RESEARCH ARTICLE

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Genotyping and Phylogenetic Analysis of Human Papillomaviruses in Formalin Fixed Paraffin Embedded Sections from Cervical Lesions in Duhok-Iraq

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Abstract

Objective: Human papillomavirus (HPV) has been identified as an important causative factor in cervical cancer development. Cervical cancer is the fourth most prevalent malignant tumor among women globally. The purpose of this study was to investigate the prevalence and genotyping sequences of HPV in formalin-fixed paraffin-embedded (FFPE) cervical tissue using conventional polymerase chain reaction (PCR), and HPV-DNA sequencing. **Material and Methods:** Retrospective cross-sectional study. Forty (FFPE) blocks with different cervical lesions were taken; patients' ages ranged from 24 to 65 years. Detection and sequencing of HPV DNA were done by conventional PCR (L1 gene), which was achieved by universal PCR primers (MY09/11 oligonucleotides). Then sequencing and phylogenetic tree was constructed. **Results:** Nine samples were found positive and detected by conventional PCR, they were identified in CIN1 and SCC at 7.5% (n=3) for each, 5.0% (n=2) KA, and 2.5% (n=1) in CIN3 cases, after sequencing were submitted to GenBank and accession numbers were obtained. The phylogenetic tree was constructed and the aligned sequences showed high homology with the nucleotide sequence of the references from the Genbank database. HPV 11, 16, 18, 22, 33, 52, and 58 were found to have little nucleotide heterogeneity and thus no amino acid heterogeneity. **Conclusion:** Sequencing and phylogenetic analysis of circulating HPV types in Duhok provides very essential data about nucleotides and amino acid heterogeneity, to reveal genetic diversity with strains included in the vaccines that have not been introduced to Iraq yet.

Keywords: Human papilloma virus- cervical lesions- molecular detection- DNA-sequencing- phylogenetic tree

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Introduction

HPV identified as an important causative factor in cervical cancer development (Jalilian et al., 2017; Kamal et al., 2021). Cervical cancer is women's fourth most prevalent malignant tumor globally (Bray et al., 2018). More than 200 genotypes of HPV have been identified based on DNA sequences (Mühr et al., 2018). HPV genotypes are divided into several categories. High-risk types (HR–HPV) are mainly 16 and 18, as well as 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82. HPV types 26, 53, and 66 were categorized as probably high-risk, and HPV types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, and 81 were categorized as low-risk types (LR-HPV) (Ehteram et al., 2019). Different studies from neighbor countries revealed that most frequent HR-HPV and LR-HPV associated with genital lesions are HPV16, HPV 6 respectively (Chalabiani et al., 2017). Infection with HPV is suspected by the presence of clinical lesions and by cytology, histology, and colposcopy, which are all subjective and often inaccurate (Liao and Li., 2019). Most cervical HPV infections are asymptomatic and the immune system naturally eliminated 70% of them within 12-24 months (Giuliano et al., 2011). The understanding that HPV is the major cause of cervical malignant tumors has led to the creation of advanced screening methods. Early diagnosis and treatment of precancerous conditions can restrain the progress of cervical cancer (Venkatas and Singh., 2020). The risk of precancerous lesions of the cervix in women is primarily explained by a cytological screening of cells. Molecular diagnostic tests that distinguish between high and low-risk HPV genotypes are highly sensitive and specific, detect HPV DNA, and are used as additional tests in cytological studies (Ghosh et al., 2014). DNA sequencing of purified DNA which is amplified by conventional PCR using consensus primers MY09/11 is another technique to detect the genotype and alignment with reference strains (Depuydt et al., 2007). Since the HPV vaccine is proved to be an efficient strategy to prevent cervical cancer in different countries therefore

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it is the next approach for control of these infections. The phylogenetic analysis wil help alignment of circulating strains with those included in HPV vaccine (Sopian etal., 2019). This study aimed to investigate the prevalence and genotyping sequences of HPV in samples obtained from FFPE cervical tissue by HPV-DNA sequencing.

