Diyala Journal for Veterinary sciences
Open Access Journal Published by College of Veterinary Medicine
University of Diyala, Iraq
P-ISSN: 2410-8863
E-ISSN:2958-6178
Vol. 2, NO. 2, June 2024

Genotyping Of Polyomavirus BK For VP1 Gene Using Gene Specific Phylogenetic Trees -Among Nephropathy Patients In Diyala Governorate

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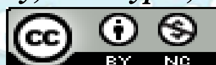
Abstract

Background BK virus-associated nephropathy (BKVAN) is an important reason for missing graft in kidney transplants
Aims This paper emphasizes exploring the genetic polymorphisms of two BK polyomavirus samples isolated from the blood of patients with nephropathy. **Methods A total of 170** blood plasma samples were collected from (130) nephropathy patients and (40) healthy blood donors. All participants were subjected to serological testing using ELISA to detect antiBKV IgM in the blood. positive antiBKV IgM samples were used for the detection of BKV DNA By RT-PCR. Two samples with the highest purity and concentration of DNA, were used to determine the genetic polymorphisms of two BK polyomavirus-.
Results: Only 20/130,(15.38%) specimens of nephropathy patients have positive BKV IgM and all healthy group was negative. A total of (40%) was positive in RT-PCR for BKVP1gene and BKtyp4 primer. The genetic polymorphisms was found that the investigated samples were clearly incorporated within the clade of serovar IV in close phylogenetic positions to many strains isolated from Kuwait with no phylogenetic distances among them.
Conclusions
BK polyomavirus-VPI amplicons could be used as markers to differentiate between BK viruses. This study emphasizes the significance of genetic variation in comprehending the molecular epidemiology and evolutionary dynamics of BK polyomavirus, which may affect the phylogenetic framework and geographical tracking of the virus currently under investigation.
Keywords: ***BK polyomavirus, Nephropathy, Genotypes, RT-PCR***



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Introduction

Nephropathy is described as problems with the structure or function of the kidney, regardless of the underlying cause or specific clinical manifestation. A grading system based on Glomerular Filtration Rate (GFR) has been developed. Chronic kidney

disease (CKD) often begins with primary kidney damage and advances through the phases to kidney failure. People are becoming more at risk of dying from cardiovascular disease (CVD) as a result of this trend [1]. Hemorrhagic cystitis in recipients of bone marrow and stem cell transplants, BK virus-associated nephropathy (BKVAN), and ureteral stenosis in patients of kidney transplants are among the clinical issues brought on by the BK virus (BKV). BK virus-associated nephropathy (BKVAN) is a significant cause of kidney transplant graft loss [2]. BKV is a small virus with a diameter of 40-44 nm and an icosahedral symmetry. Squirrel monkey renal peritubular capillary endothelial cells expressed the BKV T antigen. On the other hand, SV40 (SV40 is an abbreviation for simian vacuolating virus 40 or simian virus 40, a polyomavirus that is found in both monkeys and humans) was found in very few tissues and at very low copy counts; it was not found in blood. Polyomaviruses have a significant host limitation. Although BKV can cause tumors in rodents (such as hamsters), the virus cannot replicate in rodent cells[3]. BKV is not covered. Its genome includes about 5300 base pairs and is made up of circular, double strand DNA [4,5]. The non- coding region (NCCR), along with the early and late coding regions. The genes encoding the two non-structural proteins, the big (Tag) and small (tAg) tumor antigens, are located in the early coding region [6]. Following the initiation of viral genome replication, transcription of the late coding region occurs. It codes for a non-structural agnoprotein as well as the three viral capsid proteins, VP1, VP2, and VP3. Major capsid protein VP1 is made up of five outer loops that connect the different β-strands of the polypeptide: BC, DE, EF, GH, and HI [7]. Between the early and late regions is a hypervariable non-coding control region (NCCR), which is also referred to as a regulatory region. The NCCR includes regulatory sequences within promotors and enhancers, binding sites for T antigen and cellular transcription factors, the origin of DNA replication (ori), and other cis-acting regions [8,9]. Both the early and late regions of gene expression are regulated by transcription factors [7]. Single nucleotide polymorphisms (SNPs) in the viral protein VP1 region and the non-coding control region (NCCR) have been utilized for dividing the virus into genotypes and subtypes [10]. Jin *et al*., (1993) reported the initial genotype classification of strains, which was predicated on the genotype-specific sequence and restriction sites within the genes variable region producing the VP1 protein [11,3]. All BKPyV genotypes have very high similarity (above 95%) in the VP1 coding region, although only 61–70% similarity exists among the amino acid residues from 61E to 83R [12,6]. About 80% of BKV cases that have been reported worldwide are associated with genotype I, with genotype IV accounting for 15% of cases. Genotypes II and III are the least common. The genesis region of the virus is linked to the dispersion of its molecular variations. The most prevalent variant in Africa is subtype Ia, while Ib-1 is widely distributed in Southeast-Asia, Ib-2 in Europe, and Ic is the most prevalent form in Northeast- Asia [8,13]. Thus, the subtype of

