ACTA MICROBIOLOGICA BULGARICA Volume 40 / 1 (2024)

Human Papillomavirus Genotyping among Women and its Relationship with Cervical Cancer in Diyala Province

Duha Salah Al-awsi[1*], Ibtihal Hameed Mohsin[1], Afraa M. Al-Naddawi[2], Huda Khalil Ibraheem[3]

[1]*Department of Biology, College of Science, University of Diyala, Diyala, Iraq*
[2]*Department of Obstetrics & Gynaecology, University of Baghdad, College of Medicine, Iraq*
[3]*Followship Iraqi Board Obstetrics and Gynaecology, Albatool Teaching Hospital*

Abstract

The study aimed to identify Human Papillomavirus (HPV) and its genotypes prevalent among Iraqi women. They collected 89 cervical swab samples from diagnosed patients at Baghdad Teaching Hospital's Early Detection Clinic. Using PCR technique on 19 samples, they found HPV16 (57.89%) and HPV6 (10.52%) genotypes, while HPV-11, 18, and 45 were absent. HPV 16 and HPV 6 were common in cervical cancer among Iraqi women. Sequencing revealed nucleic acid variants in HPV-6 (124A>C) and HPV-16 (225G>T) E6 genes, resulting in silent effects on the encoded protein. These changes did not alter amino acid residues (p.74I= and p.L117=). Phylogenetic analysis showed substantial distances between their samples and other viral types, indicating distinct phylogenetic distances between type-6, type-16, and other out-group sequences.

Keywords: cervical cancer, Human Papillomavirus, genotypes, PCR, phylogenetic tree

Резюме

Настоящото проучване има за цел да идентифицира човешкия папиломавирус (HPV) и неговите генотипове, разпространени сред иракските жени. Събрани са 89 проби от цервикален секрет от диагностицирани пациентки в Клиниката за ранно откриване на инфекции в Багдадската учебна болница. Използвайки PCR техника при 19 проби са установени генотипове HPV16 (57,89%) и HPV6 (10,52%), докато HPV-11, 18 и 45 отсъстват. HPV 16 и HPV 6 са често срещани при рака на маточната шийка сред иракските жени. Секвенирането разкри варианти на нуклеинови киселини в гените E6 на HPV-6 (124A>C) и HPV-16 (225G>T), които водят до беззвучни ефекти върху кодирания протеин. Тези промени не са променили аминокиселинни остатъци (p.74I= и p.L117=). Филогенетичният анализ показа значителни разстояния между техните проби и други вирусни типове, което показва отчетливи филогенетични разстояния между секвенциите на тип 6, тип 16 и други секвенции извън групата.

Introduction

Estimates indicate that in 2020, there will be 604 000 new cases and 342 000 deaths worldwide due to cervical cancer in women. Nearly 90% of new cases and deaths worldwide in 2020 were reported from low- and middle-income countries (Bhatla *et al*., 2021). Cervical cancer is the fourth most frequent malignancy in women worldwide, with an incidence rate of 13.3% and a fatality rate of 7.3 per 100,000 women. (Bautista *et al*., 2022). Other risk factors, including smoking, extended oral contraceptive use, coinfections, multiparity, and immune-related disorders, appear to direct the infection on the path to carcinogenesis, and HPV infection alone is insufficient to proceed to cervical cancer. High-risk HPV variants are frequently linked to invasive squamous cell carcinoma, while low-risk varieties are infrequently identified in some HPV genotypes that are common around the world. Numerous studies have really shown that HR-HPVs are the most common types, with the most common genotypes being 16, 18, 59, 45, 31, 33, 52, 58, 35, 39, 51, 56, and 53 in decreasing order of prevalence. According to (Kombe *et al*., 2021). HPV 6 and HPV 11 are the most com-

*Corresponding author: alawsiduha@gmail.com
Acta Microbiol. Bulg. 2024; 40(01). https://doi.org/10.59393/amb24400102

mon LR-HPVs and are mostly to blame for genital warts. E6, E7, and E5, three early HPV proteins, are crucial to the development of cancer (Al-Hadeithi *et al*., 2022). While E5 promotes keratinocyte differentiation and immune evasion, E6 and E7 proteins are associated with functional inactivation of the key cell cycle regulators, tumor transformation suppressors, and telomerase activation (Jensen *et al*., 2013). The study aimed to identify Human Papillomavirus (HPV) and its genotypes prevalent among Iraqi women.