Materials and Methods

Study design and population

This retrospective cross-sectional study included FFPE block samples of 40 patients with different cervical lesions in the period from July 2020 to December 2021. The samples were taken from archives of histopathology departments in Duhok central lab public health and VIN private laboratories. Patients' ages ranged from 24 to 65 years (Mean: 42 years STD ± 10.5).

Sample collection

The FFPE-fixed biopsy was sectioned. Five continuous sections with a thickness of 10 µm were taken from each biopsy, with a new blade for each one to avoid cross-contamination. Two outer sections were stained with hematoxylin and eosin and examined microscopically to confirm the diagnosis that was classified as; 27.5% (n=11) and showed Koilocytotic atypia (KA), 10.0% (n=4) unremarkable (non-neoplastic) pathology negative for Intraepithelial lesions or malignancy (NILM), 20.0% (n=8) Squamous cell carcinoma (SCC) Malignant, 22.5% (n=9) Cervical intraepithelial neoplastic (CIN1), 12.5% (n=5) CIN2, and 7.5% (n=3) CIN3.

DNA extraction

For Human papillomavirus (HPV) DNA extraction, three internal sections were placed in a 1.5 ml Eppendorf tube. Xylene (1ml) was added to the microtube containing the tissue section, deparaffinized, and vortexed for 10 seconds to mix vigorously. Then centrifuged at 20,000 rpm for 2 min and the supernatant was removed. This procedure was repeated 3 times to remove paraffin residue. The pellet was then washed 3 times with 1ml of ethanol (96-100%), centrifuged at 20000 rpm for 2 min, and the supernatant was then discarded. The pellet was air-dried at 37°C for 10-15 minutes until all residual ethanol had evaporated. After tissue deparaffinization, DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions.

HPV DNA detection and sequencing

The PCR was performed using consensus MY09/11 oligonucleotide primers to amplify the 450pb sequence from the L1 region of HPV. Amplification generally consists of a denaturation step were 40 cycles at 94°C for 1 min, followed by, an annealing step for 1 min at 55°C, an extension step for I min at 72°C, a final extension of 1 cycle at 72°C for 5 min (Venceslau et al., 2014) Table1.

To confirm the presence of HPV DNA fragments, 10µL of each PCR product was visualized on a 2% agarose gel electrophoresis using RedSafe Nucleic Acid (iNtRON Biotechnology) staining.

The PCR product samples were then sent for DNA sequencing by universal PCR primers (MY09/11) to Macrogen (Macrogen Co., Seoul, Korea). Multiple alignments were performed using BioEdit (version 7.2.5.0) software and submitted in Fasta format to the GenBank database and analyzed based on the best sequence homology search was conducted using BLAST (Basic Local Alignment Search Tool), which is available at the National Center Biotechnology Information (NCBI) online web site, and accession numbers (ACCN) were obtained. The phylogenetic tree was constructed by the maximum likelihood using MEGA 11.0 software and identified the evolutionary relationships between the analyzed sequences.

Results

In this study, among (n=40) FFPE cervical specimens, the L1 gene HPV was amplified by specific primers MY09/11. Nine samples were found positive and detected by PCR (Figure 1). Patients' ages ranged from 24 to 65 years (Mean: 42 years STD ± 10.5), and participants were classified into five age groups. HPV was most prevalent in the 31–40 age group and there were no significant differences (p= 0.909) (Table 2). The virus was identified in CIN1 and SCC at 7.5% (n=3) for each, 5.0% (n=2) KA, and 2.5% (n=1) in CIN3 cases (Table 3).

Sequence analysis and phylogenetic tree

The positive PCR products were sent for DNA sequencing, then sequencing was analyzed and submitted to GenBank in which the accession numbers (ACCN), OM963133, ON210811, ON221505, ON221506, ON221508, ON221509, ON221510, ON367531, and ON191582 were obtained. The results for all samples; showed the evolutionary relations between the studied strains with the nearest similar species of the HPV genus found in the Genbank data.