the virus found in a patient can reveal details about the ethnic background of the person from whom the sample was obtained[7,8]. Evidence of BKV infection in children's tonsils and respiratory tracts supports the theory that the upper respiratory route is the main route of transmission [4]. According to several theories, BKV enters the bloodstream through the tissue of the infected tonsils, infecting peripheral blood mononuclear cells and subsequently spreading the virus to other infection sites, such as the kidney [6,14]. With an estimated 6 billion people continually infected worldwide, there is a significant infectious burden, but the route of transmission is yet unknown[15]. Exposure to BKV during early childhood can lead to lifetime infection; research has shown that 60–100% of kids were anti-BKV IgG seropositive by the time they turned ten years old [8]. Among patients, BK virus - associated nephropathy (BKVAN) varies from 1 to 15% [16]. These examples of BK virus instances imply that the clinical consequences linked with BK virus reactivations will depend on condition-specific interactions between host and viral components [6]. Clinically significant BKVAN is experienced by a significant fraction of kidney transplant recipients, viruria occurs in 30–60%, viremia in 10–30%, and BKVAN occurs in up to 10% of cases. [17,18]. After a diagnosis of BKVAN, graft loss rates have been reported to be as high as 30–50% [7]. This paper emphasizes exploring the genetic polymorphisms of two BK polyomavirus samples isolated from the blood of patients with nephropathy.

Materials and Methods

Patients

The current study was conducted in Diyala province from October 2023 to January 2024. samples were taken from the Ibn Sina Dialysis Center, the Kidney and Urology Department affiliated with the Consulting Clinic, the Oncology Center, the Urology Clinic and the main blood bank / Baqubah Teaching Hospital, and outpatient clinics specializing in kidneys and urinary tracts. One hundred seventy blood plasma samples were obtained from 40 healthy blood donors and 130 patients with nephropathy after obtaining approval according to administrative order numbered (51043) dated 25/09/2023.

Method:

Blood samples were obtained as follows: Using a sterile syringe, 3 to 5 ml of venous blood was first drawn from the bend of the arm or the back of the hand. The blood was then injected into a sterile collecting tube that had been loaded with EDTA (ethylene diamine tetraacetic acid). The tubes were left at room temperature for a maximum of four hours, or the blood was centrifuged for five minutes at 1600 rpm, in order to extract the plasma. The obtaining plasma was stored at -80 °C after being placed into a 1.5 ml Eppendorf. All participants were subjected to serological testing using the enzyme-linked immunosorbent technique (ELISA) to detect the antiBKV IgM in the blood, than all the plasma samples with positive antiBKV IgM used for the detection of BKV DNA by RT-PCR, using the primer of BKVP1 region(detiction gene of BKV)

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E-ISSN:2958-6178
Vol. 2, NO. 2, June 2024

and to determine which type it belongs to,using BK-typ1,2,3,4 primers. The first step was BKV DNA extraction, according to instructions provided by the Maxwell® 16 Viral Total Nucleic Acid Purification Kit (Promga USA), (Automated extraction). The following step was detection of BKVP1gene and determine the Genotyping of this virus. Next, the concentration and purity of the DNA in these samples were measured using the Nano-Drop spectrophotometer. This is the most practical method for estimating the concentration and purity of DNA, using the instructions provided by the manufacture company (Nabi/ Korea). Two samples with the highest purity and concentration DNA were send to Macrogen Inc. Geumchen Company, Seoul, South Korea, to explore the genetic polymorphisms of two BK polyomavirus samples isolated from the blood of patients with nephropathy.

BK Extraction of DNA from blood plasma:

Following the instructions provided by the Maxwell 16 Viral Total Nucleic Acid Purification Kit (Promga USA), (Automated extraction), genomic dsDNA was extracted from the virus sample. Then 200µl of plasma was transferred to a sterile Eppendorf tube, following the manufacturer's instructions (Promega, USA). In the 50 ml elution buffer included with the kit, the pure DNA was eluted and stored at -20°C.

BKV PCR primers

The primers used in this study are shown in the Table (1).

Table 1: The specific primer of BKV gene

Primer	Primer sequence	Annealing temp (℃)	Size nm	Reference
BKVP1-F	5′-AGTGGATGGGCAGCCTATGTA -3′	60	300	Tremolada,2010
BKVP1-R	5 ′-TCATATCTGGGTCCCCTGGA -3′	60	300	
BK-TypIV-F	5′-ACTGGGGTAGATGCTATAACAGAAGTAGA -3′	60	300	Gard,2015 (Gene bank accession number NC001538)
BK-TypIV-R	5 ′- TGGGCTATCACTSTCAAAGGCAG-3′	60	300	
BK-TypIII-F	5′- GGTAGATGCTATAACAGAGGTAGAATGC-3	60	300	
BK-TypIII-R	5 ′- TGTCTGGGCTATCACTSTCAAAGG-3′	60	600	
BK-TypII-F	5′- GGTAGATGCTATAACAGAGGTAGAATGC-3′	60	600	
BK-TypII-R	5 ′- TGTCTGGGCTATCACTSTCAAAGG-3′	60	300	
BK-TypI-F	5′-TAACCTTCATGCAGGGTCACAA-3′	60	300	
BK-TypI-R	5′-CTCCACCAACAGCAAARAAGTG-3′	60	300	

