Online ISSN: 3009-7312
Print ISSN: 3009-6391

EJCBR

Egyptian Journal of Cancer and Biomedical Research

https://jcbr.journals.ekb.eg

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EACR EGYPTIAN ASSOCIATION FOR CANCER RESEARCH
Since 2014

EGYPTIAN JOURNAL OF CANCER AND BIOMEDICAL RESEARCH (EJCBR)
FORMARLY INTERNATIONAL JOURNAL OF CANCER AND BIOMEDICAL RESEARCH (IJCBR)

RESEARCH ARTICLE

Molecular characterization of WU polyomavirus strain isolated from patients with breast cancer

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ABSTRACT

Background: Polyomaviruses (PyV) are small, circular, non-enveloped viruses with a diameter of about 40–50 nm, and have a double-stranded DNA molecule descended from the Polyomaviridae family. Until now, about 32 PyV species have been described, where more than 10 of them have been reported to cause infection. **Aim**: This study aimed to identify the sequence of the complete genome of the WU polyomavirus strain and to study its phylogenetic analysis and determine variation in the WUPyV sequence. **Materials and Methods**: Samples (n=195) were collected to identify viral DNA by real-time and conventional PCR, respectively. One purified viral DNA sample of positive cases was used for next generation sequencing (NGS) to identify the WGS. **Results**: The WUPyV sequence was registered in the NCBI GenBank under the accession number LC771087. The current study showed that the WUPyV sequence has a length of 5229 bp. The WUPyV sequence was compared to the reference sequences to determine any mutations. We found many mutations, some of which were silent, and the others caused a change in the amino acid. Ten reference strains from NCBI were used to create a phylogenetic tree for the clinical sample studied. The present results revealed that the strain LC771087.1 WU Polyomavirus showed great similarity with one sequence, EU711054.1 WU Polyomavirus strain WU/ Wuerzburg/01/03. **Conclusion**: The current study concluded that WU polyomaviruses used in the multiple sequence alignment with our strain have the same length 5229 bp, with 99.5% similarity in DNA sequences and 99.2% identity of amino acid sequences.

Keywords: Human polyomavirus; NGS; WGS; WU polyomavirus

Editor-in-Chief: Prof. M.L. Salem, PhD - Article DOI: 10.21608/jcbr.2024.278156.1344

ARTICLE INFO

Article history
Received: March 19, 2024
Revised: September 30, 2024
Accepted: November 12, 2024

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INTRODUCTION

In 1953, Polyomaviruses were first discovered in mice which had multiple tumors upon laboratory injections into newborn mice and subsequently were discovered in various types of rabbits, birds, bats, cattle, sea lions, hamsters, monkeys, and humans (Zur Hausen, 2008). The family Polyomaviridae emerged in 2000 when the International Committee on Taxonomy of Viruses formally divided the genera in Papinoviridae into two new families, Papillomaviridae and Polyomaviridae. The Polyomaviridae family includes one genus, polyomavirus (Delbue *et al.,* 2012). Human Polyomaviruses are non-enveloped particles containing approximately 5000 nucleotides with double-stranded DNA. The organization of the PyVs genome is highly conserved, which comprises three genes, the viral non-coding control regions of about 500 bp, and the early and late regions. PyVs are widespread in the population of humans. PyV infection occurs in the early of life in many cases, but most cases of infection remain asymptomatic (Gheit *et al.,* 2017).

WUPyV was discovered in nasopharyngeal aspirates by Gaynor *et al*., (2007) in a child (3 years old) with pneumonia and the virus was named after the University of Washington. Total DNA was randomly amplified, cloned, and sequenced after PCR assays yielded no detection of known respiratory pathogens. Of the total 384 reads performed, 6 reads were obtained using PCR. These transcripts were classified as viral sequences which comprise three unique regions with limited homology

compared to PyV (De Gascun, & Carr, 2013). WUPyV shares most genomic features with other human polyomaviruses, with a non-coding control region (NCCR), separated early and late coding regions on opposite strands (Bialasiewicz *et al*., 2010).

