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Molecular and cytological detection of BK polyoma virus in kidney failure patients

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Abstract
These days, the kidney transplant community widely agrees that BK virus infections are the main driver behind kidney impairment and transplant failures. A total of 110 kidney failure patients were enrolled in this study. Five ml of blood was collected from each patient, the plasma was separated, and then the plasma samples were stored until DNA extraction was performed. Molecular detection of BK was done by polymerase chain reaction (PCR). Urine samples were also obtained for cytological detection and investigation of decoy cell. The results of the PCR technique for the current study revealed that 12 patients were infected with the BK polyoma virus at a prevalence rate of 10.9 %, with 8 infections in males and 4 infections in females, with a rate of 12.5% and 8.7% respectively. The results of cytological testing and electron microscopy also demonstrated that all 8 urine samples taken from patients infected with the virus contained decoy cells and virus particles. we conclude that chronic hemodialysis could raise the chance of BK virus replication in end stage renal dialysis patients as compared to control group. It was also concluded that chronic hemodialysis may increase the likelihood of BK virus replication in patients with end-stage renal disease compared to the control group.

Keywords: BK virus, kidney failure, real-time PCR, decoy cell

التشخيص الجزيئي والخلوي لفيروس التورامي BK في مرضى الفشل الكلوي

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الخلاصة
هذه الأيام، يتفق مجتمع زراعة الكلى على نطاق واسع على أن عدوى فيروس BK هي العامل الأساسي وراء خلل وظائف الكلى وفشل عملية الزرع. شملت هذه الدراسة 110 عينة من مرضى الفشل الكلوي. جمعت 5 مل من الدم من كل مريض، وفصل البلازما، ومن ثم تم خزن عينات البلازما الى حين إجراء استخلاص الحمض النووي DNA. تم اجراء التشخيص الجزيئي بواسطة تقنية تفاعل البوليمراز المتسلسل (PCR). كذلك

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تم أخذ عينات من البول للتشخيص الخلوي وفحص الخلايا الطعمية. أظهرت نتائج اختبار PCR للدراسة الحالية إصابة 12 مريضا بفيروس التورامي البشري BK بنسبة 10.9%، بواقع 8 إصابات لدى الذكور و4 إصابات لدى الإناث، بنسبة 12.5% و8.7% على التوالي. كما أظهرت نتائج الاختبارات الخلوية والمجهر الإلكتروني أن جميع عينات البول الثمانية المأخوذة من مرضى مصابين بالفيروس تحتوي على خلايا الطعمة وجزيئات الفيروس. استنتجنا أن غسيل الكلى المزمن قد يزيد من معدل تكاثر فيروس BK لدى مرضى غسيل الكلى في المرحلة النهائية مقارنة بمجموعة السيطرة. استنتجنا أيضًا أن غسيل الكلى المزمن قد يزيد من معدل تكاثر فيروس BK في مرضى المصابين بأمراض الكلى في مرحلتها النهائية مقارنة بالمجموعة السيطرة.

1. Introduction

BK virus associated nephropathy is currently well recognized as the important aetiology of renal disorder and graft failure in people who have passed through transplantation. The BK virus is a type of polyomavirus that primarily targets human hosts. Polyomaviruses belong to the Papovaviridae family and are characterized with their small size, absence of a shielding outer layer and possession of a closed, round DNA genome inclusive of two strands. Polyomaviruses may be distinguished from papillomaviruses through their truly smaller viral particle length, as well as their specific genomic size. Polyomaviruses are extensively allotted in the environment and can be obtained from numerous organisms. The isolation of human polyomaviruses passed off in 1971 [1-3]. The list of human polyomaviruses has evolved over the past 2 decades [4]. A common childhood infection caused by BKV rarely has substantial clinical consequences, and >80% of adults have seropositive status for BKV [5][6]. The names BK and JC have been derived from the initials of the patients in whom they were initially detected. The BK virus was remoted from the urine of a kidney transplant recipient who had developed ureteral stenosis following the surgical procedure. The JC virus was isolated from the brain tissue of a patient afflicted with progressive multifocal leukoencephalopathy (PML). The nucleotide sequences of BKV and JCV exhibit a 75% degree of homology. There is no cross-reactivity within the immune reaction among the two viruses, and serologic testing can accurately distinguish among BKV and JCV by detecting specific antibodies [7]. Decoy cells may be identified in urine samples as a way of diagnosing tubular epithelial cells inflamed with BK polyoma virus. The cells display a good morphology and can be easily detected because of their basophilic nucleus that contains viral inclusion bodies [8]. Analyzed Icosahedral BK polyoma virus BKPyV viral aggregates, called "Haufen," in urine samples from renal transplant recipients identified with PVAN by using negative staining electron microscopy. According to the findings, 21 those who had obtained kidney transplants and Patients have been recognized with Polyomavirus-related nephropathy (PVAN) by biopsy and also tested superb for Haufen aggregates in their urine. This indicates a correlation among Haufen and PVAN. The findings suggest that the identity of Haufen has the ability to be hired as a noninvasive screening approach for detecting PVAN in the destiny [9]. Given the limited research conducted on BK virus in Iraq, the current study aimed to determine the molecular and cellular detection of the virus and the extent of its spread in patients with chronic kidney disease.