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P-ISSN: 2410-8863
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DNA from blood samples by using specific Real time-PCR(RT-PCR) kit for BK virus. These are supplied as lyophilized material from Kapa, USA, and were dissolved in Tris-EDTA (TE) to a concentration of 100µl (stock), in accordance with the manufacturer's instructions for a stock solution (100µl).The stock (10µl) was diluted with TE buffer (90µl) to create the working solution primer (100µM).

master mix preparation:

The KAPA SYBR® FAST qPCR Master Mix (2X) Kit was used to prepare the PCR master mix, which was then completed in accordance with the company's instructions as shown in Table (2):

Table 2: PCR master mix preparation.

Component	20 µL (Final volume)	Final concentration
KAPA SYBR FAST qPCR Master Mix (2X) Universal	10 Ml	2 x
Forward primer	1 µL	0.2µM
Reverse primer	1 µL	0.2µM
Nuclease-free water	3	--
Template DNA Sample Volume	5	1pg-100ng

After that, these PCR master mix component that mentioned above placed in standard The KAPA SYBR® FAST qPCR Master Mix (2X) Kit that containing all other components which needed to PCR reaction (except primers and template) such as a 2X master mix with integrated antibody-mediated hot start, SYBR Green I fluorescent dye, MgCl2 , dNTPs, and stabilizers. Then, all the PCR tubes transferred into Multi spin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR thermocycler (Sacace . Italy).

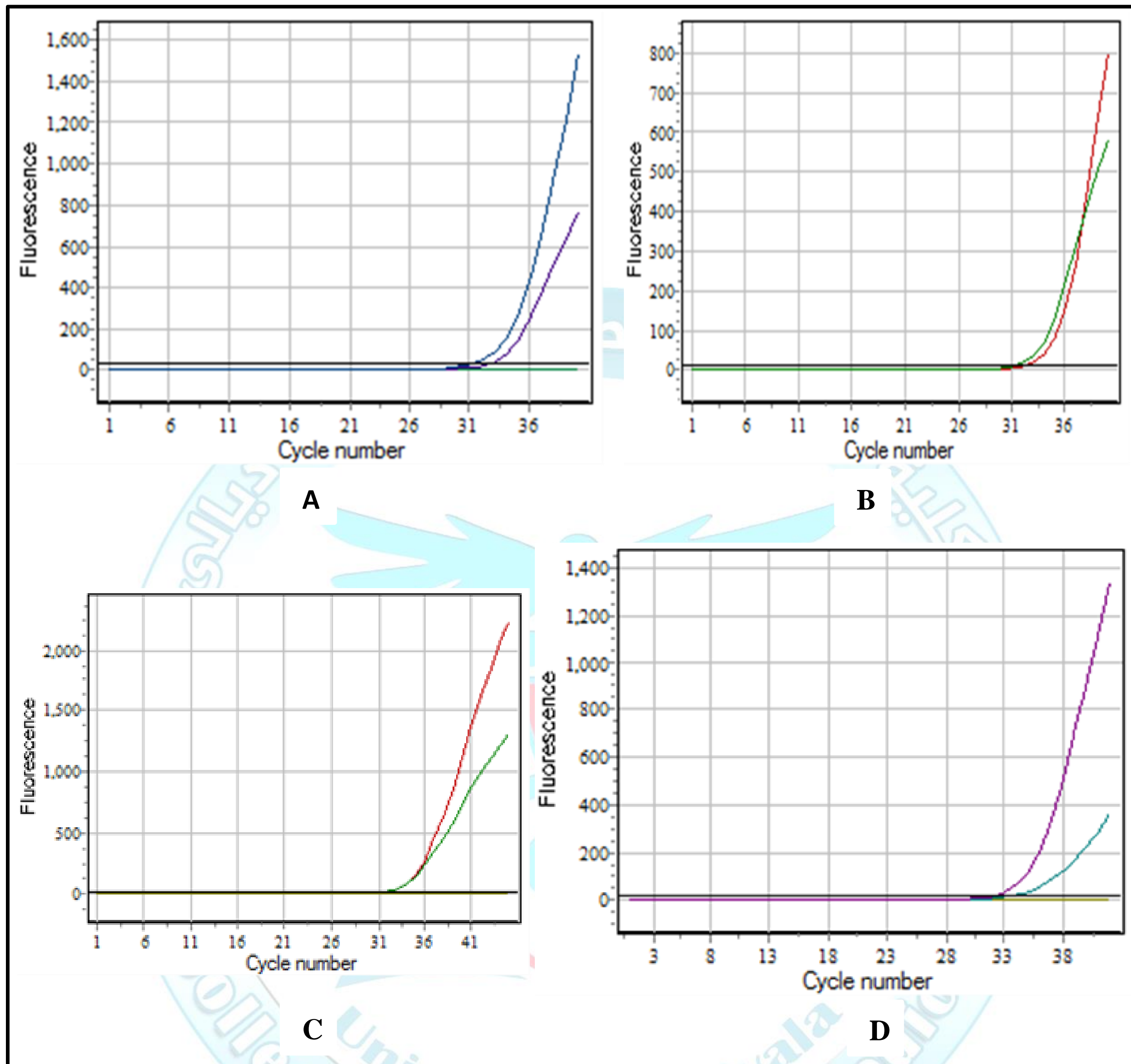


Figure (1): BK virus VP1 amplification chart according to RT-PCR in positive BKV IgM samples.