In recent years, studies have been conducted in Basrah Governorate on the most important pathogenic medical viruses, as in the studies conducted by Shihab *et al*. (2020), Salman *et al.* (2021) and Atbee *et al.* (2020). The current study aims to identify WUPyV by conventional and real-time PCRs, respectively, and identify the sequence of the complete WUPyV genome strain to analyze the phylogeny and identify variation in WUPyV sequences.

MATERIALS AND METHODS

Samples collection

In the current study, 195 samples were collected from October 2022 to the end of April 2023. They were collected from Basrah Teaching Hospital, Al-Fayhaa Teaching Hospital, Al-Sadr Teaching Hospital, and the Chest Diseases Center in Basrah City. 90 paraffin-embedded tissue samples from neoplastic and non-neoplastic lesions were obtained from the histology laboratory. These samples were retrospectively collected from the gastrointestinal tract (GIT) of the stomach, intestine, colon, jejunum, ileum, rectum, gallbladder of the patients with cholecystitis, as well as the kidneys, breasts, thyroids, tonsils, and lungs. 105 samples of nasal swabs, lung washes, and pleural fluids for respiratory infections were taken after obtaining official approvals for ethical considerations. The virus was then detected blindly using specialized primers without using standard samples.

Extraction of DNA

DNA of WUPyV was extracted using FavorPrepTM Tissue Genomic DNA Extraction Mini Kit manufactured by Taiwan and according to company instructions for 90 Formalin-fixed paraffin-embedded (FFPE) samples, while for respiratory samples, DNA was extracted according to the Biocomma Nucleic Acid Purification Kit system manufactured by Spain and according to company instructions for molecular detection of the HPyV genome in 105 samples. A nanodrop spectrophotometer (Optizen/Korea) was used to measure and evaluate the DNA quality of all the extracted samples.

Molecular identification of WUPyV

The WUPyV strains were detected by real-time PCR with SolGentTM2X Real-Time PCR Smart mix, a qPCR amplification kit with SYBR ® Green I in the mixture. The reaction was performed by mixing 10 µl of SYBR Green, with DNA (4 µl), forward and reverse primer (1 µl of each one), and 4 µl of nuclease-free water. The B-2729-forward primer (5’-CTACTGTAAATTGATCTATTGCAACTCCTA-3'), B-2808-reverse primer (5’-GGGCCTATAAACAGTGGTAAAACAACT-3') were used to amplify target gene. The real-time PCR conditions including the first step of the reaction submitted to denaturation for 10 minutes at 95˚C, after 45 cycles, the denaturation step is 20 seconds at 95 ˚C, then 58.6 ˚C for 35 seconds for the annealing step, then 72 ˚C for 45 seconds for extension step, finally, the extension step was done for 10 minutes at 72˚C. The reaction analysis was subjected to a melting curve to ensure the primer specificity.

The WUPyV strains were also detected by conventional PCR with a SolGentTM 2X Taq PLUS PCR Smart mix 1 kit from SolgGent Biotechnology [Cat. No. STD01B-M50h]. The reaction component in the 12.5 µl includes SolGentTM Taq PLUS DNA Polymerase, Buffer, dNTP, and tracking dye according to the instructions recommended by the manufacturer. The reaction was performed by mixing 12.5 µl of 2X Taq PLUS PCR Smart mix 1, 2.5 µl of Tracking dye (Band Doctor), 3 µl of DNA, 1 µl of Primers (F & R:100 pmol/ µl), and 5 µl of nuclease-free water. The B-2729-forward primer (5’-CTACTGTAAATTGATCTATTGCAACTCCTA-3'), B-2808-reverse primer (5’-GGGCCTATAAACAGTGGTAAAACAACT-3') were used to amplify target sequence 136 bp. The PCR condition was set as an initial denaturation step at 95 °C for 15 minutes, then 45 cycles, the denaturation step is 20 seconds at 95 ˚C, then 58 ˚C for 35 seconds for the annealing step, then 72 ˚C for 50 seconds for the extension step,

finally, the extension step was done for 5 minutes at 72˚C. The products of PCR were visually under a UV source on a 2% agarose gel.

