

Genotyping and Phylogenetic analysis of human Papillomaviruses from women with genital warts

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ABSTRACT

Background and Objectives. The pathophysiology of Human Papillomavirus (HPV) infections is dependent on the genotype of virus, the host immune system and the local environmental conditions. HPV infections are linked to a variety of benign and malignant disorders. Anogenital warts is a Sexually Transmitted Infection (STI) primarily caused by Low-Risk HPV (LR-HPV) genotypes 6 and 11. The aims were to Investigation of the prevalence and genotypic distribution of HPV in women with genital warts in Mosul/Iraq, HPV-DNA sequencing and Phylogenetic analysis including minimum spanning trees of amplified HPV L1 gene comparison the present

sequences with similar genotypes in ⁴ GenBank – National Center Biotechnology Information (NCBI) using mega -7- software.

¹⁷ **Materials and methods.** A total of 150 samples were collected from women with genital wart infections. ²⁶ Polymerase Chain Reaction (PCR) was performed using GP+⁵/GP+6 and MY09/MY11 consensus primers.

Results. About 84.6 % prevalence of HPV genotypes. The highest frequency (32% and 28.6%) were observed in the 19–28 and 29–38 age groups.

Conclusion. Women in Mosul, Iraq, who have genital warts are more likely to have different genotypes of HPV. HPV 11 type was found to be the most common, followed by HPV6 and HPV10. The analysis of the phylogenetic tree revealed a genetic relationship between our isolates and global isolates ranging from (10% -100%) bootstrap ratio this indicating phylogenetic relationships (divergent and convergent evolution) this was meant the diversity in origin of the various genotypes common in Mosul /Iraq.

Key Words: HPV, genital warts, DNA-sequencing, genotypes, phylogenetic tree

⁶ Abbreviations: (HPV) Human Papilloma Virus, (STI) Sexually Transmitted Infection, (LR-HPV) Low-Risk HPV, (HR-HPV) High-Risk HPV, (CC) Cervical Cancer, (NCBI) National Center Biotechnology Information, ¹ (PCR) Polymerase Chain Reaction, (IARC) International Agency for Research on Cancer, (BLAST) Basic Local Alignment Search Tool

Introduction

The pathophysiology of HPV infections is dependent on the genotype of virus, the host immune system and the local environmental conditions, HPV infections are linked to a variety of benign and malignant disorders, anogenital warts is a STI primarily caused by LR-HPV genotypes 6 and 11, however, co infections with HR-HPV genotypes can also be observed([1]. HPV has been found to play a significant role in the development of Cervical Cancer CC [2]. CC is the fourth most prevalent disease in women globally and is caused by a prolonged infection with HR-HPV specifically with HPV 16 and HPV 18 [3]. According to the most recent global burden of cancer study, roughly 600,000 incident cases and 340,000 deaths annually are caused by CC, which also poses a significant risk to global health [4]. To date about 450 HPV genotypes following their identification and sequencing have been classified as either HR-HPV or LR-HPV genotypes [5]. The International Agency for Research on Cancer (IARC) classified HR-HPV to be an oncogenic HPV genotypes, the most common HR-HPV genotypes responsible for causing cancer are HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59, which are often linked with penile cancer, the second most common genotypes linked to potential carcinogenicity are HPV 68, 26, 53, 66, 67, 70, 73, 82, 30, 34, 69, 85 and 97 which were considered to be potentially carcinogenic [6] LR-HPV genotypes included HPV genotypes 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, and 81 [7]. LR-HPV leads to minor benign

hyperproliferative lesions, while HR-HPV mostly leads to malignant lesions [8]. The most common HR-HPV and LR-HPV linked to genital lesions, according to various studies from neighboring countries, are HPV16 and HPV6, respectively [9,10]. In most patients HPV infection is asymptomatic and can be controlled by the immune system [11]. Modern screening techniques have been developed as a result of the realization that HPV is the primary cause of malignant cancers in the cervical region. CC can be prevented from progressing if precancerous symptoms are identified early and treated [12]. For accurate identification and genotyping of HPV molecular tests are necessary [13]. Additional testing in cytological investigations are molecular diagnostic tests that identify HPV DNA and differentiate between HR-HPV and LR-HPV genotypes these tests are very sensitive and specific [14]. PCR methods or gene amplification originally needed a small portion of the genome sequence an exponential amplification step is performed on a characteristic of the HPV genome sequence *in vitro*, allowing its duplication for identification purposes [15]. To identify the genotype and alignment with reference strains, another method is DNA sequencing of pure DNA amplified by traditional PCR using consensus primers GP+5/GP+6 and MY09/MY11, which yield 150 and 450 bp amplicons, respectively [16].

