ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at: www.jgpt.co.in

RESEARCH ARTICLE

Real Time PCR and Urine Cytology for Detection of BK Human Polyoma virus in-patient with Hemorrhagic Cystitis in Iraq

Faiz Kamel Al-Salami[1*], Ghanim Aboud Al-Mola[2]

1. *Department of Microbiology /College Medicine /University of Kufa*
2. *Department of Biology /College of Science for Women / University of Babylon.*

***Corresponding Author: Faiz Kamel Al-Salami**

Abstract

Hemorrhagic cystitis (HC) is considering one of the important complications in lower UT. Hemorrhage, hematuria, and dysuria are most common symptoms. In hemorrhagic cystitis, short-term hematuria can also be seen in bladder infections a result of viral infection. Viral cystitis represents another form of non-bacterial urinary tract infection touching adult and children. Human polyoma virus BK types I and V, Adenovirus types 21 and 11 and CMV viruses also can be cause of hemorrhagic cystitis. For direct detection BK polyoma virus from the aim of the present study work was to find out the relation of polyoma virus BKVS with acute syndrome consists of hematuria. This study was performed on two hundred and thirty bloody urine and blood specimens were collected from patients group comprised 170 male and 60 female age group 6 -65 years Patient samples (Urine and blood) were collected for detection of Human polyoma virus (BKVS) by using urine cytology, Real time PCR and conventional PCR technique. All bloody urine cultured on ordinary media to differentiate between bacterial and viral infection. The result of present study was found that 154 samples (67%) have positive bacterial culture which excluded and the other 76 samples(33%) give negative bacterial culture were included as suspected a viral cause of hematuria. All negative culture cases was classified as 4(5.26%) patients with glomerulonephritis, 15 (19.73%) chronic renal failure, 14 (18.42%). urinary tract infection, 27(35.5%) kidney transplantation 16 (21.5%) cystitis. urine cytology for detected decony cell (DC) showed that 13(17.1%) samples were positive for BKVS and 87(82.9%) were negative. All urine patients 76 with 25 urines samples of control were analyzed by RT-PCR for detection BVS. It was found only 5(6.67%) as a positive result. In conclusion this study report the design, development and application of real time PCR and urine cytology assay for rapid, specific and highly sensitive detection of BK virus genotype I2b.

Keywords: *Real Time PCR, Urine cytology, BK virus& Haemorrhagic cystitis.*

Introduction

Hematuria and irritative voiding considered featurest symptoms for lower urinary tract hemorrhagic cystitis. viral inflammation is recognized by the entity of distinguishable viral cause with infectious manifestation .Hemorrhagic cystitis occur due to injury of the vessels and bladder's urothelium by microorganism, medication, rays, disorder or poisin.

It is defined by injury and hemorrhage from the urothelium, which lead to hematouria and dysuria as a consequence for certain radiotherapy drugs typically cyclophosphamide or ifosfamide cause damage to the bladder, a clotting disorder that leads to bleeding. It can also be a late effect occurring years after therapy of pelvic radiation [1, 2].

Hemorrhagic cystitis is recognizing by lower urinary tract symptoms that include hematuria and irritative voiding symptoms. Viral infection is defined as a presence of an identifiable viral cause with inflammatory symptoms. It results from damage to the bladder's transitional epithelium and blood vessels by toxins, pathogens, radiation, drugs, or disease [3, 4].

Hemorrhagic cystitis is inflammation and bleeding from the lining of the bladder, which results blood in the urine and pain with urination. It can occur when certain

chemotherapy medications typically cyclophosphamide or ifosfamide cause damage to the bladder, a clotting disorder that leads to bleeding. It can also be a late effect occurring years after therapy of pelvic radiation [5, 6]. The symptoms of lower urinary tract infection include hematuria, genital or lower abdominal pain, urgency, frequency (secondary to inflammatory response and irritation of the bladder wall), pyuria, and hematospermia.