Whole genome sequences (WGSs)

The WUPyV DNA sample was sent to Apical Scientific Laboratories/Malaysia to determine the sequence using next-generation sequencing (NGS) for determining WGSs using a strategy of long PCR amplicon and one highly purified sample was selected for whole genome sequencing.

Statistical analysis

Statistical analysis was performed using SPSS, version 21. Comparison between positive samples studied according to the technique used for diagnosis was performed using the chi-square test, and differences between groups were considered statistically significant.

RESULTS

Molecular detection of WUPyV

Of the 195 samples, only 24 and 9 were positive by real-time PCR and conventional PCR, respectively. Nine samples were sent for sequencing to Macrogen/Korea to confirm the presence of the WUPyV DNA, and the sequencing of 7 samples was successful. and significant differences in WU (*P*. value = 0.009) by Real time PCR and conventional PCR.

The first sample was from a female patient (65 years old) with colon cancer, the second sample was from a female patient (27 years old) with thyroid cancer, and the third sample was from a male patient (78 years old) with breast cancer (Figures 1 and 2). The fourth sample was from a patient (38 years old) with colon cancer, and the fifth sample was from the gallbladder of a 30-year-old male patient with cholecystitis. The sixth sample was from a 38-year-old patient with non-cancerous proctitis. The seventh sample was from a 31-year-old patient with a non-cancerous inflammatory nodule in the thyroid gland.

Whole genome sequencing

One purified WUPyV DNA sample from a male patient (78 years old) with breast cancer was sent to determine the sequence of the complete genome, sequencing of the sample was successful. The WUPyV DNA has been registered in the NCBI GenBank under the accession number LC771087.1 WU Polyomavirus BASRAH88 complete genome. Using the UPGMA method, the evolutionary history of the target sample was inferred (Sneath and Sokal, 1973). An ideal tree is shown with a length of branch = 0.00711460. The tree was drawn using scale, and calculation of evolutionary distances by method of the composite maximum likelihood, with length of branches in the same units to infer the phylogenetic tree (Tamura *et al.,* 2004) and are in units of base substitutions per site. The sequences of 11 references are used in this analysis. All ambiguous positions for each sequence pair were removed (pairwise deletion option). A total of 5229 base pairs were found in the final dataset. The phylogenetic analyses are performed by using MEGA X (Kumar *et al.,* 2018).

Genome structure

According to the clinical strain assembly using the template, EU711054.1 WU Polyomavirus strain WU/Wuerzburg/01/03, the target sample in the study was found to be 5229 base pairs in length. The genome structure in the study sample is typical of WU Polyomavirus as the genome can be divided into 6 genes. The ORF map of the sample studied (Figure 3).

Analyses of genomic variation

The multiple sequence alignments (MSA) of the studied sample, LC771087.1 WU Polyomavirus BASRAH88 complete genome have been done to compare with ten reference strains taken from NCBI including; GU296392.1, GU296398.1, GU296402.1, MF094822.1, MH084642.1, EU711057.1, gi|290576719|gb|GU296396.1,GU296367.1, GU296386.1, and EU711054.1., (Figures, 4 and 5) to identify any mutations and finding any changes in the amino acid expression (Table 1). The whole genome sequencing revealed multiple mutations, some of which were silent, others caused by an amino acid change. There were 26 mutations in the LC771087.1 WUPyV sequences, 14 of them were missenses which caused changes in the amino acid, and 4 of them were silent. Interestingly, most substitutions result in an amino acid change, the position of the nucleotides 26 and 27 were mutated from

Table 1. Mutations studied sample (LC771087.1) compared with 10 references strains

