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The Molecular Detection of HPV Infection in samples of Iraqi Women with Abnormal cervical Smears

Mais N. Abdul-samad*, Nuha J. Kandala
Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq

Abstract

Human papillomavirus (HPV) types 16 and 18 cause almost 70% of cervical cancer cases worldwide. Recently, testing for high-risk HPV types have been adopted by clinical practices for the early detection of cervical cancer in conjunction with cytology tests.

Cervical swab samples were collected at the Outpatient Gynecology department of Baghdad Teaching Hospital. These samples consisted of a patient group of 50 samples, and a healthy control group of 10 samples. A papanicolaou test (abbreviated as a Pap test) was also performed for each woman to examine the epithelial cells of both the endocervix and the upper vaginal region. Total DNA (genomic, mitochondrial, and viral) was extracted from cervical swab samples for molecular studies.

HPV DNA testing was first done by using Real-Time PCR technology to target the L1 region of HR-HPV with specifically designed primers. This was followed by using AmpliSens kit for specific detection and genotyping of HPV16 and 18 with multiplex Real-Time PCR.

The results of RT-PCR detection revealed that out of 16 samples detected with high-risk HPV, 5 samples were shown to be infected with HPV-16 and 5 samples were shown to be infected with HPV-18. These results show a significant relationship between the histological outcome of the patient and persistent HPV infection.

Keywords: Cervical Cancer, HPV, Real-Time PCR, Cervical Intraepithelial Neoplasia

االكشف الجزيئي عن الاصابة بفيروس الورم الحليمي البشري في مسحات غير طبيعية لعنق الرحم لعينات مأخوذة من نساء عراقيات

ميس نمير عبد الصمد*، نهى جوزيف قندلا
قسم التقنيات الاحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق

الخلاصة

فيروس الورم الحليمي البشري (HPV) نوع 16 و 18 يسببان حوالي 70% من حالات سرطان عنق الرحم حول العالم. مؤخرا تم الجمع بين الكشف الجزيئي لثلاثة عشر نوع من الانواع الخطيرة (High-Risk types)لهذا الفيروس مع التحليل السايتولوجي في الممارسات الطبية حول العالم.

تم جمع العينات (مسحات من عنق الرحم) من العيادة الاستشارية النسائية التابعه لمستشفى مدينة الطب في بغداد. تم تقسيم هذه العينات الى مجموعتين، الاولى تكونت من 50 عينه مرضية، و المجموعة الثانية تكونت من 10 عينات سليمه لنساء لا يعانين من اي مشاكل. اختبار الفحص المبكر عن سرطان عنق الرحم تم استخامه كذلك لفحص منطقة العنق الداخلي و المنطقه المهبلية العليا. تم استخلاص الحمض النووي الرايبوزي منقوص الاوكسجين (DNA) من جميع العينات. بعد ذلك تم استخدام تفاعل البلمرة المتسلسل اللحظي-(Real Time PCR) للكشف عن منطقة) (L1) الخاصة بالانواع الاكثر الخطورة لهذا الفيروس. بالاضافه الى ذلك تم استخدام تفاعل البلمره المتسلسل اللحظي للكشف المحدد عن فيروس الورم الحليمي البشري نوع 16 و 18 باستخدام (AmpliSens Detection Kit)

*Email: missanameer@gmail.com

اظهرت نتائج تفاعل البلمرة المتسلسل اللحظي ان المجموع الكلي لحالات الاصابه بالانواع الاكثر خطورة من الفيروس بلغ عددها 16، منها 5 حالات شخصت بالاصابه بفيروس (HPV-16) و 5 حالات شخصت بالاصابه بفيروس (HPV-18) .هذه النتائج تظهر وجود علاقه هامه بين الاصابة المستمره بفيروس الورم الحليمي البشري و النتائج الهستولوجيه الناتجه عن هذه الاصابه.