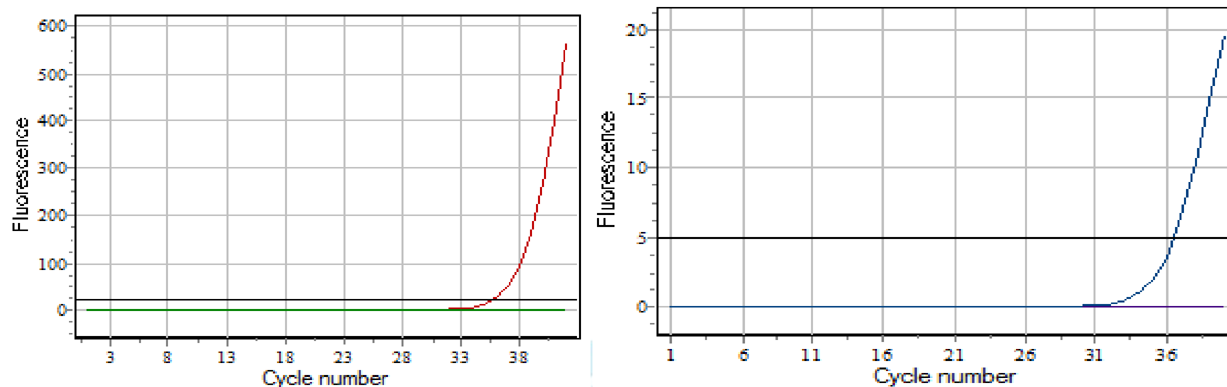


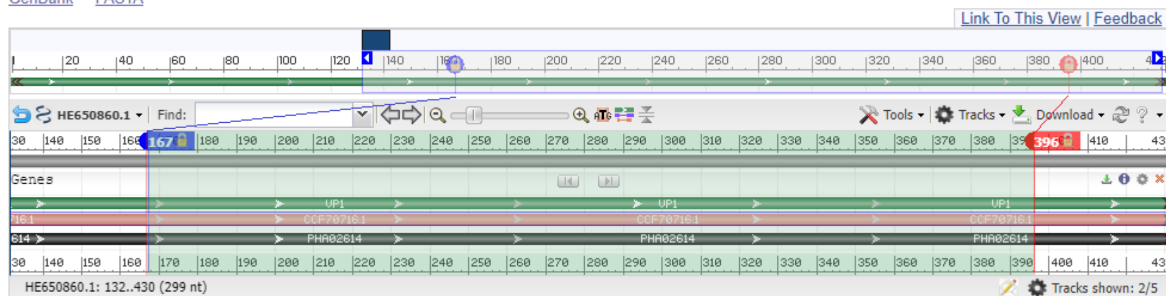
Figure (2): BKtyp4 virus amplification chart according to RT-PCR in positive BKV IgM samples.

Result:

One hundred and seventy blood plasma specimens were collected from (130) patients and (40) healthy patients underwent serological tests (ELISA) have been used to indicate BKviremia by identifying the BKV IgM in the blood. Only 20/130 specimens of nephropathy patients have positive BKV IgM and all healthy group was negative 0/40. This report focus on 20 (15.4 %) specimen with positive BKV IgM, to use for BKV DNA detection by RT-PCR technique, that founded 8/20 was positive in RT-PCR for BKV DNA and 12/20 was negative as shown in Table 3, Figure (1). And when indicated about the type of BKV was diagnostic by RT-PCR , all positive BKV DNA samples were from type IV of BKV Figure (2). Following NCBI blastn analysis, the sequencing reactions revealed the precise location of the examined samples. When comparing the sequenced samples to the intended target, which largely covered the VPI locus within the BK polyomavirus sequences, this engine displayed the highest resemblance. The precise locations and other characteristics of the retrieved PCR fragment was determined by comparing the observed DNA sequences of these viral samples with the retrieved reference sequences. The NCBI blast engine indicated the presence of 100% homology with these expected targets that covered the specified portions of the VPI sequences with the GenBank acc. no. HE650860.1 (Fig. 3).

Table 3: Positive cases of BKV according to IgG, IgM and DNA detection

Group	IgG (+)	IgM (+)	DNA (+)
Patient	98/130	20/130	8/20
Healthy	5/40	0/40	0/40



230 bp PCR amplicon legnth

Fig. 3. The exact positions of the investigated PCR amplicons that covered the PV1 locus within the BK polyomavirus genomic sequences in comparison with the reference strains of BK polyomavirus (GenBank acc. no. HE650860.1). The blue arrow refers to the starting point of these amplicons while the red arrow refers to their endpoints.