Accession number											Site	Mutations	
LC771087.1	EU711054.1	GU296386.1	gi|290576719|gb|GU296396.1|	GU296367.1	EU711057.1	MH084642.1	MF094822.1	GU296402.1	GU296398.1	GU296392.12		Type	Amino acids
G	A	A	A	A	A	A	A	A	A	A	26	Missense	Lysine to Serine
C	A	A	A	A	A	A	A	A	A	A	27		
A	T	T	T	T	T	T	T	T	T	T	82	Missense	Phenylalanine to Threonine
C	T	T	T	T	T	T	T	T	T	T	83		
A	T	T	T	T	T	T	T	T	T	T	84		
A	C	C	C	C	C	C	C	C	C	C	182	Missense	Alanine to Glutamic acid
C	T	T	T	T	T	T	T	T	T	T	1144	Missense	Tryptophan to glutamine
A	G	G	G	G	G	G	G	G	G	G	1145		
T	A	A	A	A	A	A	A	A	A	A	1147	Missense	Threonine to Phenylalanine
T	C	C	C	C	C	C	C	C	C	C	1148		
C	A	A	A	A	A	A	A	A	A	A	1149		
A	T	T	T	T	T	T	T	T	T	T	1150	Missense	Serine to Threonine
A	G	G	G	G	G	G	G	G	G	G	1174	Missense	Valine to Isoleucine
C	A	A	A	A	A	A	A	A	A	A	1371	Missense	Glutamic acid to Aspartic acid
C	G	G	G	G	G	G	G	G	G	G	1372	Missense	Glycine to Arginine
T	A	A	A	A	A	A	A	A	A	A	1428	Silent	
T	A	A	A	A	A	A	A	A	A	A	1443	Silent	
C	A	A	A	A	A	A	A	A	A	A	1651	Missense	Lysine to Arginine
G	A	A	A	A	A	A	A	A	A	A	1652		
G	A	A	A	A	A	A	A	A	A	A	1653		
A	A	G	G	G	G	G	G	G	G	G	2587	Missense	Glycine to Serine
T	A	A	A	A	A	A	A	A	A	A	3486	Silent	
A	C	C	C	C	C	C	C	C	C	C	4446	Missense	Phenylalanine to Leucine
G	C	C	C	C	C	C	C	C	C	C	4447	Missense	Proline to Alanine
T	C	C	C	C	C	C	C	C	C	C	4554	Silent	
G	G	A	A	A	A	A	A	A	A	A	4649	Missense	Asparagine to Serine

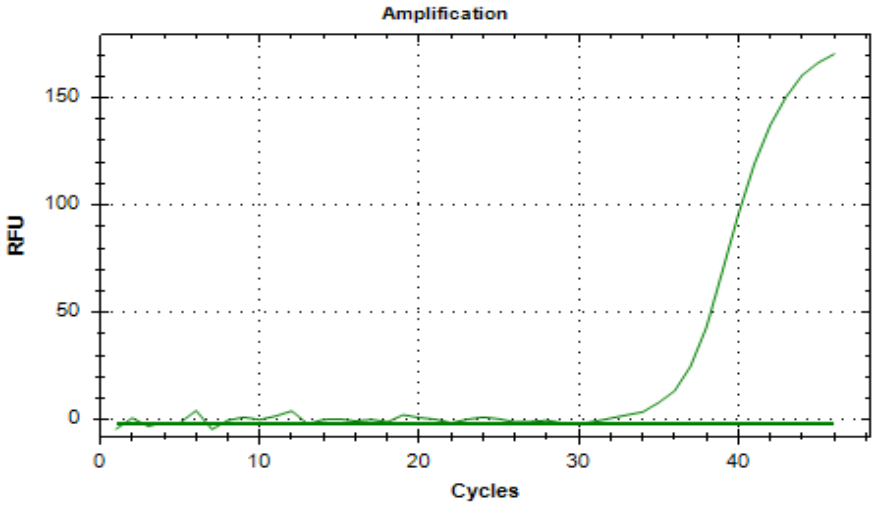

Figure 1. Representative amplification curve of WU Polyomavirus in studied sample LC771087.1 (breast cancer), the threshold was set automatically by the machine at the typical CTs range.