Material and Methods

Collection of specimens

Eighty-nine female participants with various cervical lesions participated in the study, along with 20 healthy volunteers who served as the control group. All recruited subjects ranged in age from 25 to 80. All of the cases who were included in the study visited the gynecology and obstetrics at Teaching Hospital in Baghdad, Iraq, as well as the pediatrics and gynecology clinics in Diyala and Medical City. In addition to Pap smears, all patients got comprehensive clinical examinations from a specialized gynecologist, who also conducted interviews with them all.

The final step involved making the complete nucleotide sequence of the HPV L1 gene and genes that oncoprotein E6 and E7 degrade tumor suppressor p53 and pRb. The principle of the assay was the detection of Human Papillomavirus (HPV) by conventional Polymerase Chain Reaction to determine the distribution of HPV genotype.

Extraction and genotyping of HPV_DNA DNA

According to the instructions of the Maxwell® 16 Viral Total Nucleic Acid Purification Kit (Automated extraction), genomic DNA was recovered from the virus sample.prmega, USA The simple protocol involves three main steps.

1. Lysis buffer and proteinase K are mixed to prepare a lysis solution.
2. Lysis solution is mixed with sample.
3. The lysate is added into the cartridges. Purified viral total nucleic acids are ready for analysis in approximately 45 minutes.

Primer preparation

Two pairs of PCR-specific primers were designed in this study to partially cover a portion of the E6 gene; the gene encodes the transforming protein to assess the pattern of the nucleic acids and their consequent amino acid variations in the analyzed viral sequences. NCBI-Primer blast server was used in this A highly specific online tool was used to perform this task (Ye *et al.,* 2012).

The forward and reverse primer sequences for human papillomavirus type-6 were 5- TGCAAGAATGCACTGACCAC-3 and 5-TGCATGTTGTCCAGCAGTGT-3, respectively. A PCR fragment with a length of 334 bp was created using the NCBI-Primer blast server's usual default parameters. The forward and reverse primer sequences for human papillomavirus type-16 were 5- CAGTTACTGCGACGTGAGGT-3 and 5-ACAGCTGGGTTTCTCTACGTG-3. The NCBI-Primer blast service was used to create a 349 bp long PCR fragment using the default default settings. Following PCR amplification, the presence of amplification was confirmed using agarose gel electrophoresis. The standards for the recovered DNA were the only ones used for PCR. The PCR product was directly put in 5 l to the well. Electricity was turned on for 60 minutes at 100v/mAmp. DNA moves from the cathode to the positive anode poles. The gel bands stained with Ethidium bromide were observed using the Gel Imaging apparatus. PCR amplicon sequencing for nucleic acids According to the instructions of the sequencing business (AniCon Labour GmbH), the resolved PCR amplicon was sequenced commercially beginning at the front. By contrasting the observed nucleic acid sequences of the local viral sample with the retrieved nucleic acid sequences, it was possible to determine the virtual positions and other details of the retrieved PCR fragments.

Analysis of sequencing data

Using the sequencing findings of the PCR products from the targeted sample were edited, aligned, and analyzed with the corresponding sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The sequenced sample's detected changes were given numbers in both their corresponding positions in the referencing genome and the PCR amplicon. The detected nucleic acids were assigned numbers in both their respective locations in the reference genome and the PCR amplicon. The viral sequences under investigation were entered into the NCBI-bank database with a special accession number for the examined sample.

In this investigation, two distinct PCR fragments were created, each of which partially covered the coding areas of the E6 gene in type-6 and type-16 human papillomaviruses. Human papillomavirus type-6 had two isolates (designated S1 and S2) that had the E6 gene amplified, while type-16

had ten isolates (designated S1 to S10) that had the E6 gene amplified. The amplified fragments were directly subjected to Sanger sequencing tests in order to assess the pattern of genetic polymorphism in connection to the samples that were referenced in the NCBI database. The precise type and phylogenetic distribution of the discovered variants were then assessed using a specific comprehensive tree that was built.

Result and Discussion

Molecular diagnosis of Human Papilloma (HPV) in cervical cancer patients by conventional PCR

Molecular detection was performed to determine the prevalence and distribution of the prevailing genotypes in our current study of the virus. Our current study was positive for the HPV16 genotype (57.89%) and for the HPV6 genotype (10.52%) but negative for the genotypes (HPV-11, 18, 45) (Table 1).