Introduction

Cervical cancer is caused by the presence of HPV cells in the cervix which invades the cervical epithelia and may continue to spread to other body parts [1]. Worldwide, cervical cancer is the second most common type of cancer behind breast cancer [2]. Carcinogenic types of human papillomavirus (HPV) cause most cases of cervical carcinoma and are highly abundant in young woman [3]. Vaccination against HPV infection is highly efficient and may prevent up to 90% of cervical cancers [4].

World Health Organization 2014 [2] established guidelines for cervical cancer control based upon promoting public health education and screening by pap smears, HPV detection, the visual inspection of the cervix in and colposcopy [5]. HPV cycle produces two important oncogenic products, E6 and E7 [6]. The products of these genes bind to and inactivate tumor suppressor genes, resulting in the disruption of the host cell cycle [7].

The most vital risk factor for cervical carcinoma is infection with high-risk HPV [8]. High-risk HPV constitute a group of over 15 genotypes, most significant of those are the carcinogenic HPV types 16 and 18 which are highly related to cervical cancer [9].

Precancerous conditions in the cervix are caused by a persistent HPV infection [10], and they may include the fairly reliable morphological diagnoses of cervical intra-epithelial neoplasia (CIN) which include the less histopathologically extensive CIN1 and CIN2. And the severe case of dysplasia CIN3 in which the entire layer of squamous epithelia is covered with undifferentiated cells with fixed genetic abnormalities [11].

Human papillomavirus (HPV) is one of the most common causes of sexually transmitted disease in both men and women worldwide. HPV is a member of the *Papovaviridae* family, it is a relatively small, nonenveloped virus, 55 nm in diameter [12]. It is assumed that the HPV replication cycle begins with entry of the virus into the basal layer cells of the epithelium usually via mild abrasion or microtrauma of the epidermis. Once inside the host cell, HPV DNA replicates as the basal cells differentiate and progress to the surface of the epithelium. Since HPVs encode only 8 to 10 proteins, they must employ host cell factors to regulate viral transcription and replication. HPV replication begins with host cell factors which interact with the LCR region of the HPV genome and begin transcription of the viral E6 and E7 genes [13].

Cervical cancer is a lengthy process which arises via a series of steps which start with HPV transmission and end with progression of pre-cancerous lesions [1]. In terms of histopathology, precancerous lesions may likely develop to cancer over time. However, the HPV oncogenes are essential to maintain the cancer and they must be transcribed constantly by the HPV cycle [8].

Molecular diagnosis of HPV infection is dependent upon techniques such as DNA hybridisation or nucleic acid amplification. Several polymerase chain reaction (PCR) methods have been developed to detect a broad spectrum of HPV types using either degenerate or consensus primers [13]. A second generation commercial hybridisation assay, Hybrid Capture™ (HCA II), is also available for the detection of HPV DNA in cervical swab samples, and has been used widely in epidemiological studies. However, both consensus PCR and HCA II have important limitations, neither technique can differentiate between individual types or detect infection with more than one type [14].

Rapid real time PCR can distinguish closely related sequences as it combines simultaneous PCR amplification with sophisticated computer analysis of the kinetics data generated. In the real-time PCR the amplified product is detected with the use of fluorescent dyes. These dyes are linked to PCR primers and oligonucleotide probes which bind specifically to the amplified product during thermocycling allowing for accurate detection of viral presence in the sample [15]. Thus, the use of real-time PCR technique has been implemented in screening programs as it offers a highly sensitive rout for rapid qualitative detection and genotyping of HR-HPV types in clinical samples [14].

The aim of this study is the molecular detection of HR-HPV by using Real-Time PCR Technology, and to investigate the relationship between HPV infection and variable histological stages of cervical squamous neoplasia, cervical cancer and precancerous lesions in Iraqi women complaining from

different gynecological problems, whose pap smears revealed abnormal findings using RT- PCR technique.

Materials and Methods

Materials

Sample of Clinical Collection

All samples were collected at the Outpatient Gynecology department of the Baghdad Teaching Hospital/Iraq from women complaining from different gynecological problems, whose cervical smears revealed abnormal findings. The patient group consisted of 50 samples. While a group of healthy control consisted of 10 samples collected from women with no evident gynecological problem. The samples are cells scraped from the cervix and collected from both patient and control. A papanicolaou test (abbreviated as a Pap test) and a histological examination were performed for each woman by a doctor and the results were obtained from the oncology department of Baghdad Teaching Hospital.

