similar to previous work of Daniel in USA in 2005, the mean age of his group was 45 years. This may highlight association between this particular age group and renal transplantation.

Little is known about the epidemiology of BK virus in Sudan in particular and in Africa as general. In this study the BK virus was sequenced based on T large gene and the result identified JC virus and BK virus. In my opinion one can probably BKV shows 70 to 75% sequence homology to other polyomaviruses such as JC virus (JCV) [14].

Phylogenetics analysis showed in this study that the BKV belong to and related to several strains worldwide especially from Iran the nearest one to Sudan.

Early diagnosis suggested a reduction of immunosuppressive therapy which then caused a stabilization of nephropathy and an improvement of kidney function. This result encourages us to suggest persevering with this strategy of screening to obtain an early diagnosis of patients at risk. With early diagnosis immunosuppressive therapy is modified, and BKVAN patients monitored. All of these factors contribute to achieve a better outcome from a kidney transplant. Screening is not cheap, but in cases of BKV nephropathies it is justified by the disease that can be contrasted only by an early diagnosis of viruria and viremia which must be monitored to avoid organ loss.

At the moment, the management of BK nephropathy in renal transplant patients follows two main strategies; a screening and prevention strategy and a strategy involving treatment. The former course of action, namely screening and prevention, is aimed at detecting the infection before the nephropathy starts manifesting clinically. This implies that the eventual activation of the BK virus in blood or urine must be monitored periodically, so that intervention is swift in the case of activation. The latter strategy works by reducing the dose of immuno-suppressive medication and administering antiviral agents in patients with diagnosis confirmed through biopsy or patients with a presumed diagnosis of BK nephropathy [7].

Detection of BKV DNA by PCR in urine or decoy cell urine cytology is concurrent as detection of viral replication in renal transplant recipients. And detection of BKV DNA by PCR in plasma specimens is not present in patients with low level/limited viral replication in the urinary tract. Increasing levels of viremia develop only if there is significant tissue damage with progression to BKVN. For this reason, qualitative of BKV viremia has emerged as the most specific non invasive test to confirm BKVN.

BKV screening by PCR assays may be a clinically useful non invasive test for detecting concurrent BKVN in renal allograft recipient.

5. Conclusion

Human polyomavirus BK is related to different clinical manifestations among renal transplant patients. The routine use of PCR on urine and plasma by PCR is a useful tool for the rapid and sensitive detection of reactivated BKV in asymptomatic recipients. Thus, establishing instant diagnosis may be of great value for monitoring the renal recipients, who are at possible risk for the development of BKV nephropathy.

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