Sequences were compared with the data available at the NCBI-Genbank based on the highest percentage or lowest value. The ratio of similarity to the identified strains in the study with other strains from NCBI-Genbank ranged

Table 1. Genetic Oligonucleotide Primers Used for DNA of HPV Detection (Venceslau et al., 2014)

Primers	Sequence	Fragment Size	Protocol (PCR)	Conditions (PCR)
MY09 MY11	5'CGTCCMARRGGAWACTGATC3' 5'GCMCAGGGWCATAAYAATGG3'	450pb	15 mM MgCl2 (buffer 1×), 800 µM of dNTPs, 50 pmol/µl of each oligonucleotide primer, 1.25 U from Hot Star Taq DNA polymerase	40 cycles 94°C/1min 55°C/1min 72°C/1min 1 cycle 72°C/1min

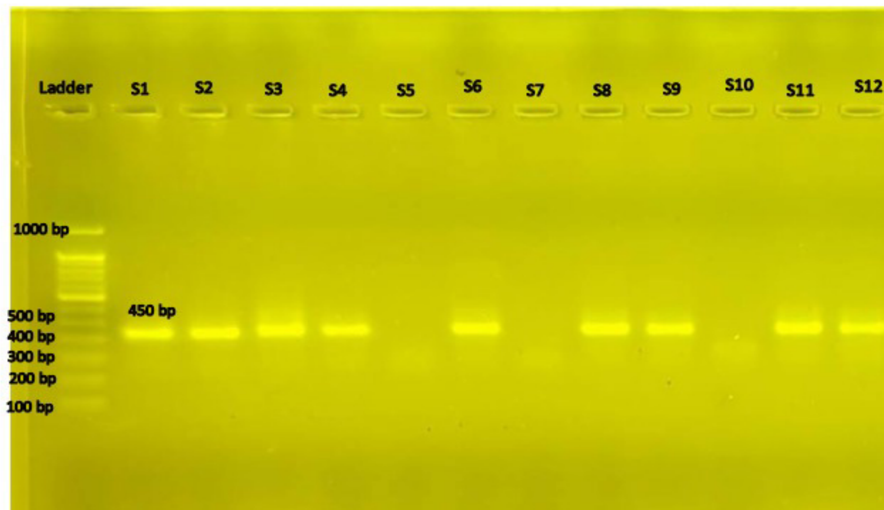

Figure 1. Agarose Gel Electrophoresis of Products of HPV PCR. Lane L, 1000bp DNA ladder; lanes 2,3,4,6,8,9,11, and 12, HPV positive samples contained 450bp amplicon from the HPV genome.

from 99-100% as in Table 4.

Two isolates of HPV16 (ON367531 and ON191582) were detected and the aligned sequence showed high homology with the nucleotide sequence of the references from the Genbank database (Figure 2). HPV18 was detected in one sample (ON210811) representing, the aligned sequence that showed high homology with the nucleotide sequence of HPV18 (MH057749) isolated in Saudi Arabia (Figure 3).

The results of the phylogenetic analysis showed that the isolated DNA of HPV33 in Duhok/Iraq (one sample) ON221508 has a kinship with those in GQ479014 Canada and GU797222 in the USA. The HPV52 identity in one case (ON221506) was the match found with the HPV52 L1 region (KT799936) isolated from Chinese patients having cervical cancer. The two HPV58 (OM963133

Table 2. HPV and Age Groups

Result	Age Groups N (%)					Total
	21-30	31-40	41-50	51-60	61-70	
Positive	1 (2.5%)	4 (10.0%)	3 (7.5%)	0 (0.0%)	1 (2.5%)	9 (22.5%)
Negative	4 (10.0%)	12 (30.0%)	9 (22.5%)	3 (7.5%)	3 (7.5%)	31 (77.5%)
Total	5 (12.5%)	16 (40.0%)	12 (30.0%)	3 (7.5%)	4 (10.0%)	40 (100.0%)