In our present investigation of the virus, molecular detection was used to as certain the prevalence and distribution of the dominant genotypes. The HPV16 genotype (57.89%) and the HPV6 genotype (10.52%) in our most recent study were both positive, however, the HPV-11,18,45 genotypes were not.

tween the reference amino acid sequences and their reported mutant equivalent was carried out using the "align" script of the BioEdit service.

Creation of an extensive phylogenetic tree

For both human papillomavirus fragments type-6 and type-16, two specific comprehensive trees were constructed in this study and the observed variants were compared with their neighbor homologous reference sequences using the NCBI-BLASTn server (Zhang *et al*. 2000). Then, a full inclusive tree, including the observed variant, was built by the neighbor-joining method and visualized as a classical rectangular cladogram and circular cladogram using the iTOL suit (Letunic and Bork, 2019). The sequences of each classified phylogenetic group in the comprehensive tree were colored appropriately to indicate the corresponding viral type incorporated within the generated phylogenetic tree.

Sequencing interpretation analysis

Concerning the E6 locus within the human papillomavirus type-6, two samples were included in the present study, as assigned as S1 and S2 samples. These samples were screened to amplify E6 gene sequences of the Human papillomavirus type-6. The variation of the E6 gene can be used

Table 1. Distribution of HPV Genotypes in cervical diseases patients were according to social factors

				Cervical diseases PCR HPV (19)	*P* value
Genotypes HPV PCR	+ve	N	HPV-6	2 (10.52%)	< 0.001
			HPV-16	11 (57.89%)	
			HPV-11, 18, 45	0 (05)	
			Total	13	
			Total	68.42%	
	-ve	N		6	
		%		31.58 %	
			Total	6	
			Total	19 (100%)	

Amino acid residues are created from several forms of nucleic acids

The amino acid sequences of the targeted E6-encoded transforming protein were downloaded from the protein data bank website (http://www.ncbi.nlm.nih.gov). The identified nucleic acid changes in the coding areas were translated into a reading frame that matched the necessary amino acid residues in the encoded protein using the Expasy online translator (http://web.expasy.org/translate/). Multiple amino acid sequence alignment be-

for human papillomavirus typing due to its possible ability to adapt to variable genetic diversity as it was seen in different viral serotypes. The sequencing reactions indicated the exact identity after performing NCBI blast for these PCR amplicons (Ye *et al*., 2012). The NCBI BLASTn engine revealed up to 99% sequence similarity between the sequenced S1 and S2 samples and the targeted reference target sequences of human papillomavirus type-6 concerning the 334 bp amplicon. The exact locations and other features of the retrieved PCR

fragments were determined by contrasting the observed and recovered nucleic acid sequences from the examined sample (GenBank accession number MK313779.1). Using the NCBI server, the targeted locus' full length was calculated, and the viral target with the highest degree of homology's start and end positions was confirmed (Fig. 1A).

Concerning the 349 bp amplicon, the NCBI BLASTn engine found up to 99% sequence similarity between the sequenced S1–S10 samples and the specified reference target sequences of human papillomavirus type-16. By comparing the observed and obtained nucleic acid sequences with those of the examined sample, it was also possible to pinpoint the positions and other details of the retrieved PCR fragments (GenBank accession number NC_001526.4). The full length of the targeted locus was discovered in the NCBI server, and the viral target's start and end positions were confirmed with the maximum level of homology. (Fig. 1B).

ment of thymine for guanine in the 124th position, or 225G>T.

Translation of nucleic acids to amino acids

The positions of the analyzed nucleic acid sequences in the transforming protein were determined through further analysis of the sequences. In the full transforming protein of the human papillomavirus type-16, it was discovered that the represented fragment stretched from the amino acid residues 43 to 158. In the full transforming protein of the human papillomavirus type-16, it was discovered that the represented fragment stretched from the amino acid residues 43 to 158 (Fig. 2). All of the ribosomal sequences under investigation were uploaded to the NCBI web server and given individual accession codes. To represent the S1 and S2 samples of the human papillomavirus type-6, GenBank OQ918080 and OQ918081 were deposited in NCBI, respectively. Regarding GenBank OQ918082, OQ918083, OQ918084, OQ918085,

