Prevalence of HPV Genotype in Cervical Cells Among Iraqi Patients with Abnormal Pap Smears

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Abstract: Cervical cancer is a leading cause of malignant related mortality among women specifically in developing countries. Research evidence has shown that Human Papilloma virus (HPV) is one of the most common sexually transmitted infections and the cause of the vast majority of cervical cancer cases. This study was designed to determine the prevalence of molecular 16 and 18 genotypes of HPV among Iraqi patients with abnormal Pap smear findings by utilizing endpoint real time PCR for qualitative detection. The study included 90 Pap smear specimens belonging to women complaining from gynecological problems and another ten specimens obtained from apparently healthy women. Samples were collected from two major Oncology hospitals in Baghdad during the period from April, 2015 to April, 2016. Total DNA (genomic, mitochondrial, and viral) isolated from the Pap smear samples for molecular studies was examined utilizing genomic DNA purification kits of Qiagen (QIAamp DNA Mini Kit / Germany). Pap smear cytological examination revealed that lesions in the forms of ASCUS, LSIL, HSIL and Squamous Carcinoma were observed in 23.3. 22, 20 and 3.3% respectively. The molecular detection method (Real Time PCR) showed that 16 and 18 genotypes were demonstrated in 23% of patients with HPV infections. An effective method has been used for detection of HPV genotypes yielding higher sensitivity by using global diagnostics kit.

Key words: HPV, Gene Line Mutations, Pap smears, Iraqi Patients

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Introduction

Cervical cancer is the fourth most common cancer among women worldwide and the leading cause of cancer related deaths (1). Although the incidence of cervical cancer in Iraq is relatively low as in most other Islamic countries, most of the cases usually present in late advanced stages. Earlier studies from Iraq have reported significant rates of cervical intraepithelial lesions (CIN) lesions among Iraqi patients complaining of gynecological problems (2,3). Therefore, promoting the level of awareness among the Iraqi population through screening is mandatory to control the disease. It has been well documented that CIN lesions could be readily detected in apparently healthy asymptomatic women through screening with Pap smear, testing for Human papilloma virus (HPV) and visual inspection with 5% acetic acid. HPV infection appears to be involved in the development of more than 90% of cases of cervical cancer and high grade intraepithelial lesions. Other related risk factors include smoking, a weak immune system, birth control pills, starting sexual life at a young age and

having multiple sexual partners (4). The study was designed to detect the prevalence of molecular 16 and 18 genotypes of HPV among Iraqi patients exhibiting abnormal Pap smear findings by utilizing endpoint real time PCR.

Subjects and Methods

This study was carried out during the period between the 1st of April 2015 until the end of April 2016 within the University of Baghdad / Institute of Genetic Engineering & Biotechnology for post Graduate Studies and the National Cancer Research Center. It included 100 Iraqi women; 90 of whom were complaining from gynecologic problems where their Pap smears revealed abnormal cytological findings (Group 1). A Healthy Control (Group 2) consisted of ten apparently healthy Iraqi women from whom Pap smears were obtained. The patients were examined in the Outpatient Gynecology department of the Medical City Teaching Hospital. For each patient, a structured questionnaire containing different demographic and clinical variables was completed. Criteria for inclusion in the study comprised the following:

- Age range between 25 to 60 years
- Having regular menstrual cycles
- No history of endocrine disease
- No use of medication or oral contraceptives.

Pap smears which were collected from the cervix of these patients, were immediately dipped in absolute methanol for a minimum period of 20 minutes then stained by Papanicolaou stain (5). That was followed by microscopical examination of the specimens by a competent cytopathologist who categorized the cellular findings according to the Bethesda System into: WNL (Within Normal Limits), ASCUS (Atypical Squamous Cells of Undetermined Significance), LSIL (Low Grade Squamous Intraepithelial Lesions), HSIL (High Grade Squamous Intraepithelial Lesions) and Carcinoma. The same specimens were then subjected for HPV detection (6).

Genomic DNA isolation

Total DNA (genomic, mitochondrial, and viral) isolated from the Pap smear samples for molecular studies was examined utilizing genomic DNA purification kits of Qiagen (QIAamp DNA Mini Kit / Germany). This kit has been used by other researchers in a similar study (7). After genomic DNA extraction, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA (8).