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Materials and methods

Sample collection

A total of one hundred fifty specimens (PBS embedded tissue, cervical swab, and cervical brush) were taken from women with genital warts who were referred to Al-Zahrawy private Hospital and Mosul General Hospital

in Mosul/Iraq from August/2023 to April/2024. And their ages range between (19- 58) years old.

²⁹ DNA Extraction

Using a QIAamp DNA mini kit, DNA was extracted from the genital warts (tissue), cervical swab and cervical brush. following the manufacturer's instructions (QIAGEN, Germany). All the extracted DNA was stored at -20 °C until analyzed by PCR.

Identification and sequencing of HPV DNA

The PCR was performed using consensus GP+5/GP+6 and MY09/MY11 oligonucleotide primers to amplify the 150 bp and 450 bp, respectively (Table 1). The steps involved in amplification typically include a step of denaturation with 40 cycles at 94 °C for 5 min and a step of annealing at 48 °C (for GP+5/GP+6 and 55 °C for MY09/MY11) for 0.45 sec. Lastly, there is an extension step lasting 0.45 seconds at 72 °C.

¹¹ Table 1. Primers used in this study

Primers	Sequence	Size (bp)	Reference
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MY09	F	5-CGTCCMARRGGAWACTGATC-3	450	[17]
MY11	R	5-GCMCAGGGWCATAAYAATGG-3		
GP+5	F	5-TTTGTTACTGTGGTAGATACTAC-3	150	[18]
GP+6	R	5-GAAAAATAAACTGTAAATCATATTC-3		

Four μ l of PCR product was analyzed by electrophoresis on 2% w/v agarose gel containing 1 μ l of Red Safe Nucleic Acid (diamond nucleic acid dye, Promega). HPV genotyping was performed by sequencing GP+5/GP+6 and MY09/MY11 PCR in Macrogen (Macrogen Co., Seoul, Korea). The software BioEdit (version 7.2.5.0) was used to perform multiple alignments. After that, the Fasta format files were uploaded to the GenBank database, where the BLAST was used to do the best sequence homology search, which is available at the NCBI online website, The phylogenetic tree was constructed by bootstrap (100X) analysis using the MEGA-11 software [19].

Results

PCR was used to amplify the HPV L1 gene using specific primers MY09/MY11 450 bp and GP+5/+6 150 bp. from 150 samples from women with genital warts. our study on molecular detection of HPV PCR, which was performed in Mosul, Iraq, Only 127 cases (84.6%) were isolated and identified (Table 2), the types of samples collected were variable among patients. In the current study, we used three types of samples (PBS-embedded tissues, endo cervical swabs, and cervical brush) to ensure the presence of viruses. Patients' ages ranged from 19 to 58 years old. and participants were classified into four age groups the variable distribution of

HPV positives across all age categories, with the highest frequency (32% and 28.6%) occurring at the 19–28 and 29-38 years age.

Table 2. Shows the frequency of HPV primers

Age Groups	PCR Results				Total	
	Positive		Negative			
	N.	%	N.	%	N.	%
19-28	48	32	3	2	51	34
29-38	43	28.6	4	2.7	47	31.3
39-48	21	14	6	4	27	18
49-58	15	10	10	6.7	25	16.7
Total	127	84.6	23	15.4	150	100

The DNA sequencing of the positive PCR products was followed by analysis. The results for all samples demonstrated the evolutionary relationships between the strains under study and the closest related species of the HPV genus found in the Genbank data. The Nucleotide Basic Local Alignment Search Tool (BLAST) program [20] was used to search for homology to the input sequences against entire sequences that are available on the sequence NCBI GenBank database. according to the present sequence alignment results, all isolates are HPV-positive, with a similarity ratio between the query and subject sequences of 93–100%, as indicated in (Table3) Based on the Capsid Protein (L1) Gene, Representative Genotyping Analysis of HPV Sample Isolates was conducted.