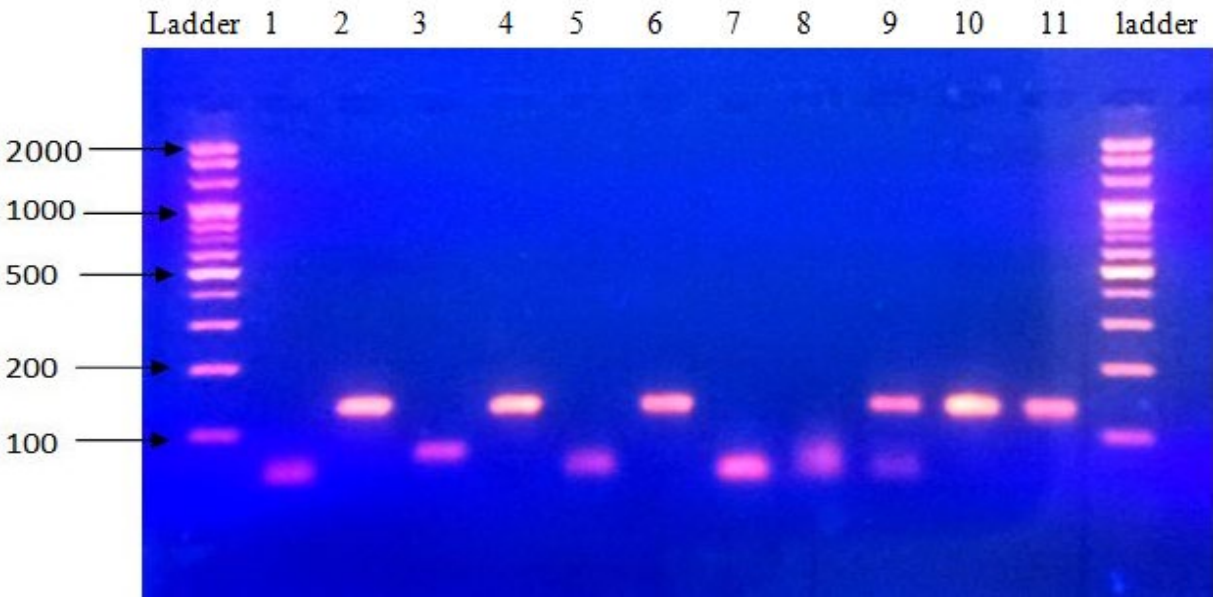

Figure 2. (2%) Agarose gel electrophoresis at (70 V) for (50 min) of PCR amplified products of WUPyV. First and last lane = DNA ladder (100-2000 bp), Lanes 2, 4, 6, 9, 10 & 11 are positive samples, Lanes 1, 3, 5, 7 & 8 are negative samples.