DNA Preparation

Total DNA (genomic, mitochondrial, and viral) was isolated from cervical swab samples for molecular studies following standard protocol for extraction assumed by many molecular studies. Genomic DNA isolation was achieved by using (AmpliSens® *HPV16/18-FRT PCR Kit*/Russia). DNA extraction kit to the all collected sixty samples (patients and control). Nanodrop instrument (Nas-99/China) was used to determine DNA concentration and purity. Furthermore, the Beta-globin gene was selected as an internal control signal to ensure presence and integrity of DNA in cervical swab samples and was detected using conventional PCR (Labnet International/USA). Presently, the housekeeping gene (Beta globin) is being used in molecular diagnosis to verify that the PCR conditions are optimum, and are thus known as amplification controls [16].

Multiplex Real-Time Polymerase Chain Reaction (PCR) for detection of High-Risk HPV

First, multiplex Real-Time Polymerase Chain Reaction (PCR) for High Risk- HPV was done by using specific primers and a probe designed specifically for our study for the detection of the L1 region of all HR-HPV Table-1. The reaction mixture consisted of 3 µl DNA template, 0.5µl of each primer, 0.5µl of probe and 10µl of BrightGreen Express 2X qPCR mastermix in total volume of 20 µl.

The template DNA was amplified for 40 cycles of denaturation programmed for 45seconed at 95°C, annealing of primers at 55°C programmed for 45 sec and extension at 72°C programmed for 45sec .Fluorescent data were acquired during each extension phase [15].

Table 1-The oligonucleotide sequences of primers and probes for RT-PCR detection of L1-HR-HPV gene

Name	Sequence(5' → 3')	Size
L1-HR_HPV	CCGTCAGGTACTTTGGAGGA CGTCCAAGGGGATCTGATCT	450 bp
L1-HR_HPV	GATGACCCATATGCCAAGCT	

Second, multiplex RT-PCR for detection of HPV 16 and18 was carried out by using specific kit for detection of High-risk HPV 16 and 18 which was designed by AmpliSens-Russia following the manufacturer's instructions. The kit contains multiple primers and probes designed specifically to target precise regions for HR-HPV etiology. A specific program designed by the kit for optimum detection was followed by RT-PCR, while measuring fluorescence continuously by using dyes specific for HPV detection channels.

The template DNA was amplified for 5 cycles of denaturation programmed for 5seconed at 95°C, annealing of primers at 60°C programmed for 20 sec and extension at 72°C programmed for 15sec, and for 40 cycles of denaturation programmed for 5seconed at 95°C, annealing of primers at 60°C programmed for 30 sec and extension at 72°C programmed for 15sec Fluorescent data were acquired during each extension phase. This program was followed according to kit's instructions for optimum detection of the target.

Statistical Analysis

The Statistical Analysis System- SAS (2012) program was utilized to effect of difference factors in the study parameters. Chi-square test was also used to significant compare between percentages in this study [17].

Results and Discussion

A total of 60 samples of cervical swabs were collected by a doctor at the outpatient gynecology department of Baghdad Teaching Hospital. These samples were divided into two groups:-

- Patient group: consisting of 50 women complaining from different gynecological problems, whose pap smears revealed abnormal findings.
- Control group: consisting of 10 healthy women with normal Pap smear results.

Histological Examination Results

Results of histological examination for each sample were obtained from the oncology department of Baghdad Teaching Hospital. The analysis of the results followed the guidelines of the cervical intraepithelial neoplasia (CIN) classification system which was evolved in 1968, to take into account the different natural histories seen with different degrees of dysplasia (ranging from CIN1/mild, to CIN2/moderate, and CIN3/severe dysplasia), CIN is graded into 3 groups, CIN 1−3 according to the degree of proliferation of atypical basal cells and the presence of mitotic figures [18]. The distribution of sample study according to the histology examination is shown in Table-2

Table 2-Distribution of the samples according to histological examination.