$\chi 2$ test used, P=0.909

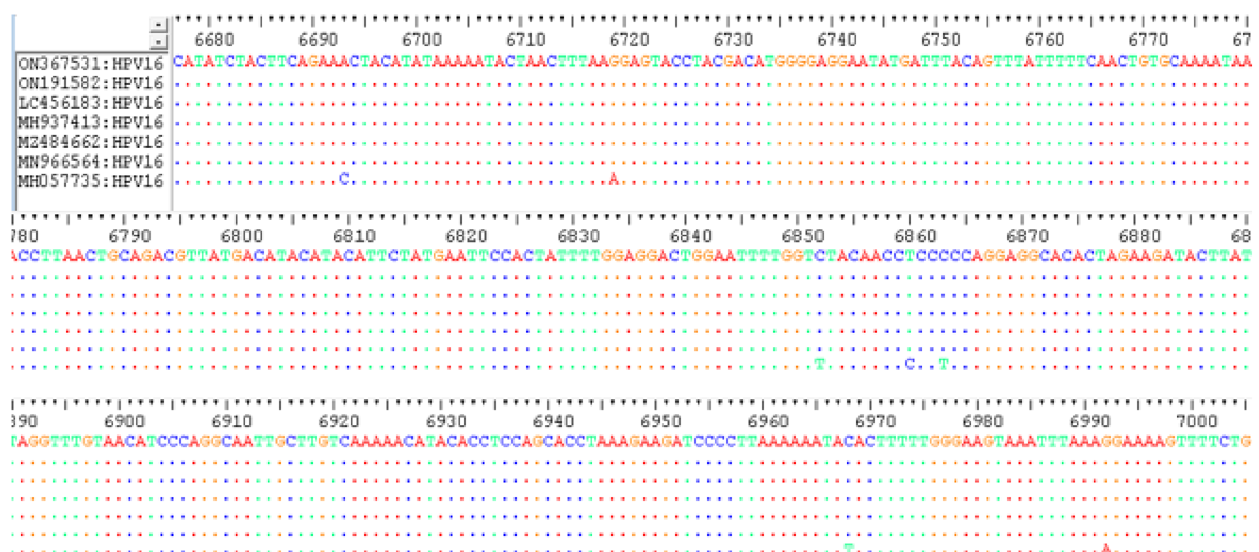

Figure 2. Two isolates of HPV16 (ON367531 and ON191582) were performed by sequence analysis after alignment (ClustalW) of the sequences with reference strains using BioEdit (version 7.2.5.0). The point mutation (base substitution) occurred at positions 6693, 6719, 6852, 6860, 6863, 6968, and 6992, to the reference sequence (ACCN: MH057735) isolated in Saudi Arabia.