In rare instances of prostatic abscess, the obstructive voiding symptom and urinary retention can be found [7]. Small infectious agent cause cystitis considered second type of urinary tract infectious agents rather than bacteria which affecting adult and young. Polyoma virus BK, Cytomegalovirus, and herpes simplex viruses and other viruses. The immunosuppressed patient submitted transplant of organ are mostly liable to infectious bladder by BK polyoma virus [8, 9].The diagnosis of HC is based on the clinical history, physical examination and the exclusion of alternative causes of painful hematuria.

Its manifestations vary from microscopic hematuria (mild) to severe bladder hemorrhage leading to clot retention and urinary tract obstruction [10, 11]. Hemorrhagic cystitis is a serious complication and the presence of blood in the urine in patients receiving these medications requires prompt treatment. Your care team will take steps during treatment with these medications to protect the bladder [12, 13].

Lower urinary tract infections (UTIs) are common among the general population and are most often caused by bacterial pathogens. Viruses are an uncommon cause of UTIs in an immune competent host; however, viruses are increasingly recognized as the cause of lower UTI, especially hemorrhagic cystitis, among immunocompromised patients. BK virus, adenovirus, and cytomegalovirus are predominant pathogens involved in hemorrhagic cystitis after stem cell and solid organ transplantation [14, 15].

Diagnosis of viral UTI is more challenging because viruses are small organisms, and they cannot be visualized with even the best optical microscope. The culture of viruses may take up to 14 to 28 days, and often it is too late to treat a patient with disseminated multi organ viral infections at that time thus, molecular and immunofluorescence techniques are used more commonly[16].

Patients

This study was performed on two hundred thirty bloody urine and blood specimens were collected from 230 selected patients to eliminate other causes of hematuria depending on the diagnosis and opinion the Urologist doctor, the patients group comprised 170 male and 60 female. The age of the patients were ranged between 6 to 65 years, who attending to different hospitals in Baghdad and Al-najaf governorate include Baghdad Medical city Teaching Hospital, Al Sadr Teaching Hospital, during period between March 2016 to February 2017.

Questionnaires were used to obtain information from the patients itself and parents or guardians accompanying the patients to hospital .Information included signs and symptoms of illness (hematuria slight pink to frank bright red blood with or without blood clots, dysuria (painful urination), burning with urination. Urinary frequency, urinary urgency, urinary incontinence (involuntary loss of urine, etc.) exclusion and induction criteria

Collection of Samples

Two hundred thirty urine samples varied in volume from 25 ml to 60 ml per patient were collected in disposable sterile containers and centrifuged at 3500 rpm for 30 minutes then examined under light microscope to determination grade of hematuria (mild, moderate and sever) and divided to three parts and distributed in Eppendorf tubes to perform bacterial culture by Inoculation urine samples on to culture media (blood agar and MacConkey agar). Than incubated at 37C for 48 hours. After that, the negative bacterial culture urine samples were used Urine for cytology detecting decony cell in urine patients and control and molecular assay for detecting BK Human polyoma virus in urine patients and control by RT-PCR.

Papanicolaou Stain for Detect Decony Cell in Urine

Papanicolaou stain for detect decony cell in urine includes both acidic and basic dyes. Acidic dye stains the basic components of the cell and basic dye stain the acidic components of the cell.

The polychromatic PAP stain involves five dyes in three solutions. Hematoxylin, Orange Green 6 and Eosin Azure. Infected cells can vary from few to abundant, and they occur as solitary cells rather than as cell clusters.

Viral DNA Extraction

Viral DNA was extracted from frozen urine samples by using Accuprep ®Genomic DNA extraction kit (Bioneer. Korea) .Transferred 200µl of urine to sterile 1.5ml micro centrifuge tube and done according to the instruction of the manufacture company .The purified DNA was eluted in 100 ul elution buffer provided with kit and store at -20℃. The extracted DNA was checked by using Nano drop spectrophotometer, and then used for preparation of Real –Time PCR (RT-PCR) master mix reaction.

Real-Time PCR

Real Time PCR was performed for detection of Human polyoma virus (BKV) DNA from urine samples by using specific qPCR kit for BK virus and this technique was carried out according to method described by [17].