The alignment results of the 230 bp samples revealed the detection of no nucleic acid variations in comparison with the referring sequences of the BK polyomavirus sequences (Fig. 4).

Diyala Journal for Veterinary sciences
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P-ISSN: 2410-8863
E-ISSN:2958-6178
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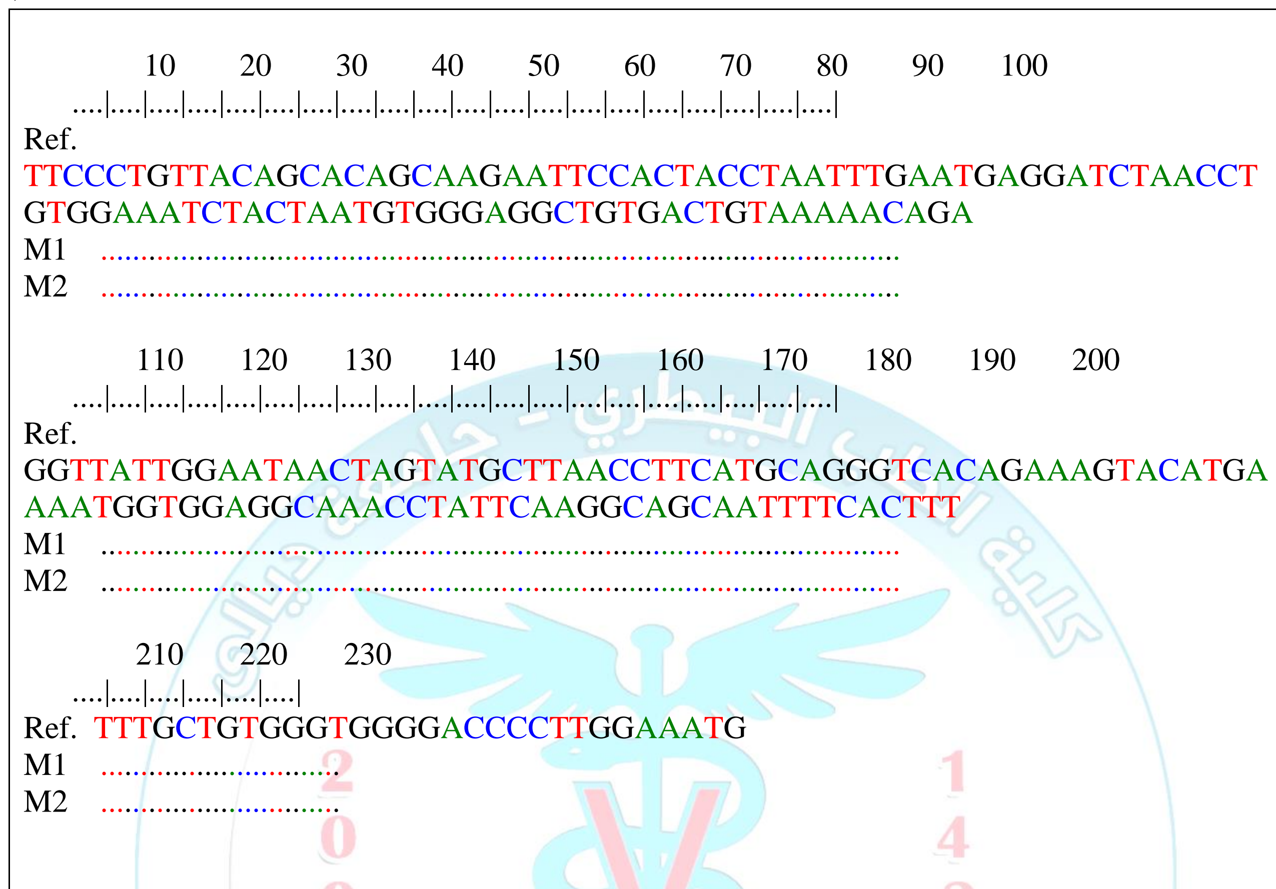

Fig. 4. DNA sequence alignment between two viral samples and the reference strains of BK polyomavirus (GenBank accession number HE650860.1) at the PV1 locus within the BK polyomavirus genomic sequences. The NCBI reference sequences are denoted by the symbol "ref," and the sample code is denoted by "D1.".

The sequences of nucleic acids under investigation were translated to their respective locations in the main capsid protein. Using the Expasy translate suite, all nucleic acid sequences from the studied sample were converted to their matching amino acid sequences. The 230 bp amplicons under investigation comprised seventy-six amino acid sequences spanning the full amino acid sequences in the main capsid protein, according to amino acid alignment of these amino acid sequences with the reference sequences (Fig. 5a). The covered amino acid residues were started from the 57th position until 132th position in the entire major capsid protein (Fig. 5b). The investigated samples of VP1 sequences were deposited in the NCBI web server, and two unique accession numbers were obtained for the analyzed sequence. Accordingly, the GenBank PP779768 and PP779769 have been deposited to represent the M1 and M2 samples of the analyzed BK polyomavirus. Based on the examined nucleic acid sequences found in the sample under analysis, an inclusive phylogenetic tree was created in the current study. This phylogenetic tree contained the investigated isolate of BK polyomavirus sequences (M1

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and M2) of BK polyomavirus sequences alongside other relative reference sequences.