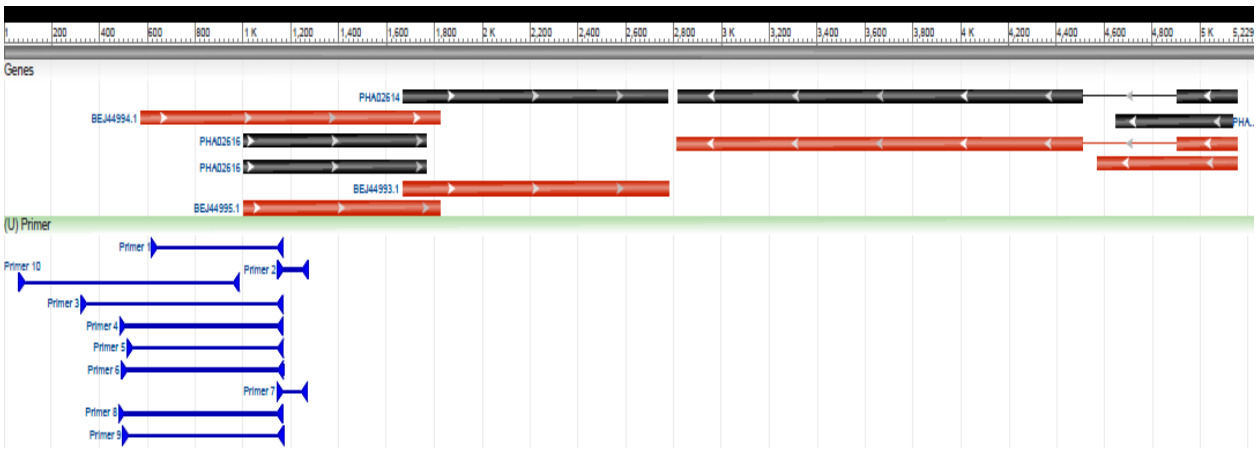

Figure 3. Map of the sample studied**.**

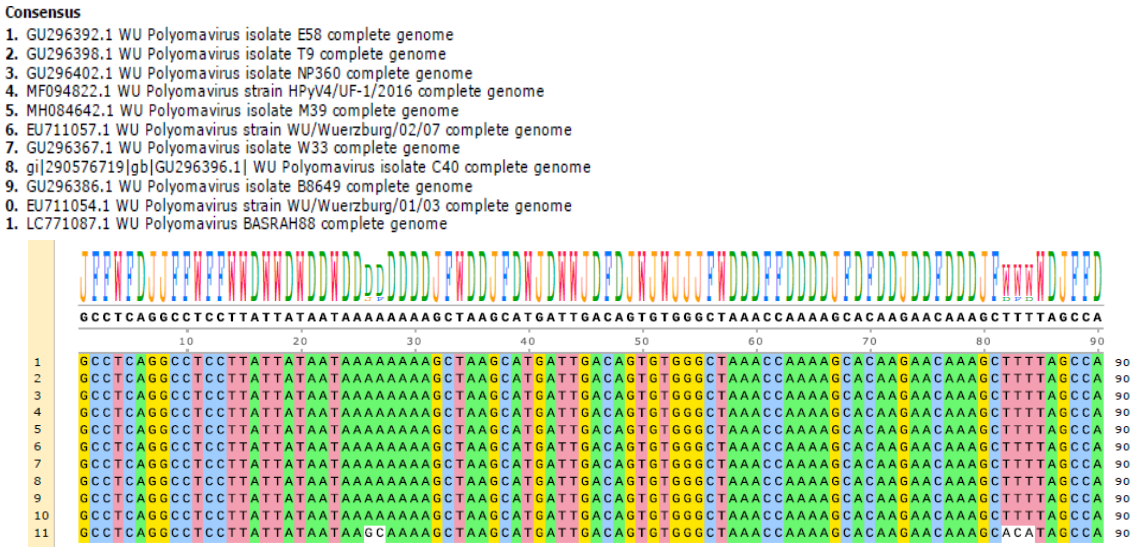

Figure 4. MSA of DNA sequences of studied sample (LC771087.1) with 10 reference WU Polyomavirus strains.

Figure 5. MSA of amino acids studied samples with 10 reference WU Polyomavirus strains.