Real-Time PCR Master Mix Preparation

Real-Time PCR master mix was prepared by using kit (Life River. China) and done according to the company instruction as in the following Table (1).

Table 1: RT-PCR master mix components:

q PCR Master mix	**Volume**
PCR Enzyme Mix	**0.4** µl
PBK Reaction mix	**35** µl
Extraction DNA	**4** µl
Internal control	**1** µl
Total	**40.4** µl

Real-Time PCR Data Analysis

q PCR date analysis was performed by calculation the threshold cycle number (CT value) that presented the positive amplification of Human polyom virus BK VP1 gene in Real-Time PCR cycle number

PCR Master Mix Preparation

PCR master mix was prepared by using (AccuPower PCR Pre Mix Kit) and this master mix done according to company instructions as following Table(2):

Table 2: PCR master mix preparation

PCR master mix	**Volume**
DNA template	5 µl
PCR water	13 µl
VP1 & agno gene Reverse primer(10 p mol)	1 µl
VP1& agno gene Forward primer(10pmol)	1 µl
Total volume	20 µl

After that, these PCR master mix component that mentioned above placed in standard AccuPower PCR Pre Mix kit that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0,KCl, MgCl2,stabilizer, and tracking dye). Then, all the PCR tubes transferred into Multi spin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR thermo cycler (Mygene. Bioneer. Korea).

PCR Thermo cycler Conditions

Polymerase Chain Reaction thermo cycler conditions by using conventional gradient PCR thermocycler system as following Table (3):

Table 3: PCR thermocycler conditions

PCR step	**Tem**	**Time**	**Repeat cycle**
Initial	**95** °C	**4 mint**	**1**
Denaturation	**95** °C	**30 sec**	**40**
Annealing	**44** °C	**30 sec**	
Extension	**72** °C	**45 sec**	
Final extension	**72** °C	**10 mint**	**1**
Hold	**4** °C	**Forever**	_
Total	**42 cycle**		

Results

Two hundred and thirty bloody urine specimens were collected from patients and subjected for culturing on blood agar and MacConkey agar. After incubation the samples at 37 ºC for 24-48 hour, it was found that 154 samples (67%) give positive bacterial culture were eliminate from the study samples, and 76 samples (33%) give negative bacterial culture (No. growth) which might belong to other causes .These samples were recorded in the study as a viral cause of hematuria for detection of BK infection in figure (2). All negative culture cases were diagnosed by urologist physician and classified as: 4(5.26%) patients underwent glomerulonephritis, 15(19.73%) chronic renal failure, 14(18.42%) urinary tract infection, 27(35.52%) kidney transplantation and 16 (21.5%) cystitis. The distribution of these study groups are listed in Table 4.

Table 4: Patients of hematuria according to the study groups

Study group	N0 of patients	Total Percentage %	NO &Percentage of positive BK infection
Glomerulonephritis	**4**	**5.26**	**1 (1.3)**
Chronic renal failure	**15**	**19.73**	**1(1.3)**
Urinary tract infection	**14**	**18.42**	**3(3.9)**
Kidney transplantation	**27**	**35.52**	**5(6.5)**
Cystitis	**16**	**21.5**	**3(3.9)**

Based on rapid test (decony cells) in urine. All negative bacterial culture specimens taken from 76 patients with hemorrhagic cystitis shows 13(17%) positive and 63(87%) negative for BKVS infection suspected other nonbacterial causes of hematuria, as shown in Figure(1) decony cells (A,B,C,D).