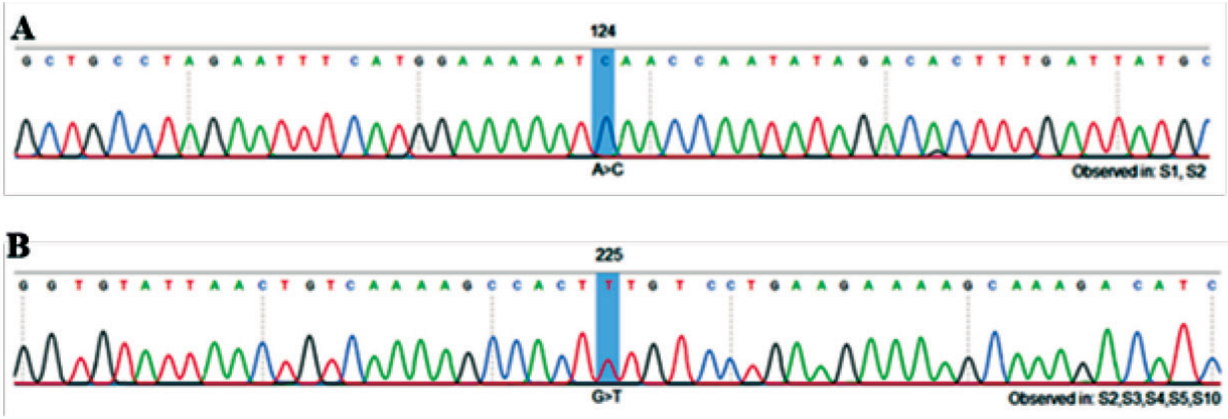

Fig. 1. The human papillomavirus types-6 and 16 in branches A and B of the chromatogram of the viral samples under investigation.
The code for the study's examined samples is denoted by the sign "S#".

The alignment results of the 334 bp samples revealed the presence of one nucleic acid variation in comparison with the most similar referring reference nucleic acid sequences of human papillomavirus type-6 (GenBank acc. no. MK313779.1). This variant was identified in both S1 and S2 samples. The alignment results of the 349 bp samples also revealed the presence of one nucleic acid variation in comparison with the most similar referring reference nucleic acid sequences of human papillomavirus type-16 (GenBank acc. no. NC_001526.4). This variant was identified in S2, S3, S4, S5, and S10 samples.

The discovered variant for human papillomavirus type 6 was indicated by the replacement of cytosine for adenine in the 124th position, or 124A>C. The discovered variant for human papillomavirus type-16 was indicated by the replace-

OQ918086, OQ918087, OQ918088, OQ918089, OQ918090, and OQ918091 S1, S2, S3, S4, S5, S6, S7, S8, S9, and S10 samples of human papillomavirus type-16, respectively, were each deposited in NCBI (Table 2).

Phylogenetic analysis

Type 6 Human papillomavirus

A thorough phylogenetic tree was created in the current study based on nucleic acid variations found in the amplified 334 bp of the E6 gene amplicons of human papillomavirus type-6 to provide a phylogenetic understanding of the actual distances between our investigated sample and the other viral types. Samples S1 and S2 under investigation within this main clade displayed a modest tilt in comparison to the other neighboring *H. papillomavirus* type-6 sequences (Fig. 3 and Table 3).

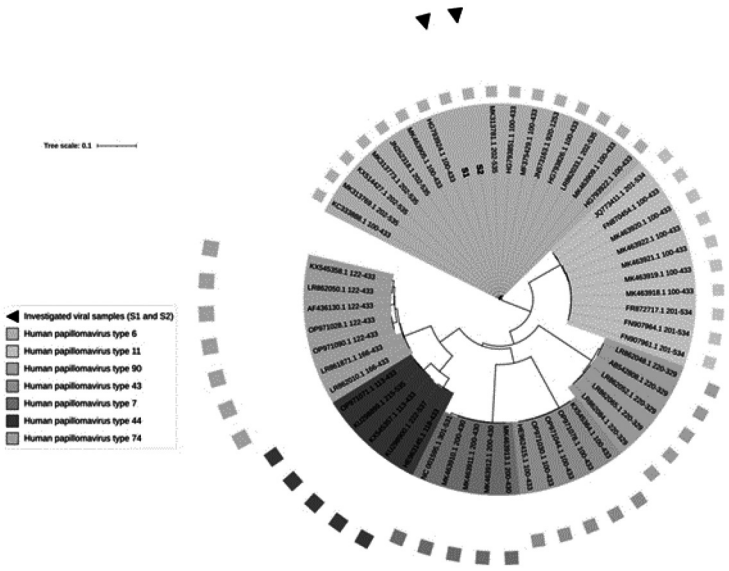

Fig. 2. The extensive circular cladogram evolutionary tree of human papillomavirus type-6 genomic different E6 gene segment variants.