2. Materials and methods

2.1 study group

The present study included 110 dialysis patients, 64 males and 46 females, of various ages between 15-64 years, who visited the dialysis center in Kirkuk governorate.

2.2 Sample collection

Five ml of venous blood was taken from study patients, the blood was placed in EDTA tubes and subsequently, plasma samples were obtained using the process of centrifugation at a speed of 3000 revolutions per minute for a duration of 5 minutes. Plasma samples were distributed in microcentrifuge tubes and stored at a temperature of (-20 degrees Celsius) until they were used to diagnose the virus. Urine samples were also collected from patients for the purpose of detecting decoy cells as well as for direct diagnosis using a transmission electron microscope.

2.3 Molecular diagnosis:

2.3.1 DNA extraction and gel electrophoresis

After extracting nucleic acid from the plasma using a special extraction kit, the extraction products were then transferred using an gel electrophoresis to ensure the presence of nucleic acid in the product and to ensure that the extraction steps were carried out correctly and accurately, as a defect in the extraction steps leads to the absence of virus nucleic acid in the extraction products. This, in turn, can lead to a misdiagnosis, as real-time PCR results may yield false negatives. This phenomenon is illustrated in Figure 1, highlighting the potential for inaccurate diagnostic outcomes.

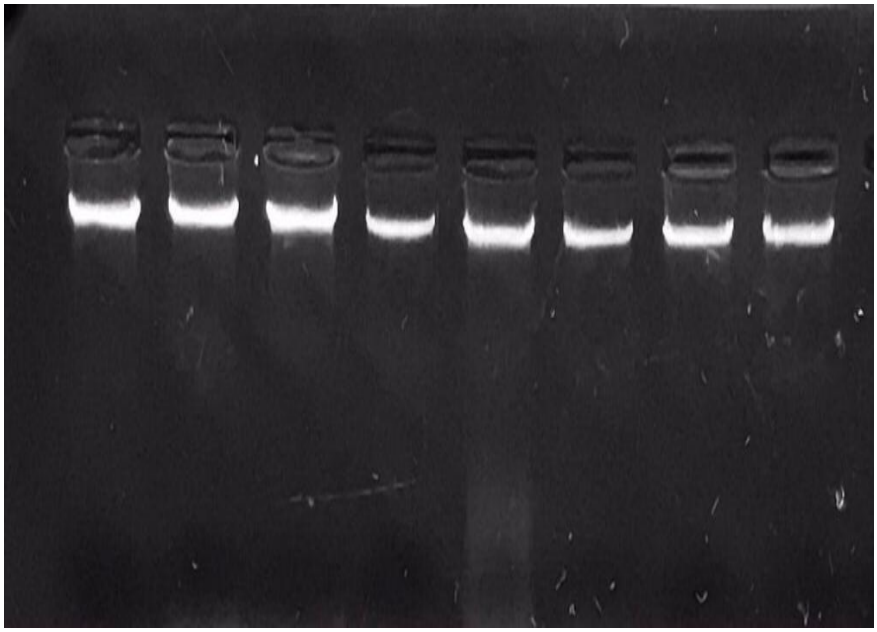


Figure 1: showed DNA extraction products

2.3.2 Detection of BK polyoma virus by Real-time PCR

Following extracting DNA from plasma, primers were designed and supplied from Macrogen laboratories provided primers in a lyophilized state. The lyophilized primers were reconstituted in nuclease-free water to achieve a final concentration of 100 pmol/µl, resulting in the formation of a stock solution. To prepare a working solution of these primers, 10µl of primer stock solution, previously stored at -20 °C, was combined with 90µl of enzyme-free water. This resulted in a functional primer solution with a concentration of 10 picomoles per microliter.