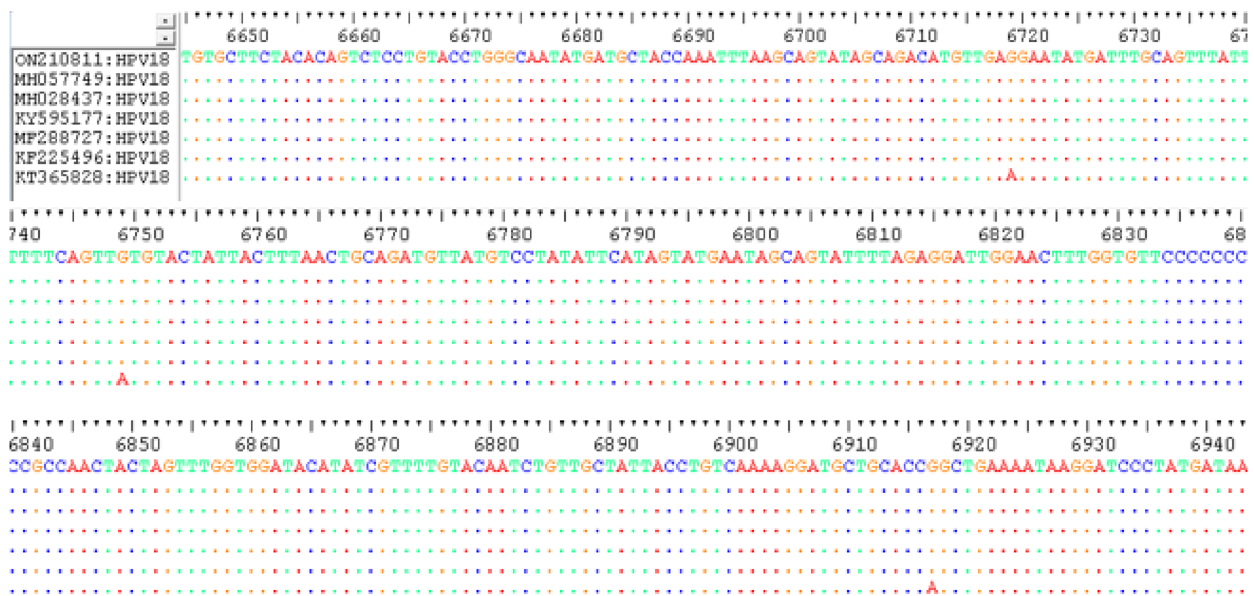

Figure 3. One Isolate of HPV18 (ON210811) was Performed by Sequence Analysis after Alignment (ClustalW) of the Sequences with Reference Strains Using BioEdit (version 7.2.5.0). The point mutation (base substitution) occurred at positions 6719, 6749, and 6917 to the reference sequence (ACCN: KT365828) isolated in Iraq.

and ON221509) identity matches were found with the HPV58 L1 region sequence (MT267729) isolated from Iran among patients with cervical cancer. The HPV11 (ON221510) identity match was found with L1 region sequence HPV11 (KU721777) isolated from China patients, while, HPV22 isolated in one case (ON221505) was found to match identity with HPV22 isolated from macules on the chest of an Italian epidermodysplasia verruciformis patient (Table 4).

A phylogenetic tree was constructed using the genomic sequence deposited under the ACCN, OM963133, ON210811, ON221505, ON221506, ON221508, ON221509, ON221510, ON191582, and ON367531 and analyzed against HPV genotype references sequences. Bootstrap test analysis was performed (1000 replicates) to estimate the reliability of the obtained branching patterns in the phylogenetic trees (Figure 4).

Discussion

There are 12.2 million females over 15 years of age in Iraq who are at risk of developing cervical cancer. Current estimates that 286 women are diagnosed with cervical cancer each year and 193 die from the disease. Cervical cancer is the 15th most common cancer in Iraqi women and the 12th most common cancer in women between 15-44 years of age (Bruni el al., 2021). Human papillomavirus (HPV) distribution varies in different populations, countries, and even in different parts of a country. Data on the burden of HPV on the Iraqi population are not yet available. However, in West Asia, including Iraq, an estimated 2.5% of women in the general population will always be infected with HPV 16/18 in the cervix at a given time, and 72.4% of (invasive cervical cancers will result in HPV 16 or HPV18 (Bruni el al., 2021).

The prevalence and detection of HPV in cervical cancer specimens using various technical tools have been published in many reports from Iraq, but an analysis of genetic diversity based on the L1 region in this study was done for the first time.