The triangle in black indicates the analysed viral variants. All of the figures corresponded to the referred species' GenBank entries. The number "0.1" at the top of the tree indicates the full degree of scale range for all tree-categorized creatures (6). The letter "S#" stands for the code for the sample that is the subject of the examination.

Table 2. Comparing the pattern of the mutations found in the human papillomavirus type-6 and type16 PCR products to their respective NCBI reference sequences (GenBank accession numbers MK313779.1 and NC_001526.4, respectively)

No.	Sample	Native	Allele	Position in PCR fragment	Position in the protein	Variant summary
1	S1, S2	A	C	125	74	125A>C (p.74I=)
2	S2, S3, S4, S5, S100	G	T	225	117	225G>T (p.L117=)

The silent effect of the identified variant is denoted by the sign "=".

Type-16 Human papillomavirus

The genetic diversity of human papillomavirus type-16 taken from ten patients with cervical cancers was also analyzed using phylogenetic tree construction. Human papillomavirus type 16 is a well-studied and clinically significant type of HPV that is strongly associated with the development of cervical cancer and other types of anogenital and oropharyngeal cancers. While HPV-16 is highly prevalent, it exhibits a relatively low level of genetic diversity compared to other viruses. This is likely because HPV-16 has a relatively small genome (~8,000 base pairs), a slow mutation rate, and a predominantly clonal mode of transmission (Mobini Kesheh *et al*., 2022).

Despite its low genetic diversity, HPV-16 still exhibits some variations in its genetic sequences that can be used for phylogenetic analysis. Phylogenetic analysis of HPV-16 sequences from around the world has identified five distinct lineages or subtypes, each with different geographical and ethnic distributions. Phylogenetic analysis of HPV-16 sequences from around the world has identified five distinct lineages or subtypes, each with different geographical and ethnic distributions. The most common HPV genotypes in invasive cervical cancer worldwide are 16, 18, 31, 33, 35, 45, 52, and 58 (Wang *et al*., 2022).

The distribution of HPV subtypes also exhibits regional differences, with HPV16 being the predominant type in Beijing and Jiangsu province, while HPV52 was the most common type in Shanghai and Zhejiang province. In a study of HPV-positive cases, HPV16 was the most prevalent subtype, followed by HPV52 and HPV58. These findings highlight the importance of understanding the distribution of HPV subtypes in different regions to inform the development of effective prevention and treatment strategies. Additionally, phylogenetic analysis has been used to identify specific amino acid changes in the E6 oncoprotein of HPV-16 that were associated with differences in cervical cancer risk (Xi *et al*., 2007).

Accordingly, studying the phylogenetic anal-

Table 3. The location and size of the 247 bp PCR amplicon utilised to amplify a section of the E6 gene's coding regions in the type-6 and type-16 human papillomavirus genomes

Amplicon	Sequences of the reference locus (5'–3)'	length
Sequencing of the E6 gene in the human papillomavirus type-6	TGCAAGAATGCACTGACCACTGCAGAGATTTATTCA TATGCATATAAACAGCTAAAGGTCCTGTTTCGAGGC GGCTATCCATATGCAGCCTGCGCGTGCTGCCTAGAA TTTCATGGAAAAATTAACCAATATAGACACTTTGATT ATGCTGGATATGCAACAACTGTTGAAGAAGAAACTA AACAAGACATTTTAGACGTGCTAATTCGGTGCTACCT GTGTCACAAACCGCTGTGTGAAGTAGAAAAGGTAAA ACATATACTAACCAAGGCACGGTTCATAAAGCTAAA TTGTACGTGGAAGGGTCGCTGCCTACACTGCTGGAC AACATGCA	334 bp
E6 gene sequences found in type-16 human papillomavirus	CAGTTACTGCGACGTGAGGTATATGACTTTGCTTTTC GGGATTTATGCATAGTATATAGAGATGGGAATCCAT ATGCTGTATGTGATAAATGTTTAAAGTTTTATTCTAA AATTAGTGAGTATAGACATTATTGTTATAGTTTGTAT GGAACAACATTAGAACAGCAATACAACAAACCGTTG TGTGATTTGTTAATTAGGTGTATTAACTGTCAAAAGC CACTGTGTCCTGAAGAAAAGCAAAGACATCTGGACA AAAAGCAAAGATTCCATAATATAAGGGGTCGGTGGA CCGGTCGATGTATGTCTTGTTGCAGATCATCAAGAAC ACGTAGAGAAACCCAGCTGT	349 bp