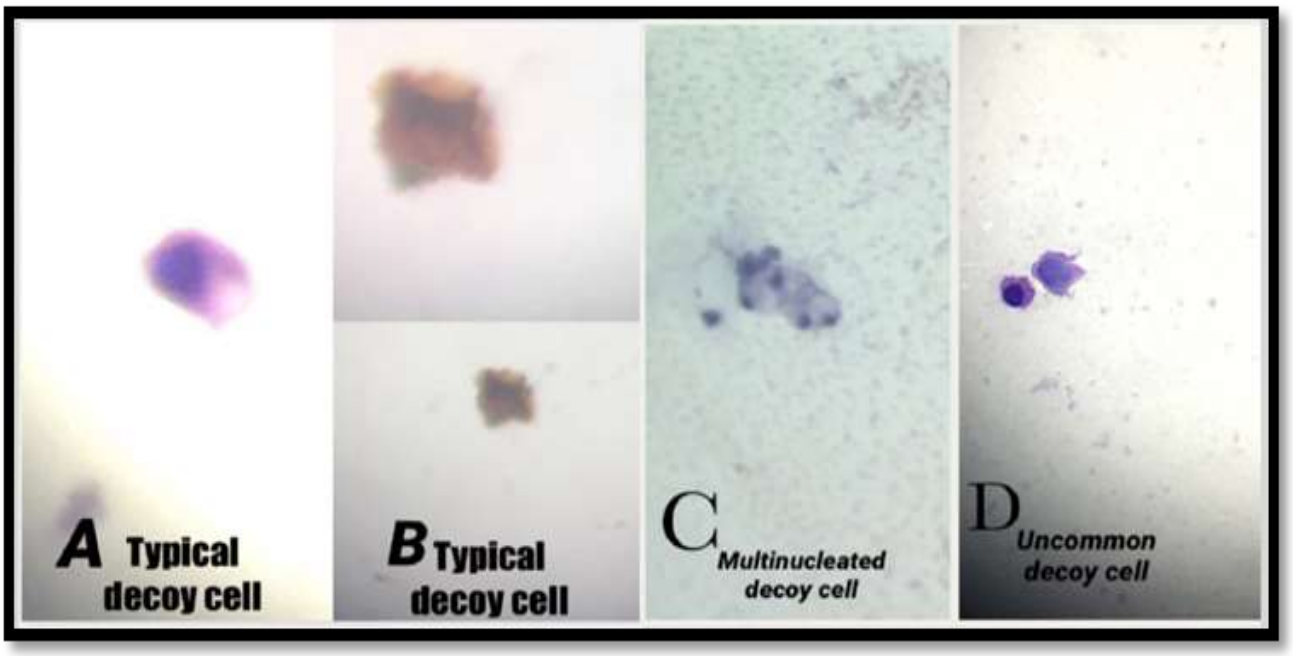

Figure 1: types of decony cells in urine

RT-PCR technique was done by using forward vp1 primer (AAATGGGGGATCCAGATGAT) and revers primer (GGAACATTTTCCCCTCCTGT) were used for the detection of BKV in this studied was based on the fluorogenic 5 nuclease assay during the PCR reaction. This cleavage results in the fluorescent signal generated by the cleaved reporter dye which is monitored Real time PCR by the PCR detection system via using the complete sequence of Human polyoma virus VP1 gene (Gen Bank: KY488564, KY488565, KY488566, KY488567) from NCBI Gene-Bank data base and Primer online and provided through (Bioneer company, Korea).To confirm further presence of BKV and to exclude the possible contamination with other viruses. All urine patients 76 suffering hematuria with 25 urine samples of control (no hematuria) were analyzed by RT-PCR for detection BKVS .It was found that 5(6.6%) positive result from 76 total patients suffering hematuria. Figure (2), (3) and Table (5), (6).