A) Translated PCR amplicon

```
          10        20        30        40        50        60        70
....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
Ref.
PCYSTARIPLPNLNEDLTCGNLLMWEAVTVKTEVIGITSMLNLHAGSQKVHENGG
GKPIQGSNFHFFAVGGDPLEM
M1   .......................................................................
M2   .......................................................................
```

B) The entire protein

>CCF70716.1 major capsid protein, partial [Betapolyomavirus hominis]
GGVEVLEVKTGVDAITEVECFLNPEMGDPDNDLRGYSLRLTAETAFESDSPDRK
MLPCYSTARIPLPNLNEDLTCGNLLMWEAVTVKTEVIGITSMLNLHAGSQKVHE
NGGGKPIQGSNFHFFAVGGDPLEMQGVLMNYRTRYP

Fig. 5. Amino acid residues alignment of the detected variations of the VP1 encoded pa major capsid protein within the investigated human-infecting BK polyomavirus. The positions of the identified variants are shown in PCR amplicons in branch A and the entire protein in branch B. The grey highlights refer to the amplified region of the encoded recombinase.

The constructed phylogenetic tree included thirty-four aligned nucleic acid sequences and revealed the presence of four major serovars, among which our investigated samples were classified. Insights gleaned from the PV1-based tree provided clear phylogenetic information about our investigated virus. This tree was presented in a circular cladogram format (Fig. 6), which highlighting a specific phylogenetic distribution of the integrated sequences.

It was found that both investigated M1 and M2 samples were incorporated within the serovar IV. The number of incorporated samples within this specific clade was eleven. It is important to highlight that, within this particular group, our examined isolates fit in the neighborhood of nine reference sequences deposited from Kuwaiti (GenBank acc. no. HE650860.1, HE650856.1, HE650855.1, HE650847.1, HE650851.1, HE650850.1, HE650849.1,

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P-ISSN: 2410-8863
E-ISSN:2958-6178
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HE650864.1, and HE650876.1) origins within this clade.

Notably, all the listed clades were found to occupy variable distinctly separated phylogenetic positions from each other in the tree. The significant phylogenetic distinctions observed among all three genotypes highlighted the diverse evolutionary pathways within the BK polyomavirus.

Based on the VP1 amplicons, serovar I is considered the likely ancestor from which the other serovars have evolved, as indicated by its position adjacent to the root of the tree. In contrast, serovar IV, followed by serovar II, are the most recent manifestations of BK polyomavirus, respectively. These serovars likely represent the most recent evolutionary developments in comparison to the other serovars included in the study of BK polyomavirus.

The neighbor-joining tree currently generated has provided highly informative genetic distances among all examined samples of M1 and M2. Notably, the specific phylogenetic positioning of the analyzed samples may suggest a particular role for the VP1fragment in the adaptations of these BK polyomavirus sequences to the clinical sources from which they were derived. Consequently, the VP1-based amplicons currently in use are effective in discerning genetic variations between the D1 sample and other related samples within the same clade. This underscores the utility of VP1-specific primers for identifying the phylogenetic clustering of the BK polyomavirus isolate being studied.