Real Time PCR for qualitative detection and genotyping of HPV

The PCR-based methods have been used successfully for the detection and typing of genital HPV genotypes in clinical specimens including Pap smear findings. For Real time amplification with Rotor-Gene 3000/6000/Q, Tubes were transferred into the carousel of Rotor-Gene 3000/6000/Q. The first tube was placed of strip N°1 in A1 and followed by the others so that the first tube of the strip (mix "16, 18, 31") was in position 1 or 5 (A5, B1, B5, C1 etc). for that method, software 1.7 Build 67 was utilized.
 The Program Rotor-Gene 2000/3000 was applied as follows:

A. Reaction Volume (μl): 13
B. Hold: 95º - 15 min
C. Cycling: 45 cycles
    95º – 15 sec
    60º – 30 sec

Fluorescence detection was obtained at 60°C on the channels Fam (Green), Joe (Yellow), Rox (Orange) and Cy5 (Red). The adjustment was made according to the fluorescence channel sensitivity: Channel Setup, Calibrate (Gain Optimisation for Rotor-Gene 6000), Auto Gain Calibration (Optimisation) Setup, Calibrate Acquiring (Optimise Acquiring) and selected perform Calibration (Optimisation). Before 1-st Acquisition, the calibration was performed for the first tube on the channels Fam (Green), Joe (Yellow), Rox (Orange) and Cy5 (Red) and the selected target sample range 4FI – 8FI. In the column Name program was inserted positioning the tubes in the carousel of the Rotor-Gene 2000/3000.

Results and Discussion

Age distribution

The age of all women was categorized as those who were less than 30 years old *versus* those equal to and over 30. Table 1 revealed that 28% of the study groups women in general were less than 30 years old while 72 % were equal or over 30; the difference in the age of women in both groups was not significant. The results agreed with those reported by Wright in 2014 (9) who displayed that the aging is a risk factor for persistent infection. In this study, the rate of persistent of high-risk infections for women older than age 40 was 50%, compared with a persistence rate of 20% in women younger than age 25.

Pap Smear Cytological Findings

The distribution of the study sample according to the cytology examination is illustrated in (Table 2).

Table (1): Distribution of the Study Groups according to Age.

Group NO.	Age group (years)	Study group		The sub groups			
				Group 1		Group 2	
		No.	%	No.	%	No.	%
1	Less than 30	28	28	26	28.88	2	20
2	Equal and over 30	72	72	64	71.12	8	80
Total		100	100	90	100	10	100

Table (2): Distribution of the Study Sample according to Cytological Examination.

Cytology	Number		Percentage (%)	
	Group 1	Group 2	Group 1	Group 2
ASC-US	21	0	23.33	0
HSIL	18	0	20	0
LSIL	20	0	22.22	0
WNL	28	10	31.11	100
Squamous Carcinoma	3	0	3.33	0
Total	90	10	100	100

WNL: With normal limited, **ASC-US:** Atypical squamous cells of undetermined significance,
LSIL: Low-grade squamous intraepithelial lesion and **HSIL:** High-grade squamous intraepithelial lesion.

All ten Pap smear specimens obtained from Group 2 (apparently healthy women) showed cellular changes within normal limits (WNL). On the other hand, Group 1 revealed that Pap smears exhibiting lesions in the forms of WNL, ASCUS, LSIL, HSIL and Squamous Carcinoma were observed in 31.1, 23.3. 22,20 and 3.3% respectively. According to the Bethesda Terminology (5), specimens exhibiting evidence of koilocytotic atypia, suggestive of HPV infection, are categorized within LSIL together with mild dysplasia or CIN I.

The severity of the diagnosis was based on the degree of replacement of the normal stratified epithelium with mitotically active basal-like epithelium ($\leq1/3$ = CIN1, $\leq2/3$ =CIN2, $>2/3$ = CIN3). CIN was viewed as a stepwise progression, with a high probability of transition from the more minor to more serious cancer precursors (10). The frequencies of the demonstrated Pap smear findings in the present study are higher than what was reported in previous studies from Iraq obviously attributable to the fact that all patients included in Group 1 were symptomatic patients who had serious gynecological problems in the form of chronic persistent infections, cervical erosions, dysparunia and bloody vaginal discharge (2, 11).