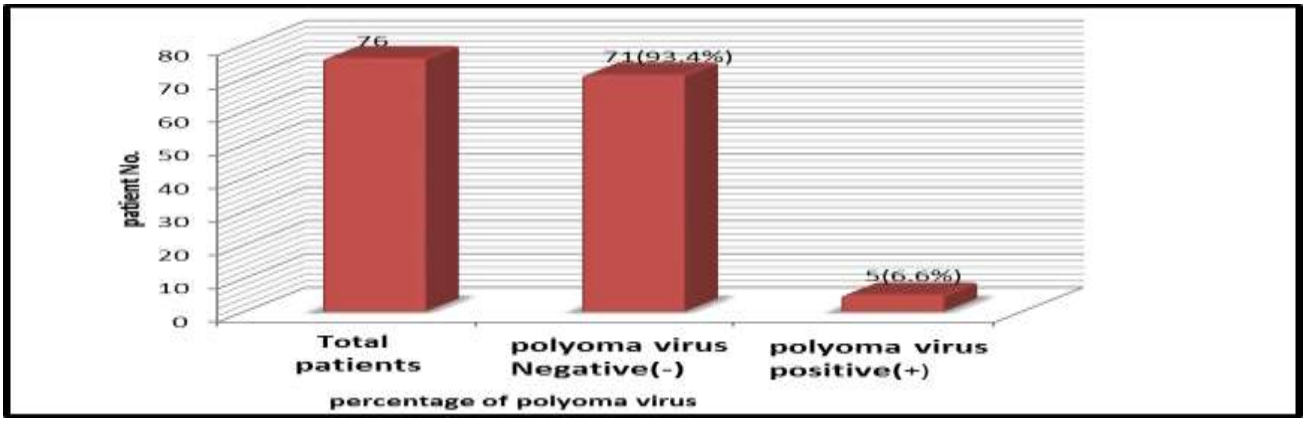

Figure 2: percentage of HPV (Bk) by using RT-PCR technique in hematuria patients

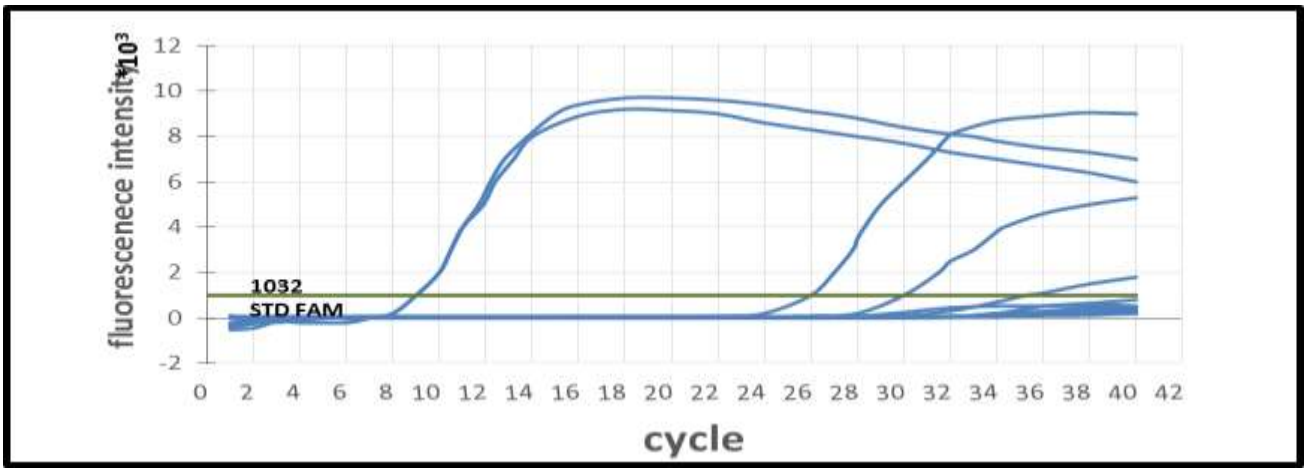

Figure 3: Real-Time PCR amplification plot for VP1 gene in polyoma virus positive and negative hematuria patient samples by using Taq Man probe (FAM dye) reaction

Table 5: Real-Time PCR threshold cycle (CT) of polyoma virus in positive and control sample. Where, positive samples CT: 9.05 -35.54 and NA: Non-amplification as control samples.