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P-ISSN: 2410-8863
E-ISSN:2958-6178
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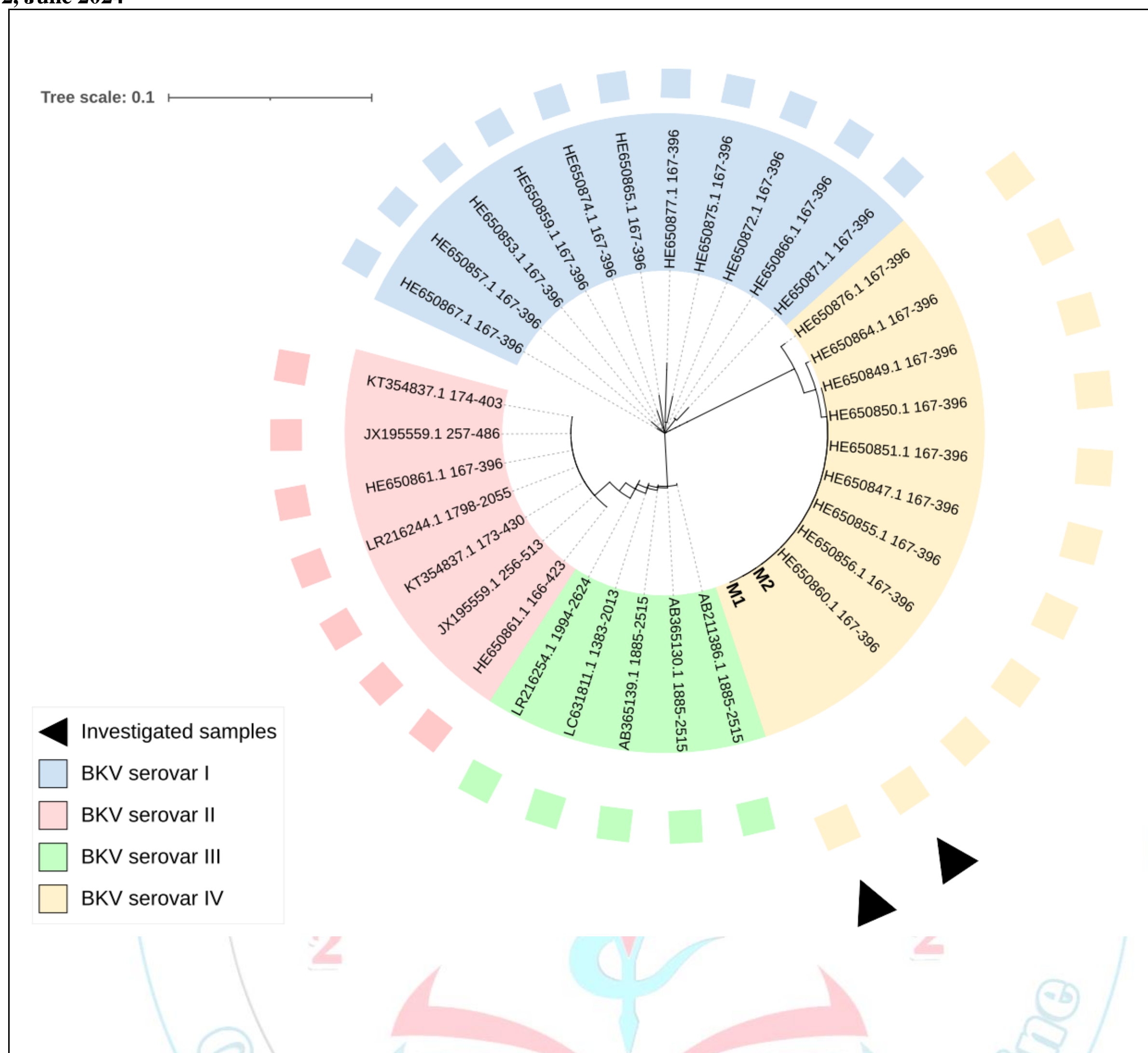

Fig. 6. The complete circular phylogenetic tree illustrating the genetic variants in the VP1 locus discovered in two samples of BK polyomavirus. Which genotypes have been included are shown by the numbers with different colors. The scale on the left side of the structure indicates the degree of phylogenetic positions among the viral species listed in the tree. The notation "M1 and 2" indicates the code of the sample that is being examined.

Discussion:

In order to swiftly identify each genotype and subgroup of BKVs, PCR technology was developed. According to, because of its extreme sensitivity when compared to other techniques (cultivation), it requires an active virus and a protracted cultivation time of up to six weeks [19]. In the present study ,all positive BKV IgM blood specimen were prepared for analyzed by PCR for detection BKV DNA. The results of current study showed that, 8/20 specimen was positive for BKVP1 RT-PCR. The number of sample types used for analysis contributes to the differences in results between the serological test for antiBKV IgM and the

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P-ISSN: 2410-8863
E-ISSN:2958-6178
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RT-PCR molecular test for BKV DNA. The variety of calibrators, extraction techniques, and PCR methods used further hinders the comparability of data from molecular assays for these pathogens. [20,21,22]. One genetic fragment, partially covering the VP1 within this virus was selected in this study. The amplified fragment was made up to 230 bp and was subsequently exposed to Sanger sequencing to resolve the pattern of its genetic diversity for the analyzed BK polyomavirus isolates (assigned M1 and M2). The NCBI blastn engine indicated the presence of 100% homology with these expected targets that covered the specified portions of the VP1 sequences with the GenBank acc. no. HE650860.1 (Fig. 3). Following the placement of the 230 bp amplicons' sequences within the BK polyomavirus's VP1 locus, the specifics of these sequences were emphasized, beginning with the forward primer's location and ending with the reverse primer's position within the same targeted BK polyomavirus sequences (Table 4).

Table 4. The length and position of the 230 bp PCR amplicons used to amplify the PV1 locus within the genomic sequences of BK polyomavirus in relation to the reference strains of the virus (GenBank accession number HE650860.1).

* Refers to the forward primer sequences (placed in a forward direction)

Targeted gene	Sequences (5′-3′)	Length
VP1 of BK polyomavirus	*TTCCCTGTTACAGCACAGCAAGAATTCCACTACCTAATTTGAATGAGGATCTAACCTGTGGAAATCTACTAATGTGGGAGGCTGTGACTGTAAAAACAGAGGTTATTGGAATAACTAGTATGCTTAACCTTCATGCAGGGTCACAGAAAGTACATGAAAATGGTGGAGGCAAACCTATTCAAGGCAGCAATTTTCACTTTTTTGCTGTGGGTGGGGACCCCTTGGAAATG**	230 bp

** Refers to the reverse primer sequences (placed in a reverse complement direction)

The constructed phylogenetic tree included thirty-four aligned nucleic acid sequences and revealed the presence of four major serovars, among which our investigated samples were classified. Insights gleaned from the PV1-based tree provided clear phylogenetic information about our investigated virus (Fig. 6).