Real Time PCR for qualitative detection and genotyping of HPV

Real Time amplification test for qualitative detection and genotyping of Human Papillomavirus (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59,66 and 68) in the urogenital swabs and biopsies (Sacace, Italy). Relevant approaches yielding comparable results were practiced by other studies (11, 12).

Table (3): HPV Genotypes Detection Dyes utilized in the Study.

FAM	JOE	ROX	Cy5
16	31	18	IC
39	45	59	IC
33	35	68	56
58	52	66	51

By analyzing the results of real time PCR for qualitative detection and genotyping of HPV it was revealed that there were four Quantitation data for cycling channels (Per dye figure) (Figures 1 , 2, 3, 4) .
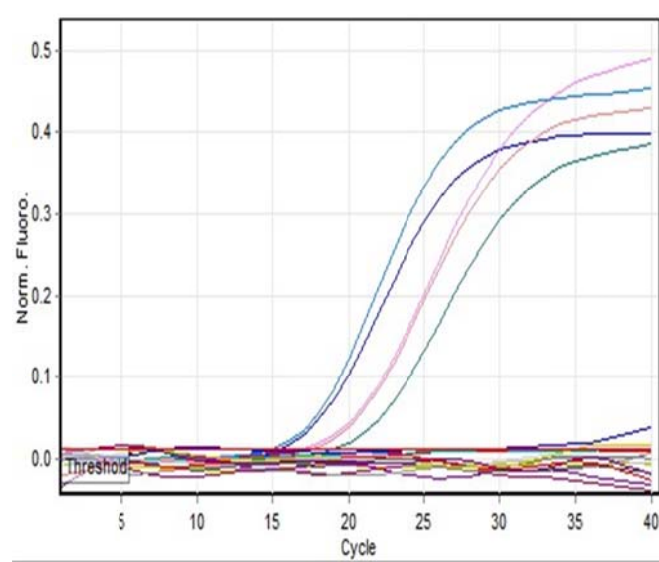

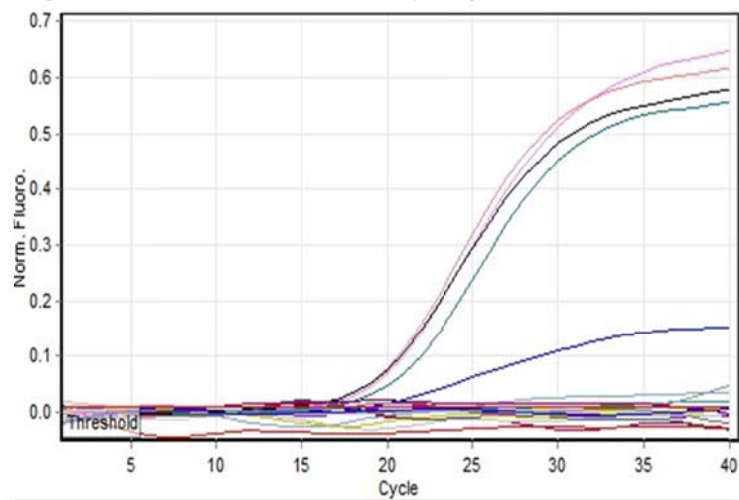**Figure (1): Quantitation data for cycling Green channel (FAM)**