Histology	Number	Percentage (%)
Normal	10	16.66
Non-cancerous conditions	31	51.66
CIN I	7	11.66
CIN II	4	6.66
CIN III	3	5.00
Carcinoma	5	8.33
Total	60	100%
Chi-Square (χ^2)	---	11.483 **
P-value	---	0.0001

**This high value of Chi-Square indicates a relatively high incidence of sample distribution in the environment.

A study conducted by **Mezaal, (2012)** [19] showed similar results in histological distribution of samples collected from Iraqi women with abnormal Pap smears.

For grading of CIN the squamous epithelium is divided into 3 thirds. The atypical basal and parabasal cells involve the basal third in CIN 1, the basal and the middle third in CIN 2 and more than two thirds in CIN 3. In particular, CIN 1 and 2 are not well defined since the presence of mitosis as well as koilocytotic changes are considered further diagnostic criteria. For the diagnosis of CIN 3 the presence of mitoses in the superficial third of the epithelium is considered helpful. In contrast to CIN 1 and also CIN 2, CIN 3 lacks a significant amount of koilocytes [18].

DNA Extraction

Total DNA (genomic, mitochondrial, and viral) was isolated from cervical swab samples for molecular studies and it showed a concentration ranging from 0.8–3.7 µg/ µL (Figure-1)

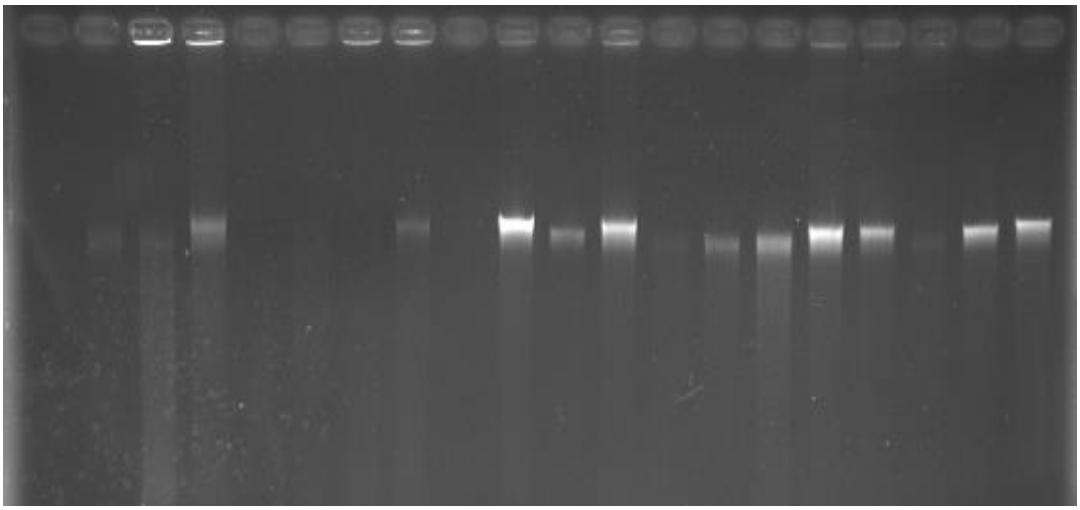

Figure 1-Total DNA bands on 1% agarose gel at 100 volt for 20min. DNA sample were extracted from Pap smear samples .*(beta-globin)*

Detection of Internal Control Beta-globin Gene by conventional Polymerase-Chain Reaction technique

The Beta-globin gene was selected as an internal control signal to ensure presence and integrity of DNA samples. First PCR technique was used to detect the (158bp) target. All 60 samples were detected following the extraction process and they all showed identical cohesive bands for the *beta-globin* gene, Figure-2