Table 1: Components of primer

Primer	Sequences
Forward primer (20 mer)	**5`-TTTGACAGGAGGGGAAAATG-3`**
Revers primer (20 mer)	**5`-GGCCTCTCCACTGTTGTGTT-3`**
Probe (20 mer) 5`FAM-3`BHQ1	**5`-GTGTGGGGCCTCTTTGTAAA-3`**

Assay procedure

The thermal protocol consists of an initial denaturation step to activate the HotStarTaq DNA Polymerase, followed by a two-step amplification cycle, and concluding with a terminal hold. As shown in table 2. Data is captured in real time throughout the second stage of the amplification cycle

Table 2: Programming the Fluorine Detection System

Step	Degree	Duration	Cycle number
Initial denaturation	95°C	15:00 min.	1
Denaturation	95°C	00:30 min.	
Annealing andelongation (Datacollection)	54°C	01:30 min.	50

Data analysis

At the conclusion of the thermal protocol, the Fluorine Detection System software autonomously establishes the baseline cycles and the threshold. The data received from the set standards is used to plot the standard curve, which shows the relationship between the Threshold Cycle and the Log Starting Quantity.

2.4 Detection of viral particle by transmission electron microscope

The Valentine Method was employed, although the Valentine Method requires more effort, the results are often better when implemented correctly as follows [10].

1- Two sterile microcentrifuge tubes were labeled appropriately for the name of each sample and 1 ml of sterile sample was taken and transferred to a labeled tube.

2- The samples were centrifuged for 10 minutes at ≥14,000×g to pellet the debris and 500 µl of the supernatant was transferred into a second labeled tube. The contents of the second tube were used for the following EM staining steps.

3- A paper towel was placed on the seat and A 6-inch strip of parafilm was cut, the paper backing was removed, and placed on a paper towel (with the paper side facing up).

4- The samples were gently pipetted onto the tube to evenly suspend the particles and 10 µl of sample lysate was applied to Parafilm. Subsequently, 40 µl of diluted TEM solution (5 mM MgSO4) was added to achieve a 5x dilution of urine, followed by gentle mixing by pipetting.

5- Next to each drop of urine, 40 µl of 2% w/v uranyl acetate was placed on the parafilm. Care was taken when pipetting the uranyl acetate solution to avoid spots or droplet formation.

2.5 Detection of decoy cell

The Papanicolaou stain method was used as follows: Five ml of urine was transferred a test tube and centrifuged at 1300 xg for 5 minutes. The slide was allowed to air dry, and the smear was fixed with 95% ethanol for 15 minutes. Harris Hematoxylin stain was applied for 1-3 minutes, followed by a rinse with tap water. The slide was then allowed to air dry and examined under a microscope at 100X using oil

3. Results and discussion

Dialysis patients were divided into four groups according to the history of dialysis, as follows: a group who have been on dialysis since the year and less than one year (≤ 1), a group for more than one year and less than two years (1 < 2), a group of two years or more and equal to or less than three years (2 ≤ 3), and a group of more than three years (≥3 years). The results of the current study indicated that 23 samples, representing 20.9%, fell into the group (≤ 1), while 31 samples, accounting for 28.2%, were categorized in the group (1 < 2). Additionally, 20 samples, or 18.2%, were included in the group (2 ≤ 3), and 36 samples, comprising 32.7%, were classified in the group (3 >). This is illustrated in Figure 1.