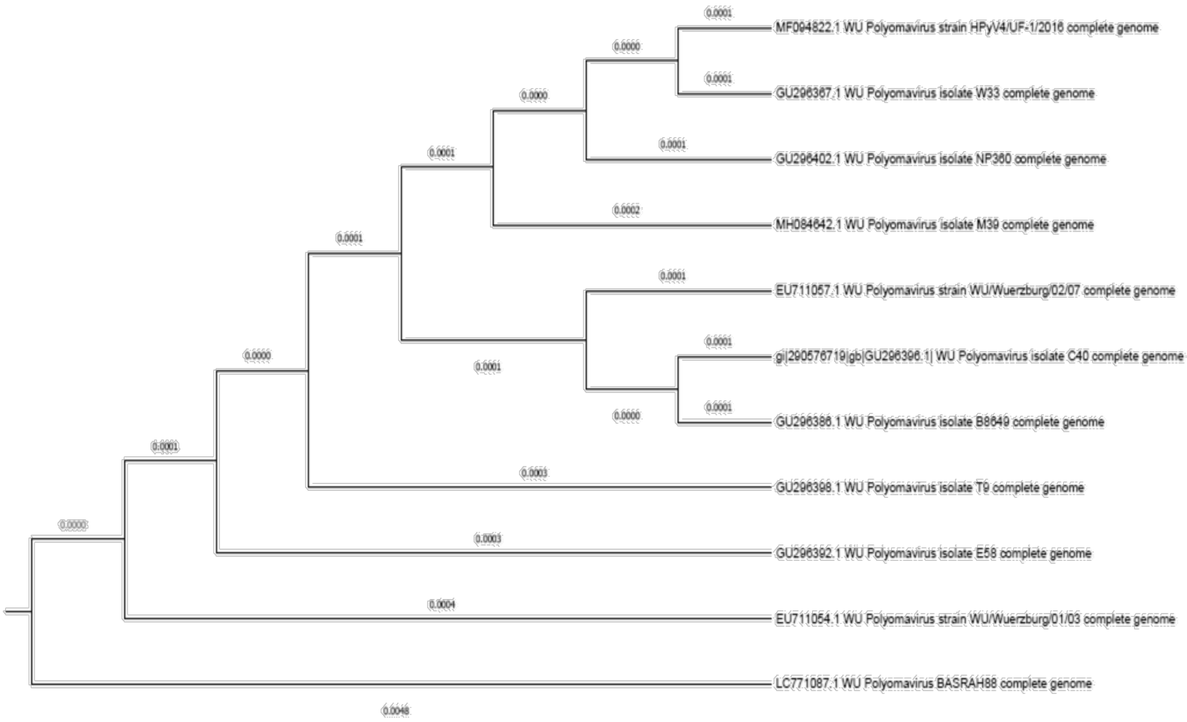

Figure 6. Phylogenetic analysis of WUPyV strains.

A-to-G and A-to-C substitutions, respectively, leading to a change in Lysine to Serine. On the other hand, the LC771087.1 WU Polyomavirus sequence Substitutions are often very different from other reference strains. EU711054.1 WU Polyomavirus strain WU/ Wuerzburg/01/03 shared studied sample with the same mutations in positions 2587 and 4649 were mutated from G-to-A and A-to-G substitutions, respectively, leading to a change in amino acids Glycine to Serine and Asparagine to Serine, respectively.

Phylogenetic analysis

Using the maximum composite likelihood method, a phylogenetic tree consisting of the complete nucleotide sequences of the target clinical sample with 10 reference strains was constructed, and the evolutionary distances were highlighted. As shown in Figure 6, the sample studied was compared with the reference strains at the level of evolutionary relationships and the genetic distance between the 11 WUPyV strains was calculated.

All samples showed similarity in the sequences. 8 main clades were found through phylogenetic tree analysis, and the current results showed that studied strain LC771087.1 WU Polyomavirus showed great similarity with one sequence, EU711054.1 WU Polyomavirus strain WU/ Wuerzburg/01/03, with genetic distance or sequence divergence 0.0048 reflecting a close relationship of these strains.

DISCUSSION

This study focused on collecting 3 types of samples taken from 195 patients. During the study, WU polyomavirus was detected in 9 out of 195 patient tissue samples at a rate of about 4.6%, which is consistent with most of the research published by Gaynor *et al.* (2007). They found that the number of infected samples reached 37 out of 1,245 samples. This percentage represents 3.0%, as it was first discovered in 2007 in respiratory samples taken from children. In the current study, the virus was isolated from adults with breast, thyroid, and colon cancer, and this may be evidence of the involvement of WUPyV as a causative factor in the occurrence of cancer in infected patients, although the ability of WUPyV to cause disease has not yet been proven and is still a matter of debate.

Polyomaviruses are a growing family that infects fish, birds, rodents, humans, and non-human primates. The discovery of new species is accelerating because of the development of modern molecular diagnostic tools during the past decade (Gheit *et al*., 2017). A new member of the Polyomavirus genus in the family Polyomaviridae, Washington University Polyomavirus (WUPyV), consists of a small, non-enveloped dsDNA called HPyV4 by the International Committee on Taxonomy of Viruses. The WUPyV was first discovered by Gaynor *et al.* (2007) and isolated from acute respiratory tract infection (ARTI) from pediatric patients in 2007. It is worth noting that the infection with this virus occurs in the early stages of life, and serological studies indicate its incidence at a high rate, with the infection rate reaching high levels of more than 69%. Globally, WUPyV DNA is found in respiratory samples: frequently 0.35-27.5% (Csoma *et al*., 2018). Serological studies show the high seroprevalence of WUPyV in the adult group, thus suggesting the onset of infection occurs early in life. Indeed, Nguyen and colleagues used VP1 antibodies in the ELISA test to report an 80% seropositivity for WUPyV (Nguyen *et al*., 2009). At the early stage of life (18 months), the child becomes infected with WUPyV in about 50%, indicating widespread of the WUPyV. However, the WUPyV pathogenesis remains unclear (Dinwiddie *et al*., 2016). When conducting molecular tests, we find that the infection rate decreases significantly by about 4.6 %, that the presence of the virus's genetic DNA is low compared to the seroprevalence. This indicates the role of the immune system of infected people against viral infection. In some studies, the detection of WUPyVs and symptoms have been reported only in co-infections or immunocompromised individuals (Dinwiddie *et al*., 2016).