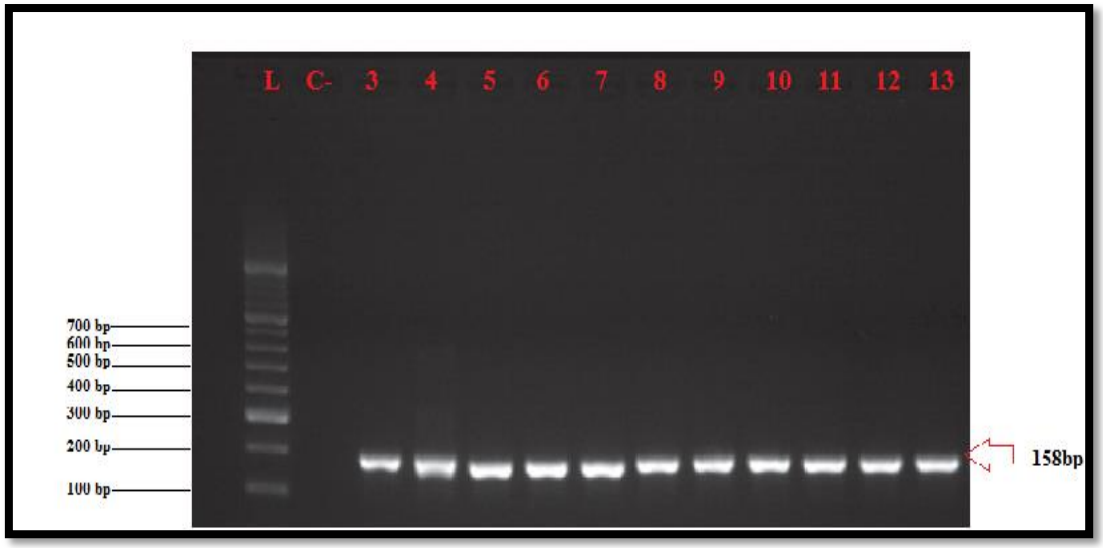

Figure 2- Gel electrophoresis products (158bp) for the internal control gene (beta-globin), on 1.5% agarose at 70V for 1.5h, bands were visualized under U.V. light following EB staining . Lane (1): Ladder (100-1500bp) ; Lane (2): Negative control ; Lanes (3-13): Different samples showing bands for the internal control gene**.**

Multiplex Real-Time PCR for Detection of High-risk HPV

This study utilized Multiplex Real-Time PCR (Biometera/Germany) technique for the qualitative detection of HR-HPV by targeting the L1 region of HR-HPV with a specific set of primers and probe designed specifically for our study. The results show that out of a total of 60 samples including (50 patients and 10 controls), 16 samples were positive for high-risk HPV. ROX fluorophore channel was used (Figure-3) to detect the L1 region of HR-HPV in the samples.