We found HPV DNA in just 22.5% of the specimens in our research, which is substantially lower than in previous PCR investigations. This result might be explained by inadequate DNA extraction from fixation. Fixing pathology specimens and embedding them in paraffin wax are critical stages in preparing tissues for microscopic examination and long-term storage

The MY09/MY11 consensus primer, which targets the conserved 450bp conserved sequence of the HPV L1 region, is one of the most commonly used in conventional PCR assays, and previous studies have shown that it has some limitations, especially a low sensitivity (Gravitt et al., 2000; Depuydt et al., 2007; Venceslau et al., 2014), therefore only nine cases were detected that

Table 3. Human Papilloma Virus Results and Morphologic Diagnosis

Results	morphologic diagnosis N (%)						Total
	NILM	KA	CIN1	CIN2	CIN3	SCC	
Positive	0 (0.0%)	2 (5.0%)	3 (7.5%)	0 (0.0%)	1 (2.5%)	3 (7.5%)	9 (22.5%)
Negative	4 (10.0%)	9 (22.5%)	6 (15.0%)	5 (12.5%)	2 (5.0%)	5 (12.5%)	31 (77.5%)
Total	4 (10.0%)	11 (27.5%)	9 (22.5%)	5 (12.5%)	3 (7.5%)	8 (20.0%)	40 (100.0%)

$\chi 2$ test used, p= 0.471

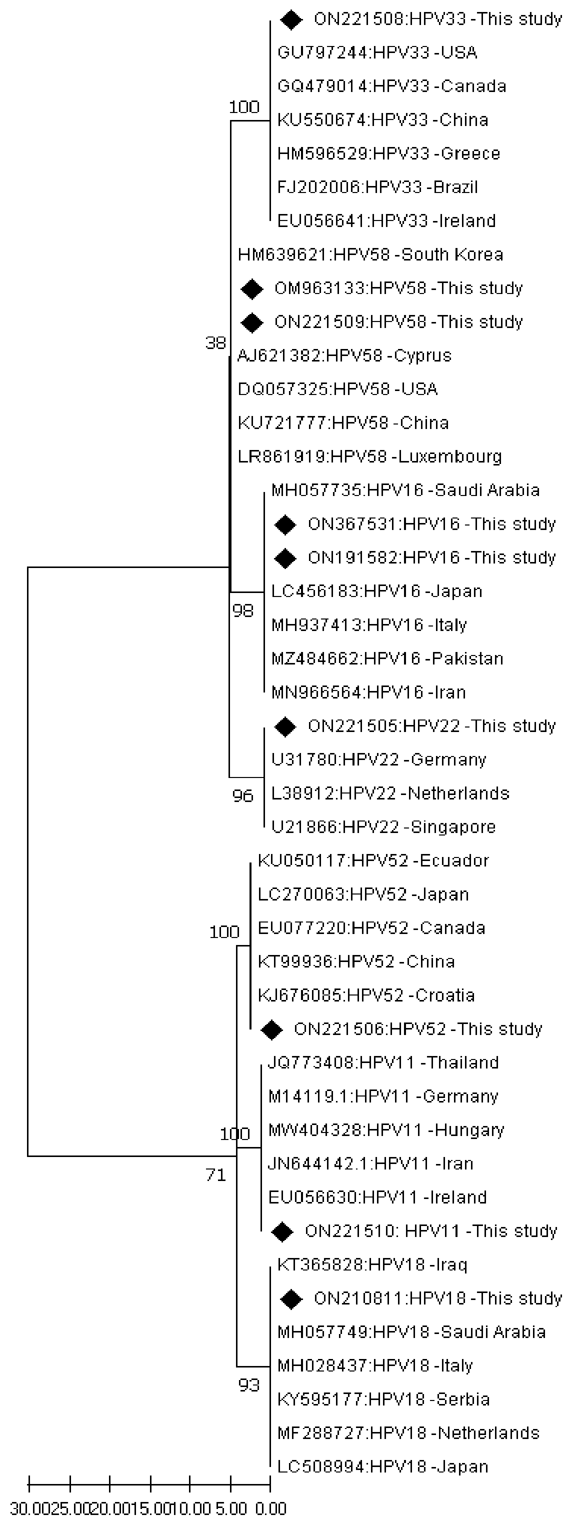

Figure 4. Phylogenetic Analysis of L1 Gene Using Selected HPV: The phylogenetic tree was constructed by the maximum likelihood method of MEGA 11.0 software, the percentage of the replication tree in which the relevant taxa are clustered together in the bootstrap test (1000 replications) is shown next to the branches.