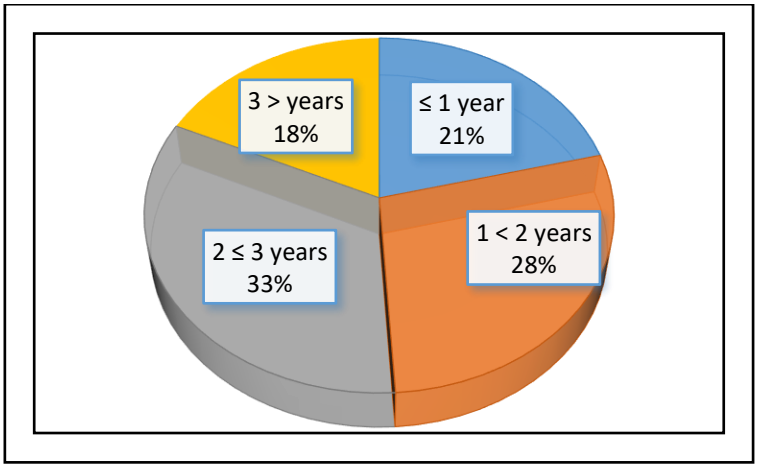


Figure 1: Percentage of samples by dialysis duration.

The samples were further divided up into three groups based on how often the patients who required dialysis per week - once, twice, or three times. The results showed that 40 samples were dialysis once a week at a rate of 36.4%, and 36 samples were dialysis twice a week at a rate of 32.7%, while 34 samples were dialysis three times a week at a rate of 30.9%. As shown in Figure 2.

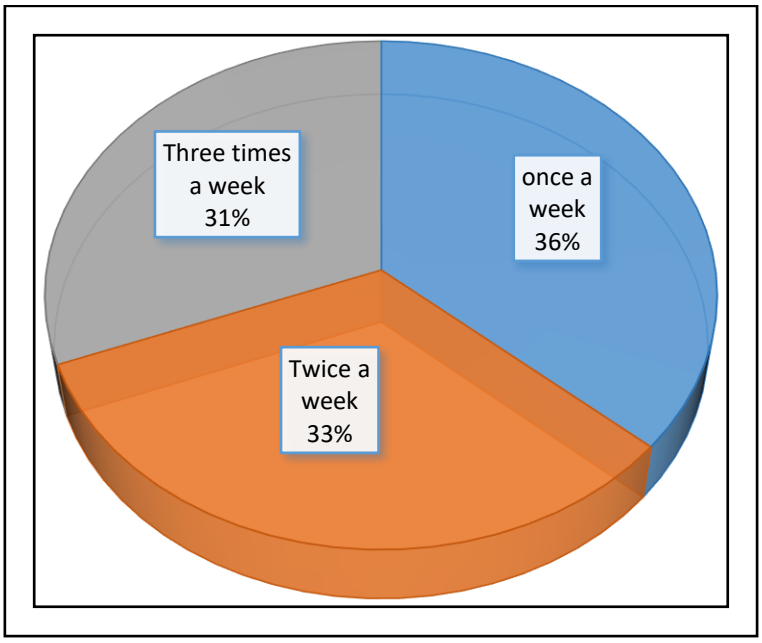


Figure 2: Percentage of samples by dialysis interval.

3.1 Molecular diagnosis:

The results of molecular detection (real-timePCR) as shown in Figure 4, indicated that 12 patients were infected with the BK polyomavirus.