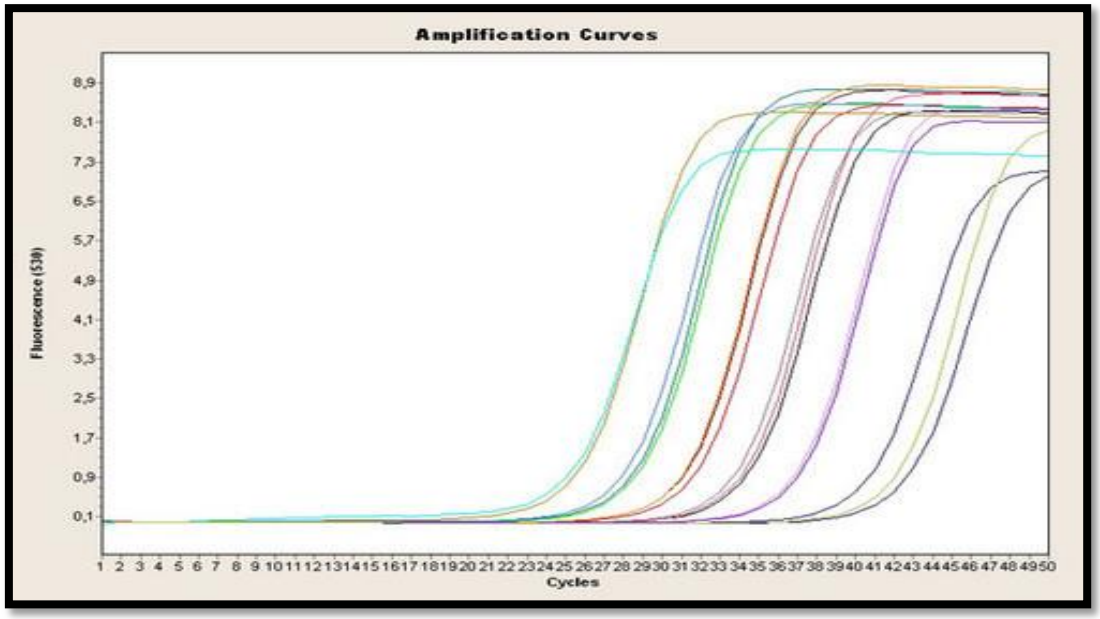

Figure 3- Amplification curves in semi-logarithmic view obtained from target DNA. Each curve represents a sample positive for HR-HPV based on CT values. HPV High Risk (HR) as general detection without genotyping, channel for RoX fluorophore. C+ curve: Positive for internal control.

Real Time PCR for qualitative detection and genotyping of HPV

The AmpliSens Kit for detection of HR-HPV 16 and 18 was used for the qualitative detection and genotyping of those two types, considering these two genotypes are proven the most abundant in cases ranging from cervical abnormalities to cervical carcinoma [9].

The HPV High Risk 16/18 Detection kit includes two vital steps, DNA isolation from samples and multiplex Real Time amplification of the sample. The kit used three channels for reading three different types of dyes (FAM, ROX and JOE) to detect both HPV16, HPV18 and the internal control gene Beta-Globin respectively, as shown in Table-3.

Table 3-Channels used by AmpliSens kit rof detection of HPV 16, 18 and the internal control gene.

Fluorescence channel	***Color***	***Target***
FAM	Green	HPV 16
ROX	Orange	HPV 18
JOE	Yellow	Internal control

Then CT values were assessed through the report of the device Thermal cycler multigene optimax (Labnet International-USA) for each channel to confirm whether each sample was positive or negative and the type of HPV-HR. By analyzing the results of real-time PCR for qualitative detection and genotyping of HPV16 and 18 respectively, it was revealed that there were two Detections data for cycling channels (Per dye figure) as shown in Figures-(4, 5) :