means this method is less sensitive to detect multiple infections compared to other methods. Remmerbach et al, reported that HPV detection was higher for GP5+/GP6+ oligonucleotides than for MY09/MY11 oligonucleotides

Table 4. Representative Genotyping Analysis of HPV Sample Isolates based on Capsid Protein (L1) Gene According to a Phylogenetic Tree and NCBI –BLAST Genotypes Identity (%) Analysis

No.	Accession Number	Country	%Identities	Author, year
1	ON221510:HPV11	Duhok/Iraq		Current study, 2022
2	M14119	Germany	100.00%	(Dartmann et al., 1986)
3	JN644142	Iran	100.00%	(Eftekhaar et al., 2017)
4	MW404328	Hungary	100.00%	(Nagy et al., 2021)
5	JQ773408	Thailand	100.00%	(Chansaenroj et al., 2012)
6	EU056630	Ireland	99.74%	(Menton et al., 2009)
7	ON191582: HPV16	Duhok/Iraq		Current study, 2022
8	ON367531	Duhok/Iraq	100.00%	Current study, 2022
9	LC456183	Japan	100.00%	(Galati et al., 2019)
10	MH937413	Italy	100.00%	(Hirose et al., 2019)
11	MZ484662	Pakistan	100.00%	(Minhas et al., 2022)
12	MN966564	Iran	100.00%	(Mahmoudvand et al., 2022)
13	MH057735	Saudi	97.97%	(Sait et al., 2019)
14	ON210811: HPV18	Duhok/Iraq		Current study, 2022
15	MH057749	Saudi	100.00%	(Sait et al., 2019)
16	MH028437	Italy	100.00%	(Frati et al., 2020)
17	KY595177	Serbia	100.00%	(Kovacevic et al., 2019)
18	MF288727	Netherlands	100.00%	(van der Weele et al., 2018)
19	LC508994	Japan	100.00%	(Yamaguchi-Naka et al.,
20	KT365828	Iraq	99.05%	(Al-Malkey M. K, 2018)
21	ON221505: HPV22	Duhok/Iraq		Current study, 2022
22	U31780	Germany	99.74%	(Kremsdorf et al., 1984)
23	L38912	Netherlands	99.62%	(Berkhout, 1995)
24	U21866	Singapore	99.60%	(Chan et al., 1995)
25	ON221508:HPV33	Duhok/Iraq		Current study, 2022
26	GU797244	USA	100.00%	(Boyd et al., 2011)
27	GQ479014	Canada	100.00%	(Cornut et al., 2010)
28	KU550674	China	99.71%	(Chen et al., 2016)
29	FJ202006	Brazil	99.71%	(Raiol et al., 2009)
30	HM596529	Greece	99.12%	(Ntova et al., 2012)
31	EU056641	Ireland	99.12%	(Menton et al., 2009)
32	ON221506:HPV52	Duhok/Iraq		Current study, 2022
33	KJ676085	Croatia	100.00%	(Zhang et al., 2014)
34	KT799936	China	100.00%	(Zhang et al., 2016)
35	EU077220	Canada	100.00%	(Gagnon et al., 2007)
36	KU050117	Ecuador	100.00%	(Bedoya-Pilozo et al., 2018)
37	LC270063	Japan	100.00%	(Tenjimbayashi et al., 2017)
38	OM963133: HPV58	Duhok/Iraq		Current study, 2022
39	ON221509	Duhok/Iraq	100.00%	Current study, 2022
No.	Accession Number	Country	%Identities	Author, year
40	KU721777	China	99.76%	(Wang et al., 2016)
41	LR861919	Luxembourg	99.76%	(Latsuzbaia et al., 2020)
42	DQ057325	USA	99.76%	(Calleja-Macias et al., 2005)
43	AJ621382	Cyprus	99.28%	(Neophytou P.I., 2004)
44	HM639621	South Korea	99.04%	(Chan et al., 2011)