Table 3. Genotyping Analysis of HPV Sample Isolates based on Capsid Protein (L1) Gene and make homology against entire sequences, which are available on the sequence, NCBI GenBank database

No.	Genotypes	Ident per %	Accession Number	Country	Year
5	HPV6	98.79	ON053201.1	Iran	2022
6		99.24	KC706454.1	Saudi Arabia	2013
1	HPV 10	98.32	OP971088.1	South Africa	2022
14	HPV11	96.23	KM501531.1	Thailand	2014
15		99.03	KR674044.1	Kenya	2015

1. Human papillomavirus type 6 isolate 7 major capsid protein L1-like (L1) gene, partial sequence
 Sequence ID: [KC706454.1](#) Length: 403 Number of Matches: 1

2. Range 1: 2 to 398 [GenBank](#) [Graphics](#)

Score	Expect	Identities	Gaps	Strand
715 bits(387)	0.0	394/397(99%)	2/397(0%)	Plus/Plus
Query 14	AATTACCTCCC-AAAAC	TAAGGTTCTTATAGGGATCTGGCTTTTCCTTTTCAGGAGTGGG		72
Sbjct 2A.....		61
Query 73	CTTTTGACAGGTAATGGCCTGTGACTGCACATACCTATAGGTATCTTCTAATGTACCATT			132
Sbjct 62		121
Query 133	TGGGGGAGGCGATAACCAAAGTTCAGTC TTCAAAAACAGAGGGATTTCATTGTGTGAAT			192
Sbjct 122		181
Query 193	ATAGGCCAT TACTTCAGCAGACAATGTAATGCTACATAATTGAAAAATAAATGTAAATC			252
Sbjct 182		241
Query 253	ATACTCTCCACATGACGCATGTACTCTTTATAATCAGAATGGTGTATGTGGAAGATGT			312
Sbjct 242		301
Query 313	AGTTACGGATGCACATAATGTCATGTTGGTACTGCGTGTGGTATCTACCACAGTAACAAA			372
Sbjct 302		361
Query 373	CAGTTGATTACCCCAACAAATACCATTATTAT-ACCC		408	
Sbjct 362G.....G.....	398	

the genetic variation in the nucleotide sequence which was relatively few is due to the occurrence of some point mutation (insertion, deletion, and base substitution) as (Figure 1)

Figure 1. The isolate of HPV 6 (HPV No. 6) using MY09/11 primer was performed by gene sequence alignment with reference strains in NCBI. **The point mutation** (deletion and base substitution) occurred at positions 13, 389, and 394 to the reference sequence (ACCN: KC706454.1) isolated in Saudi Arabia.

A phylogenetic tree (Figure 2) was built using sixteen typical HPV L1 gene sequences from the current study and thirty-one sequences of other HPV genotypes that were obtained from the NCBI nucleotide databank based on percentage similarity. Using the MEGA-11 program and bootstrap (100X) analysis, the phylogenetic tree was created [19].

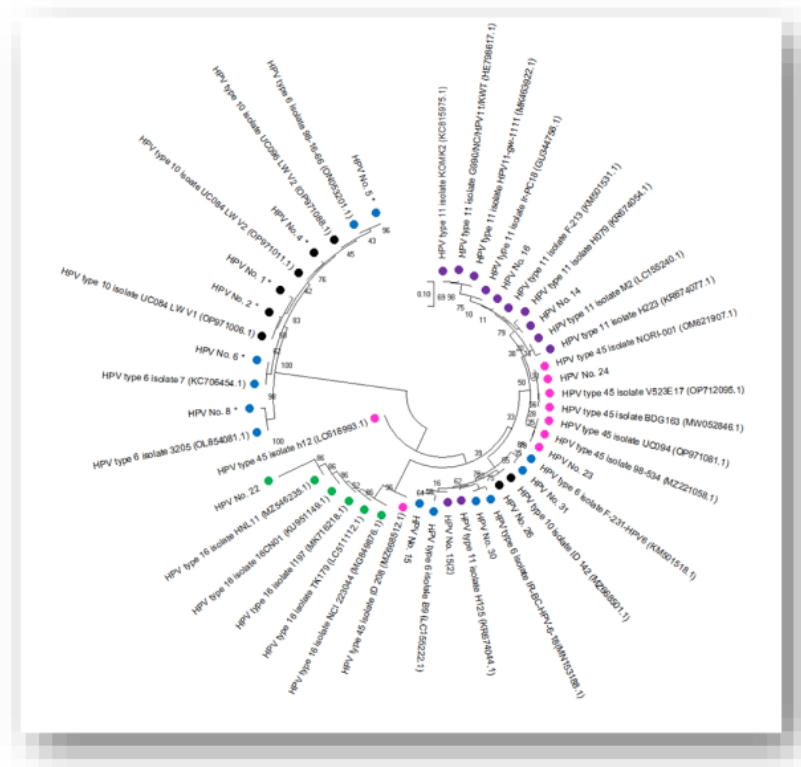


Figure 2. Neighbor-joining phylogenetic trees showing the relationship between our results indicate as (HPV No.) based on MY09/11 sequences with an asterisk and GP+5/+6 using MEGA-11 software with a scale length of 0.1 and the reference HPV types obtained from the NCBI Genbank database each color indicate different genotypes.