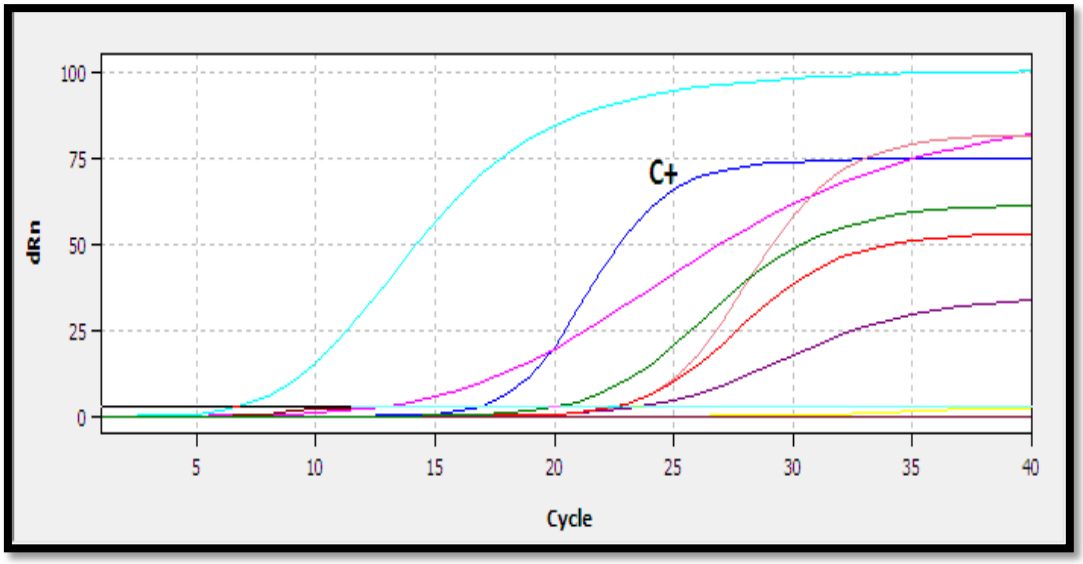

Figure 4- HPV Genotyping , channel for FAM fluorophore (HPV 16).Each curve represents a sample positive for HPV 16, C+ curve: Positive for internal control

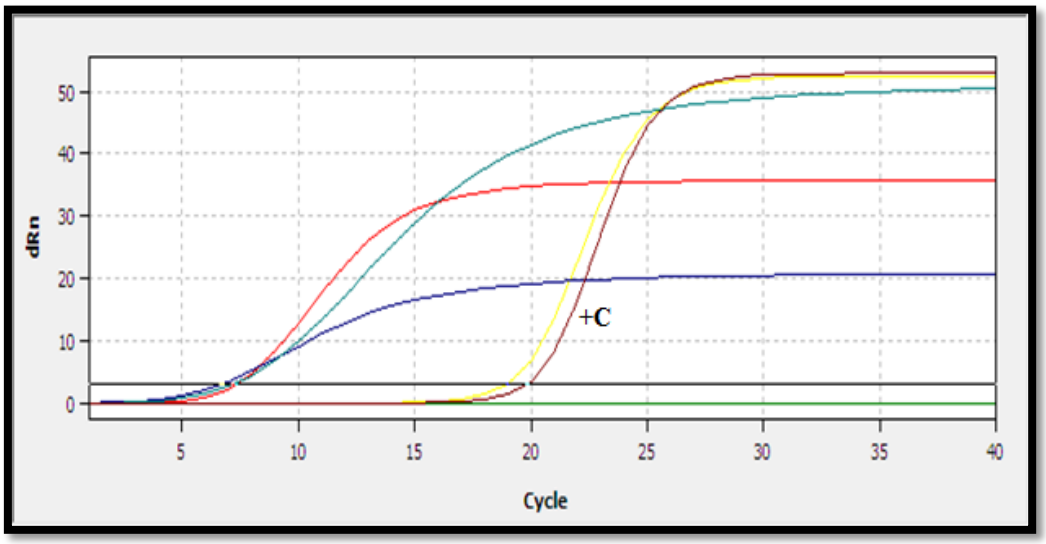

Figure 5- HPV Genotyping , channel for Rox fluorophore (HPV 18). Each curve represents a sample positive for HPV 18, C+ curve: Positive for HPV-18.

The results of RT-PCR detection revealed that out of the 16 samples detected with high-risk HPV, 5 samples were shown to be infected with HPV-16 and 5 samples were shown to be infected with HPV-18 Table-4, these results agree with multiple studies which show that infection with high-risk HPV types 16 and 18 are the most abundant among cases of persistent infection ranging from mild inflammation to growing intraepithelial lesions [21].

Table 4-Ratios of infection with HR-HPV 16, 18 or other types.

HPV type	n=	%
HR-16	5	8.33
HR-18	5	8.33
Other HR-HPV	6	10
Negative	44	73.33
Total	16	26.66

A study by **Sudolska *et al*,. (2011)** [21] utilized this method in the detection and typing of HPV types 16/18, various mycoplasma and ureaplsasma species from cervical smear samples of women with genital warts and cervical swaps by using Real time PCR.

Relationship between High-Risk HPV detection and histological findings of the samples

More than 95% of cervical cancer is associated with oncogenic human papillomavirus (HPV) infection, of which there are at least 13 different high-risk HPV (HR-HPV) types [22].