Based on the analyzed nucleic acid sequences obtained in the examined sample, an inclusive phylogenetic tree was created in the current investigation. The examined isolate of BK polyomavirus sequences (M1 and M2) were included in this phylogenetic tree along with other relative reference sequences. Given the existence of four primary serovars of BK polyomavirus, a direct comparison was made between our samples and the well-documented reference serovars to accurately determine the genotyping of the M1 and M2 samples within these main viral groups.

Consequently, within the phylogenetic tree that was constructed, our viral isolate was directly compared with several representative reference samples from each of the four primary viral serovars, ranging from serovar I to serovar III. This comparison facilitated a more precise understanding of the actual evolutionary distances between them. To enhance this analysis, diverse representatives of BK polyomavirus serovars I, II, II, and IV were incorporated into the tree, based on the VP1 sequences.

Common serovars of BK polyomavirus were used as reference sequences in the tree, aiding in the determination of the evolutionary relationships among the viral samples studied. Utilizing a neighbor-joining method, four distinct phylogenetic clades were identified, to represent the referred serovars I to IV, respectively. Within these groups, our investigated BK polyomavirus samples were specifically placed within serovar IV, illustrating their genetic positioning and relationship with closely related strains.

It was found that both investigated M1 and M2 samples were incorporated within the serovar IV. The number of incorporated samples within this specific clade was eleven. Within this specific clade, it deserves to be noted that our investigated isolates were suited in the vicinity of nine reference sequences deposited from Kuwaiti (GenBank acc. no. HE650860.1, HE650856.1, HE650855.1, HE650847.1, HE650851.1, HE650850.1, HE650849.1, HE650864.1, and HE650876.1) origins within this clade. The reason for the close phylogenetic positioning of these samples toward these isolates originated from the entire homology with these reference sequences. On the other hand, eleven reference samples were incorporated to represent serovar I, which represents the closest clade toward the roots of the tree. This clade of serovar I has a much more diverse phylogenetic distribution than that found in the other clades. This is due to the wide biological diversity of serovar I compared with the other serovars. In addition to serovars IV and I, five and seven samples were also incorporated to represent III and II, respectively. The region of origins of all incorporated sequences is presented in Table 5.

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Table 5. The details of the incorporated strains in the BK polyomavirus sequences within the currently generated phylogenetic tree. The shown colors refer to the specific genotype within each generated phylogenetic clade.

No.	GenBank	Serovar	Country
1.	HE650877.1	I	Kuwait
2.	HE650867.1	I	Kuwait
3.	HE650872.1	I	Kuwait
4.	HE650866.1	I	Kuwait
5.	HE650865.1	I	Kuwait
6.	HE650859.1	I	Kuwait
7.	HE650853.1	I	Kuwait
8.	HE650875.1	I	Kuwait
9.	HE650874.1	I	Kuwait
10.	HE650871.1	I	Kuwait
11.	HE650857.1	I	Kuwait
12.	HE650861.1,166-423	II	Kuwait
13.	HE650861.1,167-396	II	Kuwait
14.	LR216244.1	II	Australia
15.	KT354837.1,173-430	II	Argentina
16.	KT354837.1,174-403	II	Argentina
17.	JX195559.1,256-513	II	Spain
18.	JX195559.1 257-486	II	Spain
19.	LR216254.1	III	Australia
20.	LC631811.1	III	Japan
21.	AB365139.1	III	Japan
22.	AB365130.1	III	Japan
23.	AB211386.1	III	Japan
24.	HE650860.1	IV	Kuwait
25.	HE650856.1	IV	Kuwait
26.	HE650855.1	IV	Kuwait
27.	HE650847.1	IV	Kuwait
28.	HE650851.1	IV	Kuwait
29.	HE650850.1	IV	Kuwait
30.	HE650849.1	IV	Kuwait
31.	HE650864.1	IV	Kuwait
32.	HE650876.1	IV	Kuwait

CONCLUSION

Based on the phylogenetic positioning of clinical samples examined relative to Kuwaiti viral samples of serovar IV, VP1 amplicons demonstrated highly sensitive differentiation among the analyzed viral sequences. According to the study, BK polyomavirus-VP1 amplicons could serve as markers to distinguish between these pathogenic viral isolates. To enhance understanding of the biological diversity of these viruses in the Iraqi population, the study recommends the inclusion of more samples across broader research areas. This study emphasizes the significance of genetic variation in comprehending the molecular epidemiology and evolutionary dynamics of BK polyomavirus, which may affect the phylogenetic framework and geographical tracking of the virus currently under investigation.

Conflicts of interest

The authors declare that they have no potential conflict of interest concerning the authorship or publication of this article.

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