Discussion

The anogenital HPV burden varies across different geographic groups and even within the same country's diverse areas. and also genital warts were considered HPV-related geographic regions and/or population subgroups [21,22]. The highest frequency (32% and 28.6%) occurring at the 19–28 and 29-38 years age, So there was a tendency for women in the third and fourth decades to have greater viral infections. because this time sexual activity begins and marriage becomes more popular. as well as sinful illicit sex [23,24] HPV infections are transient, which explains the progressive decline in HPV infection rates among middle-aged and older people and the potential that certain tested and immunized women may eventually have a stronger immune system, which would, in most circumstances, eliminate the virus cases [25, 26]. Among the most widely used primers in traditional PCR experiments is the MY09/11 consensus primer, which targets the 450 bp conserved sequence of the HPV L1 region. Its drawbacks, particularly its low sensitivity, have been demonstrated by earlier research [27-29]. This was enhanced by missing a positive case [30]. Because the GP-PCR method amplifies tiny DNA fragments, it is possible to explain why the detection of HPV was higher for GP+5/GP+6 oligonucleotides than for MY09/MY11

oligonucleotides [31]. Viruses evolve genetically through a variety of mechanisms including Evolutionary fitness, Recombination, and mutations; Successful reproduction is referred to as evolutionary fitness. Viruses can go through natural selection-based evolution to maximize their fitness since they are self-replicating creatures with a genotype/phenotype relationship. ²² The prevalence (frequency) of an HPV type in the host population can be used to determine its fitness. Prevalence is a function of both ²⁰ incidence (rate of successful transmission to new hosts) and persistence (length of productive infection). HPV genomes have low mutation rates because they replicate using host DNA polymerases [32]. The analysis of the phylogenetic tree (Figure 7) revealed a genetic relationship between our isolates and global isolates ranging from (10% -100%) bootstrap ratio this indicating phylogenetic relationships (divergent and convergent evolution) with types related to the same genotype (intra typic) and with types related to the different genotype(extra typic), The sequences from Iran, South Africa, Kenya, France, Pakistan, China, and Indonesia have coevolved with various HPV types were grouped to many clusters with our isolates of five HPV genotypes 6,10,11,16 and 45 (each genotype has a distinct color). The genotype clustering suggests a taxonomical separation of HPV types, and the intra-typic variations also demonstrate a very low level of genomic diversity. ² The phylogenetic tree showed that HPV 6 (HPV No 6) isolated in this study was close match to reference strain (ACC. OL854081.1) globally isolate of China 2021 while HPV11 (HPV No.16) showed the lowest similarity with global isolates of Iran 2009 (ACC. GU344758.1), China 2019 (ACC. MK463922.1) and Kuwait 2012 (ACC. HE798617.1). These findings suggest that nucleotide heterogeneity. The potential for viral isolates from other nations that were spread by the entry of infected individuals into the

country might be the reason for the discrepancy in replicate ratios between our local isolates. This, however, is in line with research by [33-35] which highlights the significance of nucleotide polymorphism and mutational changes in viral genes rather than the fact that geographic distance does not entirely influence the distribution of HPV types. The variation analysis corroborates a study by Bernard *et al.* 2010 [36], which found that differences in the genetic makeup of the women from whom HPV was isolated could account for disparities in HPV strains. Similarly, the study by Xi *et al* 2003 [37], which found that differences in the genetic makeup of the host contribute to disparities in HPV strains.

Conclusion

According to the current study, women in Mosul, Iraq, who have genital warts are more likely to have different strains of HPV. HR HPV varieties are very low. LR HPV type 11 was shown to be the most common HPV genotype, followed by HPV types 6 and 10. The partial L1 region's sequencing revealed that it was extremely conserved across all sequences that have been published. The prevalence of different HPV types in this study emphasizes the need for more research on the factors that influence HPV infection and its associated risk factors, including previously unrecognized factors like race and ethnicity, income and socioeconomic status, marital status, sexual partners, education level, and use of contraceptives. The information is important for the creation of novel HPV screening tests as well as for evaluating the impact of upcoming vaccinations on infections with varying degrees of severity. The report also emphasizes the necessity of routine HPV screening to prevent the problem from getting out of control and leading to cervical cancer. Additionally, prompt and

accurate HR-HPV diagnosis may help in ¹ the early detection and prevention of cervical cancer in the area.

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