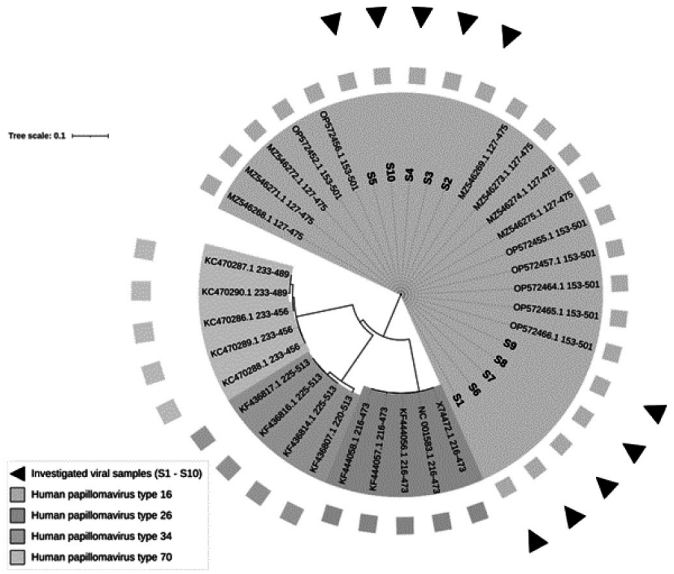

Fig. 3. The complete circular cladogram evolutionary tree of the human papillomavirus type-16's E6 gene sequences.

It is noteworthy that the study's use of the E6 gene sequences has provided additional evidence for the presence of the accurate identification of the true type of this viral organism. This in turn provides more evidence that the E6 gene-specific primers now in use may accurately define the human papillomavirus under investigation and its phylogenetic locations.

ysis of HPV-16 can provide valuable insights into the genetic diversity and evolution of the virus, as well as its clinical and epidemiological significance. However, it is important to use phylogenetic analysis in conjunction with experimental and clinical studies, to fully understand the biology and pathogenesis of HPV-16 infections.

Phylogenetic trees are a powerful tool for visualizing the genetic relationships between different types of human papillomaviral sequences. Several studies have used phylogenetic analysis to investigate the genetic diversity of human papillomavirus type-16 isolates obtained from different clinical sources. These studies have identified different lineages or subtypes of HPV-16, which have different geographical and ethnic distributions.

For example, a study conducted in Saudi Arabia found that HPV-16 isolates from Jeddah were

distributed in six lineages, including A1, A2, A4, B1, C, and D2 (Sait *et al.,* 2019). This study found that HPV-16 and HPV-18 are known as high-risk types associated with the development of cervical cancer. Thus, phylogenetic analysis has been a useful tool for understanding the genetic diversity and distribution of HPV-16, which can inform the development of effective prevention and treatment strategies.

Conclusion

The study found that HPV-16 and HPV-6 were the most prevalent genotypes, suggesting a heightened risk of cervical cancer among the women examined.

Sequencing the E6 gene emerged as a robust method for identifying and characterizing clinical samples of both HPV-6 and HPV-16, offering advantages like high sensitivity, specificity, low cost, and quick results.

The proximity of the investigated HPV-6 samples to European strains and HPV-16 samples to Asian strains suggests a likely European and Asian clinical origin, respectively, indicating the geographical source of these HPV types in the studied samples.

Acknowledgments

My gratitude and thanks to Diyala University, College of Sciences, as well as to Baghdad Teaching Hospital staff for supporting me in the collection of samples.

References

Ahmed, S., A.Vajeeha, M. Idrees, A. Hussain, R. Munir, G. Zaidi, K. Zahid, R. Ahmed, Z. Fatima, S. Rafique, N. M. Achakzai (2023). Genotypic distribution of human papillomavirus and phylogenetic analysis of E6 and E7 gene of HR-HPV variants isolated from Pakistani population. *Medicine* **102**: e32651. https://doi.org/10.1097/MD.0000000000032651.