Well	Fluor	Target	Content	Sample	C q	C q Mean	C q std. Dev
A1	FAM	VP1	Unkn	Human polyoma virus	25.95	25.95	0
A5	FAM	Agno	Unkn	Human polyoma virus	9.08	9.08	0
A6	FAM	Agno	Unkn	Human polyoma virus	9.05	9.05	0
A8	FAM	VP1	Unkn	Human polyoma virus	30.11	30.11	0
B2	FAM	VP1	Unkn	Human polyoma virus	35.54	35.54	0
C3	FAM	VP1	NTC	Human polyoma virus	0.00	0.00	0
D3	FAM	VP1	Neg Ctrl	Human polyoma virus	0.00	0.00	0

Table 6: Real-Time PCR endpoint analysis for Human polyoma BK virus in positive hematuria patient samples. Where unkn: unknown patient samples, NTC: non-template control and Neg Ctrl: negative control

Well	Fluor	Content	Sample	End RFU	Call
A1	FAM	unkn	Human polyoma virus	$9.7*10^3$	(+)positive
A5	FAM	unkn	Human polyoma virus	$9.2*10^3$	(+)positive
A6	FAM	unkn	Human polyoma virus	$9.05*10^3$	(+)positive
A8	FAM	unkn	Human polyoma virus	$5.3*10^3$	(+)positive
B2	FAM	unkn	Human polyoma virus	$1.8*10^3$	(+)positive
C3	FAM	NTC	Human polyoma virus	$0.520*10^3$	
D3	FAM	Neg Ctrl	Human polyoma virus	$0.270*10^3$	

Discussion

The urine cytology for detected decony cells one the early identification of BKV infection. Also it agreement with the study done by [18].Who stated that the activation and replication of polyoma virus was detected by identification of decony cell, and it has high sensitivity [19]. Demonstrated a highly significant association between BKV positivity and decony cells positivity in pap-stained urine cytology smear.

The (decoy cells) are easily identified and measured by Pap dye so the detection of it considered morphologic sign for the reactivation of BKVs. Four type of decony cell can be identified rapidly for diagnosis of BKVs in patient with Hemorrhagic cystitis because that HBKVs infection is difficult to diagnose clinically alone. Decoy cells often contain polyoma-BK-viruses.

The activation and replication of polyoma virus was detected by identification of decony cell, and it has high sensitivity [19]. Demonstrated a highly significant association between BKV positivity and decony cells positivity in pap-stained urine cytology smear. The (decoy cells) are easily identified and measured by Pap dye so the detection of it considered morphologic sign for the reactivation of BKVs. Four type of decony cell can be identified rapidly for diagnosis of BKVs in patient with Hemorrhagic cystitis because that HBKVs infection is difficult to diagnose clinically alone [20].

The RT-PCR need effected and equitable technique. The inhibition of amplification of the nucleic acid considered the major limit step for PCR-based tests which happened through substances in specimen [21].PCR technique developed to diagnose all genotype & subgroup of BKVs in short period. Due to its high sensitivity. By comparing to other method (Culture), require active virus and long duration to cultivation that may reach to 6weeks. Mention by [16].In the present study ,all negative bacterial culture growth 76 specimen bloody urine and 25 control were prepared for analyzed by PCR for detection BKVS. The results of present study showed that, a well characterized done by [8].

Who compare the sensitivity of PCR for purified DNA of urine sample which is increased markedly while urine sample without extraction have lower sensitivity due to presence of inhibitory substances which lead to false negative so new method of PCR developed to control the large concentration of these inhibitors [22, 23].The value of RT-PCR used as an indicator for viral load in urine. All patients with positive PCR for human polyoma virus had mainly hematuria symptoms with hemorrhagic cystitis ranging from mild to severe hematuria.