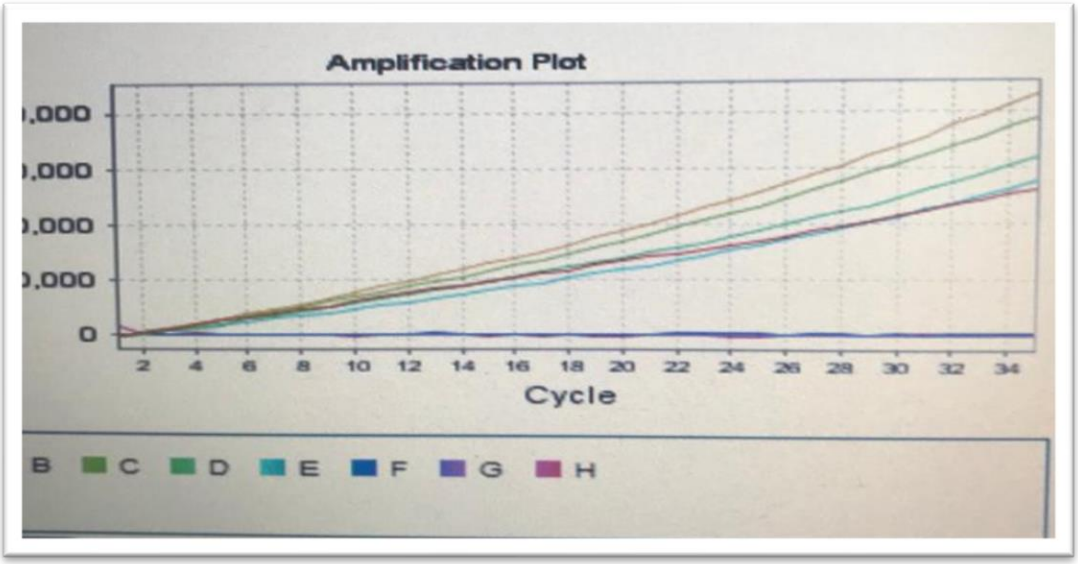


Figure 3: Amplification plot of BK polyoma virus by real-timePCR

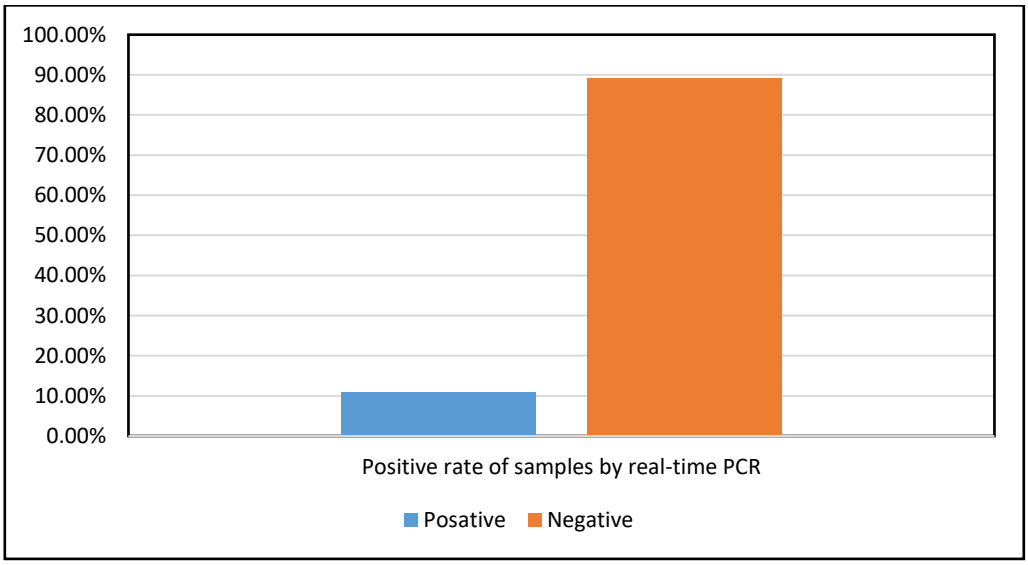


Figure 4: Positive rate of samples by real-time PCR

According to results, 4 out of the infected individuals were female, and 8 out of the infected individuals were male. This indicates that 8.7% of the female population and 12.5% of the male population were contaminated. As shown in table 3.

Table 3: positive rate among study groups by real-time PCR

Sex group	Positive		Negative		Total	
	No	%	No	%	No	%
Males	8	66.7%	56	57.1%	64	58.2%
Females	4	33.3%	42	42.9%	46	41.8%
Total	12	100%	98	100%	110	100%
Chi-Square = 0.399 df = 1 P value = 0.528 P > 0.05 Non-significant (Ns)						

The results of the current study confirmed that chronic hemodialysis could raise the chance of BK virus replication in end stage renal dialysis patients. The origin of BK virus DNA in serum is uncertain, however, it is likely that it arises from either intact viral particles or viral DNA migrating from tubular epithelial cells through exposed basement membranes to peritubular capillaries. Alternatively, it is also plausible that serum BK virus DNA originates from the replication of BK virus in tissues outside of the kidneys. Nevertheless, additional research is necessary to compare the genetic lineages of viruses found in urine and serum samples.