Al-Hadeithi, Z. S., S. A. Jasim, O. D. Salahldina (2022). Detection and genotyping of Papillomavirus by Real-Time PCR in Iraqi patients. *Arch. Clin. Infec.t Dis.* **17**: e121143. https://doi.org/10.5812/archcid-121143.

Bautista-Valarezo, E., B. Vega Crespo, R. Maldonado-Rengel, M. E. Espinosa, V. A. Neira, V. Verhoeven (2022). Knowledge and perceptions about cervical cancer and HPV screening in women in Rural areas of Ecuador: A qualitative research study. *Int. J. Environ. Res. Public Health* **19**: 11053. https://doi.org/10.3390/ijerph191711053.

Bhat, P., S. R. Mattarollo, C. Gosmann, I. H. Frazer, G. R. Leggatt (2011). Regulation of immune responses to HPV infection and during HPV-directed immunotherapy. *Immunol. Rev*. **239**: 85-98. https://doi.org/10.1111/j.1600-065X.2010.00966.x.

Frati, E. R., S. Bianchi, A. Amendola, D. Colzani, F. Petrelli, G. Zehender, E. Tanzi (2020). Genetic characterization of variants of HPV16, HPV18 and HPV52 circulating in Italy among general and highrisk populations. *Mol. Med. Rep.* **21**: 894-902. https://doi.org/10.3892/mmr.2019.10847.

Johansson, C., S. Schwartz (2013). Regulation of human papillomavirus gene expression by splicing and polyadenylation. *Nat. Rev. Microbiol*. **11**: 239-251. https://doi.org/10.1038/nrmicro2984.

Kombe Kombe, A. J., B., Li, A. Zahid, H. M. Mengist, G. A. Bounda, Y. Zhou, T. Jin (2021). Epidemiology and burden of Human Papillomavirus and Related diseases, molecular pathogenesis, and vaccine evaluation. *Front. Public Health.* **8**: 552028. https://doi.org/10.3389/fpubh.2020.552028.

Letunic, I., P. Bork, (2019). Interactive Tree Of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Res*. **47:** W256–W259. https://doi.org/10.1093/nar/gkz239.

Mobini Kesheh, M., M. Barazandeh, A. Kaffashi, M. K. Shahkarami, S. A. Nadji (2022). Genetic diversity of HPV 16 and HPV 18 based on partial long control region in Iranian women. *Can. J. Infect. Dis. Med. Microbiol*. **2022**: 4759871. https://doi.org/10.1155/2022/4759871.

Sait, K., R. Turki, A. M. Abuzenadah, O. H. Jiffiri, A. Bohmaidah, S. S. Sohrab (2019). Genetic diversity and phylogenetic analysis of HPV 16 & 18 variants isolated from cervical specimens of women in Saudi Arabia. *Saudi J. Biol. Sci*. **26**: 317-324. https://doi.org/10.1016/j.sjbs.2018.05.005.

Wang, X., S. Han, X. Li, X. Wang, S. Wang, L. Ma (2022). Prevalence and distribution of human papillomavirus (HPV) in Luoyang city of Henan province during 2015-2021 and the genetic variability of HPV16 and 52. *Virol. J.* **19**: 37. https://doi.org/10.1186/s12985-022-01759-5.

Xi, L. F., L. A. Koutsky, A. Hildesheim, D. A. Galloway, C. M. Wheeler, R. L. Winer, J. Ho, N. B. Kiviat (2007). Risk for high-grade cervical intraepithelial neoplasia associated with variants of human papillomavirus types 16 and 18. *Cancer Epidemiol. Biomarkers Prev*. **16**: 4-10. https://doi.org/10.1158/1055-9965.EPI-06-0670.

Ye, J., G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen, T. L. Madden (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**: 134. https://doi.org/10.1186/1471-2105-13-134.

Ye, M., S. Li, P. Luo, X. Tang, Q. Gong, B. Mei (2022). Genetic variation of E6, E7, and L1 genes of human papillomavirus 51 from central China. *J. Med. Virol*. **94**: 2811-2823. https://doi.org/10.1002/jmv.27603.

Zhang, Z., S. Schwartz, L. Wagner, W. Miller (2000). A greedy algorithm for aligning DNA sequences. *J. Comput. Biol*. **7**: 203-214. https://doi.org/10.1089/10665270050081478