which can be explained by the small size of the DNA fragments amplified by the GP-PCR system (Remmerbach et al., 2004). The sensitivity and specificity of the PCR method depend primarily on the choice of oligonucleotide primers to be used, the number of base pairs in the product amplified by PCR, the performance of the DNA polymerase used in the reaction, the amplified DNA-HPV type spectrum, and the ability to recognize different types of infection (Iftner and Villa, 2003).

Considering the age, although statistically not significant, higher HPV rates were observed among young women (31–40 years of age). It represents the highest incidence at marriage age and after starting sexual activity due to some cultural customs and religious beliefs such as not having illegitimate sex, increased marriage age, and lack of immunity to HPV in the absence of a national vaccination program (Oztürk et al., 2004; Muderris et al., 2019). The gradual decrease in HPV infection rates among middle-aged and older individuals can be explained that young women's infections with HPV are transient and the immune system would eliminate the virus in most cases (Garbuglia et al., 2020; Ferrall et al., 2021).

Considering different morphologies, HPV DNA was identified in those with abnormal morphology. In Erbil-Iraq, 71% of cases with abnormal morphology were HPV DNA positive with the absence of this DNA among negative cases (Ismail et al., 2014), while in a previous local study (Duhok-Iraq), 46.2% of NILM cases and 53.8% of abnormal Pap cases were positive for HPV (Pity et al., 2019). Nevertheless, heterogeneous findings have been obtained among unremarkable and abnormal cases in different neighboring countries such as Saudi Arabia (28.6% and 53.1%) respectively and Kuwait (36.8% and 63.2%) respectively (Al-Awadhi et al., 2013; Al-Ahdal et al., 2014).

The highest identity (100.0%) of HPV16 detected in this study was observed with HPV16 (LC456183, MH937413) reported from Japan and Italy respectively. In addition, comparative nucleotide sequence analysis of the HPV18 L1 gene with retrieved HPV18 from Genbank showed variable sequence similarity ranging from 100.00% in Saudi Arabia to 99.05% in Iraq. This demonstrates that there was very little nucleotide heterogeneity, and therefore no amino acid heterogeneity, concerning HPV 16, 18, which is a very important result obtained from this study to align the circulating viruses with that included in the vaccine which is not introduced to Iraq yet.

In conclusions, the current work extends previous observations by providing baseline data on the circulation of HPV types among Duhok's women, and their association with normal and abnormal cytology with age. Primer configurations used in HPV DNA amplification are important to determine the sensitivity and specificity of PCR-based assays. This study provides very important information and a practical approach to genetic diversity and phylogenetic analysis, which can be very helpful in linking epidemiological studies as well as the natural history and evolution of HPV in Iraq and deciding the type of vaccine that would be introduced in the future.

Author Contribution Statement

OAA: Table development, editing the manuscript and writing; GAA: conceptualization, editing the manuscript and writing review; PIS: supervision, visualization, and review, all authors have read and approved the final version.

Acknowledgements

Ethical Approval

Ethical approval to carry out the study was obtained from the Medical Ethics Committee of the Duhok General Director of Health on 20/July/2020- Reference number (20072020-3). This study was approved by the scientific committee of the college of Medicine/ University of Duhok as Ph.D. project.

Data Availability

The data appeared in this research can be obtained from the corresponding author of the article

Conflict of Interest

We declare no conflict of interest associated with this research and there has been no significant financial support for this project that could influence its outcome.

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