In this study, we focused on the histological findings for each patient in our attempt in finding a significant relationship between ultimate histopathological outcome and the detection of HR-HPV infection

The results showed that all five cases of cervical carcinoma tested positive for HR-HPV presence, including one positive for (+HPV16) and two cases positive for (+HPV18). Furthermore, all three cases of cervical intraepithelial neoplasia type 3 (CIN3) also tested positive for HR-HPV presence with (HPV18) detected in two cases. While cases of cervical intraepithelial neoplasia types 1 and 2 were heterogeneous as 71.42% of CIN1 HPV testing results were negative for HR-HPV, and 25% of CIN2 testing results negative for HR-HPV.

Meanwhile, non-cancerous conditions included in this study comprised 31 cases, that ranged from mild inflammation of the cervix, atypical squamous cells of undetermined significance (ASCUS) or Low-grade squamous intraepithelial lesion (LSIL). The results for HR-HPV detection in this group proved a very low ratio of HR-HPV infection which consisted of only three cases of infection {including 1 (+HPV16), 1 (+HPV18) and 1 positive for general HR-HPV}. Accordingly, all cases of the control group tested negatively for any type of HR-HPV (Table-5).

Table 5-Relationship between HPV and Histological results (Total No. = 60)

Group	Type of HPV	No. (%)	P-value
Carcinoma (No. 5)	HPV-16	1(20%)	0.0026 **
	HPV-18	2 (40%)	
	HR	2(40%)	
CIN III (No. 3)	HPV-16	1(25%)	0.0001 **
	HPV-18	2(75%)	
CIN II (No. 4)	HPV-16	2 (50%)	1.00 NS
	HR	1(25%)	
	Negative	1(25%)	
CIN I (No. 7)	HR	2(28.57%)	0.0023
	Negative	5(71.42%)	
Non-cancerous conditions (No. 31)	HPV-16	1 (3.22%)	0.0001 **
	HPV-18	1 (3.22%)	
	HR	1(3.22%)	
	Negative	28(90.32%)	
Control (No. 10)	Negative	10 (100%)	--
Total (No. 60)	HPV-16	5(8.33%)	0.0001 **
	HPV-18	5(8.33%)	
	HR	6(10%)	
	Negative	44(73.33%)	
** (P<0.01).			

NS : Non-significant value.

These results show a significant Relationship between HR-HPV infection and histological findings and they support the claim that carcinogenic types of HPV are associated with progression of lesions to invasive cervical cancer [23].

A study conducted by **Howitt *et al*,. (2017)** [24] proved that HR-HPV types 16 and 18 have been detected in almost 80% of cases ranging from cervical intraepithelial neoplasia grade 3 (CIN3) to cervical carcinoma in Malawi.

Conclusion

The study included 50 women with various gynecological problems, whose Pap smear results were abnormal, in addition to a group of (10) healthy women to serve as a control group. Histological examination results were obtained from the oncology department of Baghdad Teaching Hospital, these results ranged from cases of mild cervical inflammation, to various degrees of cervical intraepithelial neoplasia (CIN), and finally (5) cases of cervical cancer. The focus of our analysis was to combine histology and HR-HPV types 16 and 18 in order to investigate the role of HPV infection in ultimate histopathological outcome. Molecular detection of HR-HPV was investigated by targeting the specific L1 and E6 regions in the HPV genome, the results showed that HR-HPV types were detected in (16) samples out of a total of (50) and that out of the (16) cases diagnosed with HR-HPV infection, (5) tested positively for (HPV-16) and (5) tested positively for (HPV-18). These results show that about (62%) of these HR-HPV infections were caused by either HPV type 16 or 18 which proves that most HPV infections are caused by these two types of the virus.

This study also focused on the histological findings for each patient in the attempt of finding a significant relationship between ultimate histopathological outcome and the detection of HR-HPV infection, and the study found a significant relationship between these two factors with all cases of cervical cancer and cervical intraepithelial neoplasia type 3 (CIN3) testing positively for HR-HPV infection, meanwhile lower ratios of infection were found in CIN2 cases and other non-cancerous conditions. These results support the claim that carcinogenic types of HPV are associated with progression of lesions to invasive cervical cancer.

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