The occurrence rates of polyomavirus infection differ depending on the population studied and the method of detection employed. The current study found a prevalence of infection in patients with CKD of 9.2%, which is less than the prevalence found in a study conducted in the metropolitan region of Belém, involving the general population, where the virus was present in 33% of the samples analyzed [11].

The results of a study conducted in Iraq showed that 51.66% of dialysis patients were infected with the BK polyoma virus [12]. And study conducted in Iran found that 3.3% of dialysis patients had BKV viruses in their plasma [13]. During research conducted in north-east Poland, it was shown that 10.3% of individuals who had received a kidney transplant had BK virus in their blood and urine. The main determinants that had a substantial impact on the incidence of BK viremia and viruria were the post-transplantation length and the specific immunosuppressive drug regimen [14]. Kaneko et al. found that 13.5% of the samples in the control group tested positive for BK polyoma virus, while 24.3% tested positive for JC virus. Within the group of individuals suffering from renal sickness, 33.3% were found to have tested positive for BK virus, whereas an equal number tested positive for JC virus. Kaneko et al. found in 2005 that the occurrence of BK polyoma virus infection in the urine of patients with chronic renal disease (CRD) was much higher than in healthy individuals. [15]. The data in Table 4 shows the 20 to 29 age group had the highest rate of infection for males. For females, the group with the highest incidence was 40 to 49 years old.

Table 4: Positive rate among study groups by real-time PCR technique according to patients' age and sex group.

Age groups	Male positive		Male negative		Female positive		Female negative		Total	
	No	%	No	%	No	%	No	%	No	%
< 20	0	0%	2	3.6%	0	0%	0	0%	2	1.8%
20 – 29	4	50%	6	10.7%	0	0%	6	14.3%	16	14.5%
30 – 39	2	25%	10	17.9%	0	0%	7	16.7%	19	17.3%
40 – 49	1	12.5%	18	32.1%	2	50%	10	23.8%	31	28.2%
50 – 59	1	12.5%	12	21.4%	1	25%	14	33.3%	28	25.5%
≥ 60	0	0%	8	14.3%	1	25%	5	11.9%	14	12.7%
Total	8	100%	56	100%	4	100%	42	100%	110	100%
Chi-Square = 16.478 df = 15 P value = 0.351										
P > 0.05 Non-significant (Ns)										

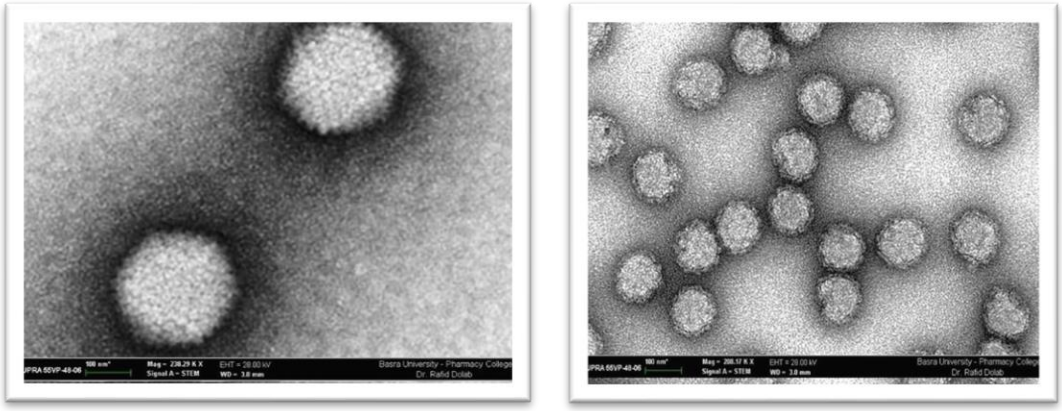
3.2 Direct detection

Transmission electron microscopy (TEM) is a microscopy technique that employs an electron beam to traverse an object and generate an image [16]. Since some dialysis patients were unable to produce urine, we were limited to collecting only 10 urine samples. The

results of the TEM showed that virus particles were visible in 8 samples, at a rate of 80%. The results also showed that in all samples where virus particles were present, nucleic acid was detected in the plasma, and the two samples in which virus particles were not observed gave negative result for real-time PCR, as shown in table 5 and Figure 5.