Through amplification of the WUPyVs, the PCR technique showed low viral DNA loads in each positive sample, in which amplification was delayed, indicating latency with continued infection more than virus replication. It may be transmitted through respiratory, oral, and fecal routes. Dormant infection is activated if the

human body is immunosuppressed. WUPyVs are thought to persist in patients with poor immune function (Babakir *et al*., 2013). It is worth noting that infections with WUPyV occur in immunocompromised patients or those who suffer from some chronic diseases, and this indicates a high rate of recovery from viral infections because of the strength of the immune system of those infected.

In human polyomaviruses, the degree of identity between the amino acid sequences of the VP1 protein is high. For example, BKPyV identity shares 78.2% with JCPyV of VP1 amino acid sequence, while the identity of WUPyV with KIPyV shares about 65% of the amino acid sequence, and lower similarity to BKPyV (28%), JCPyV (27%), and SV40 (28%) (Babakir *et al*., 2013).

By performing whole genome sequencing and phylogenetic analysis, it was found that the WUPyV has the same typical genomic organization, but with a difference in some nitrogenous bases (mutations) from all WU polyomaviruses used in the multiple sequence alignment (MSA) of approximately 0.5%. By comparing the amino acid expression of WUPyV proteins with the expression of reference strains using multiple sequence alignment (MSA), it was found that there are some differences in these proteins (Table 1) with a high percentage of identity of approximately 99.2%, and this indicates the occurrence of many different mutations. In the sequence there are 26 mutations, 14 of which are missenses, and 4 of which are silent.

Bialasiewicz *et al*. (2010), it was found that the total genomic variation of the 64 WUPyV strains decreased by 0 to 1.2%, with several islands of dense diversity in the VP1, VP2, and L-Tag N-terminal regions. Variation is more significant in the VP1 region at the levels of both nucleic and amino acids and to a greater extent. Less than that, in the VP2 and S-Tag genes.

Dinwiddie *et al.* (2016) performed a sequence alignment to NC_009539, and the study revealed a total of 6 variants, 2 of which were non-synonymous. It discovered one amino acid variant in the L-Tag and one in the VP2 protein.

CONCLUSIONS

All WU polyomaviruses used in the sequences were aligned with our strain that has the same length 5229 bp, with 99.5% similarity in DNA sequences and 99.2% identity of amino acid sequences. The WU Polyomavirus DNA has been registered in the NCBI GenBank under the accession number LC771087.1 WU Polyomavirus BASRAH88 complete genome.

ACKNOWLEDGMENT

We would like to acknowledge the authorities in the College of Life Sciences and Basrah Teaching Hospital, Al-Fayhaa Teaching Hospital, Al-Sadr Teaching Hospital, and the Chest Diseases Center in Basrah City for providing all the facilities for sample collection and consulting of the clinicians.

CONFLICT OF INTEREST

We confirm that there are no known conflicts of interest associated with this publication and there has been no financial support for this work that could have influenced its outcome.

FUNDING

The work was entirely funded by the Ph.D. Akeel Abd Ali Al-Abadi.

ETHICAL APPROVAL

We followed the certified protocols and obtained ethical approval (No. 1010 in 5/10/2022) from the health office and the research unit in Basrah Health Department.

AVAILABILITY OF DATA

The data is available for any further communication.

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