References

1. Heim A, Ebnet C, Harste G, Pring-Akerblom P (2003) Rapid and quantitative detection of human adenovirus DNA by real-time PCR. J .Med. Virol., 70(2):228-239.
2. Hofl CA, Eron LJ, Washecka RM (2004) Hemorrhagic Adenovirus cystitis after Srenal transplantation. Transplant Proc., 36: 3025-3027.
3. Alkan I, Jeschke S, Leeb K, Albquami N, Janetschek G (2006) Treatment of radiation-induced hemorrhagic cystitis with laparoscopic cystoprostatectomy. Urol. Int., 77 (2): 190-2.
4. Egli A, Infanti L, Dumoulin A (2009) Prevalence of polyomavirus BK and JC infection and replication in 400 healthy blood donors. J. Infect Dis., 199: 837-846. Polo C., Perez J8.
5. Hoffman NG, Cook L, Atienza EE, Limaye AP, Jerome KR (2008) Marked variability of BK virus load measurement using quantitative real-time PCR among commonly used assays. J. Clin Microbiol., 46: 2671-2680.
6. Doerries K (2006) Human polyomavirus JC and BK persistent infection. Adv. Exp. Med. Biol., 577: 102-16.
7. Jeffers LK, Madden V, Webster-Cyriaque J (2009) BK virus has tropism for human salivary gland cells in vitro: implications for transmission. Virology, 394: 183-193.
8. Robinson C, Echavarria M, Murray PR, Baron EJ, Jorgensen J, Pfaller M, Landry ML (2007) Adenoviruses. (Eds.), Manual of Clinical Microbiology, (9th ed., 1589).
9. Miano M, Faraci M, Dini G Bordigoni P (2008) Early complications following

haematopoietic SCT in children. Bone Marrow Transplant, 41(2): S39-S42. PubMed: 18545243

10. Anoop P, Shaw BE, Riley U (2001) Clinical profile and outcome of urotheliotropic viral hemorrhagic cystitis following hematopoietic stem cell transplantation: A 7-year tertiary center analysis. Hematolog., 16: 213-220.
11. Chakrabarti S, Mautner V, Osman H, Collingham KE, Fegan CD, Klapper PE, Moss PA, Milligan DW (2002) Adenovirus infections following allogeneic stem cell transplantation: incidence and outcome in relation to graft manipulation, immunosuppression, and immune recovery. Blood, 100(5):1619-1627.
12. Astegiano S, Bergallo M, Terlizzi ME (2011) Combined measurement of serum DNA and urine VP1 messenger RNA in monitoring BK virus replication in kidney graft recipients. Transplant Proc., 43: 1052-1054.
13. Avidor Y, Nadu A, Matzkin H (2000) Clinical significance of gross hematuria and its evaluation in patients receiving anticoagulant and aspirin treatment. Urology, 55(1):22-4.
14. Randhawa PS, Khaleel-Ur-Rehman K, Swalsky PA, Vats A, Scantlebury V, Shapiro R, Finkelstein S (2002) DNA sequencing of viral capsid protein VP-1 region in patients with BK virus interstitial nephritis. Transplantation, 73: 1090-4.
15. Kurosaki K, Miwa N, Yoshida Y (2004) Therapeutic basis of vidarabine on Adenovirus-induced hemorrhagic cystitis. Antivir. Chem.
16. Tremolada S, Akan S, Otte J et al (2010) Rare subtypes of BK virus are viable and frequently detected in renal transplant recipients with BK virus-associated nephropathy. Virology, 404: 312-8.
17. Andriole VT (1994) Fleroxacin the newly introduced once-daily quinoline Int. J. Antimicrob. Agents, 4 (2):S1-12 S6 2011.
18. Singh HK, Reisner H, Derebail VK, Kozlowski T, Nickeleit V et al (2015) Polyomavirus nephropathy: quantitative urinary polyomavirus-Haufen testing accurately predicts the degree of intrarenal viral disease. Transplantation, 99: 609-615.
19. Mori Y, Miyamoto T, Kato K (2012) Different risk factors related to Adenovirus or BK virus-associated hemorrhagic cystitis following allogeneicstem cell transplantation. Biol. Blood Marrow Transplant, 18: 458-465.
20. Boldorini R, Brustia M, Veggiani C (2005) Periodic assessment of urine and serum by cytology and molecular biology as a diagnostic tool for BK virus nephropathy in renal transplant patients Acta Cytol., 49: 235-243.
21. Bergallo M, Costa C, Gribaudo G (2006) Evaluation of six methods for extraction and purification of viral DNA from urine and serum samples New Microbiol., 29: 111-119.
22. Hirsch HH, Steiger J (2003) Polyomavirus BK. Lancet Infect Dis., 3: 611-23
23. Shimizu H, Mitamura K, Okuyama Y Hirai, Ushijima H (2001).