Table 5: Percentage of Viral particle detected by (TEM)

BKPyV Antibodies	Viral particles				Total	
	Positive		Negative			
	No	%	No	%	No	%
IgM (+) and IgG (-)	6	75%	0	0%	6	60%
IgM (+) and IgG (+)	2	25%	2	100%	4	40%
Total	8	100%	2	100%	10	100%
Chi-Square = 3.750 df = 1 P value = 0.053 P < 0.05 Significant (S)						



(a) **(b)**

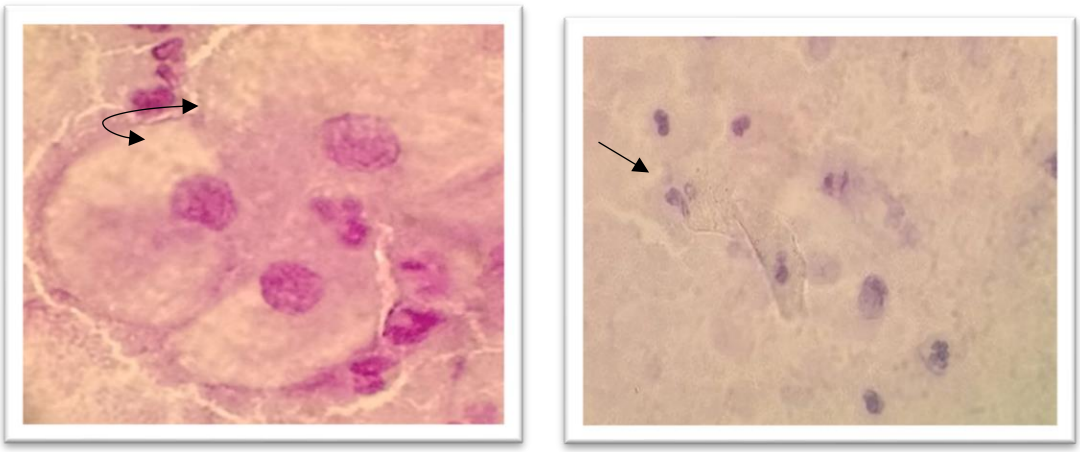
Figure 5: BK polyomavirus particles under the transmission electron microscope (a) *Mag=238.29 kx* (b) *Mag=208.17 kx*

3.3 Detection of decoy cells

The presence of decoy cells can be visualized in a urine sample by employing Papanicolaou staining. The cells frequently have an enlarged nucleus containing a basophilic inclusion, encircled by chromatin that imparts a ground-glass or gelatinous appearance. At times, the nuclear inclusion has a vesicular morphology, characterized by clustered chromatin and a halo encircling it. Cells derived from the urothelium that mimic neoplastic cells exhibit distinctively enlarged and altered nuclei, together with irregularly shaped cell bodies [17]. The findings of the present study revealed that out of 10 urine samples tested for decoy cells, 8 samples (80%) were positive for the presence of decoy cells, while 2 samples (20%) were negative and did not contain detectable decoy cells. It was observed that the 8 positive urine samples were found to harbor viral particles. It was detected by an electron microscope. These eight samples also had virus DNA detected in the serum .While the two negative samples did not detect virus particles by electron microscopy and they also did not detect DNA in the plasma. As shown in table No 6 and figure 6.

Table 6: percentage of Decoy cells detected by Papanicolaou stain

BKPyV Antibodies	Decoy cells				Total	
	Positive		Negative			
	No	%	No	%	No	%
IgM (+) and IgG (-)	6	75%	0	0%	6	60%
IgM (+) and IgG (+)	2	25%	2	100%	4	40%
Total	8	100%	2	100%	10	100%
Chi-Square = 3.750 df = 1 P value = 0.053 P < 0.05 Significant (S)						



(a) **(b)**

Figure 6: Decoy' cells under compound microscope (a) 100X, (b) 40X

Conclusion

This study concluded that 10.9% of dialysis patients were infected with the BK polyoma virus. The study also showed a positive relationship between diagnosing the virus using PCR technology and the presence of virus particles as well as its decoy cells in urine. It demonstrated the efficiency of cytologic diagnosis of the virus from urine samples.

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