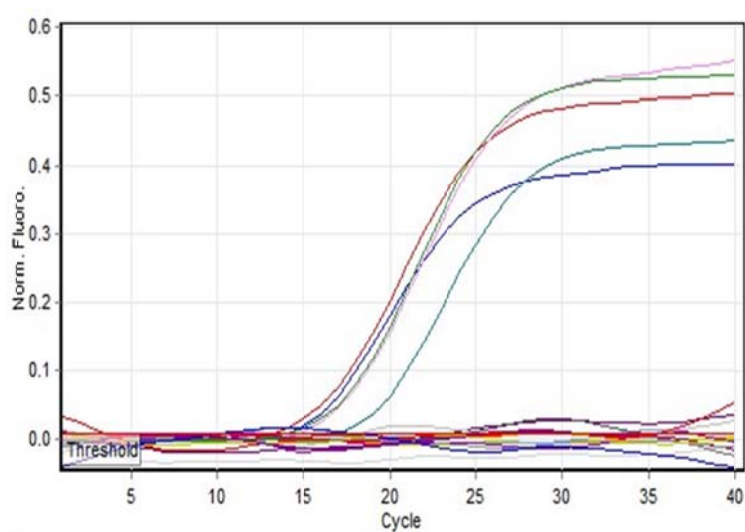**Figure (2): Quantitation data for cycling Yellow channel (JOE).**

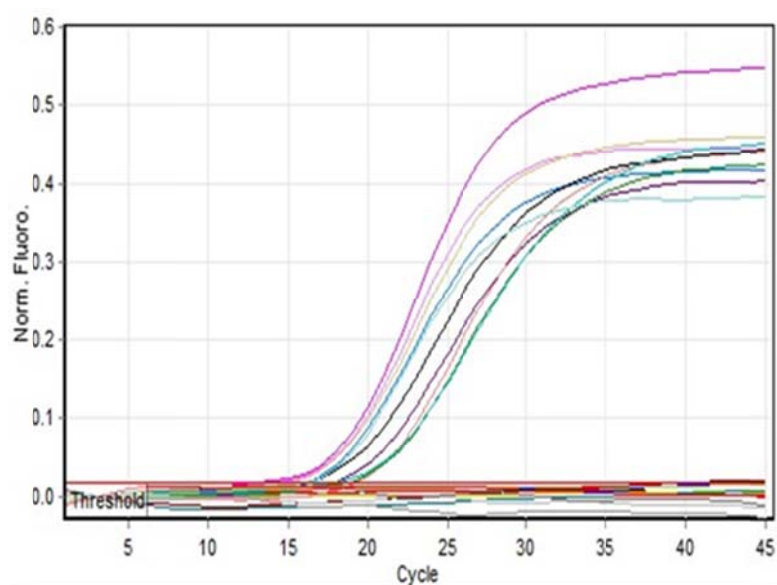**Figure (3): Quantitation data for cycling Orange channel (ROX).**

Figure (4): Quantitation data for cycling Red channel (Cy5).

Figure (5): Final results analyzed by using the software HPV Genotype 14 Real-TM (ENG) v.1.3 Sacace.

After these steps, we assessed the ct values through the report of the device - Rotergen for each channel. Then the software (HPV Genotype 14 Real-TM (ENG) v.1.3 Sacace) was initialized in our device (Laptop). By inserting ct values for the computer program, we obtained the final result for each sample whether positive or negative and the type of HPV-HR (Figure 5) .The results of this study indicated that the frequency of positive and negative results for Genotypes 16 and 18 were (23/100) and (77/100) respectively as shown in the (Table 4).

Table (4): Frequency of HPV within the Study Sample according to RT-PCR

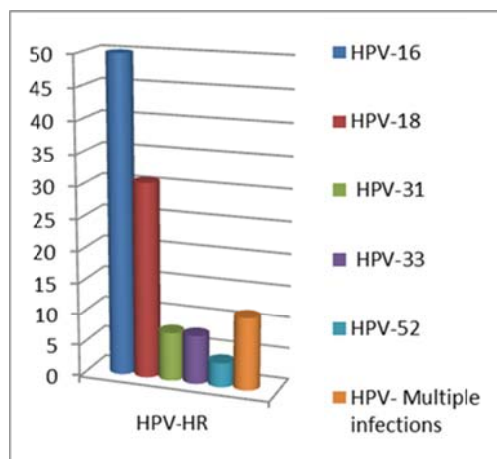| RT-PCR	Number	Percentage (%)
Positive	23	23.00
Negative	77	77.00
Total	100	100%
Chi-square value	---	12.952 **
P-value	---	0.0014
** ($p<0.01$).		

Figure (6): Frequency of the Various HPV-HR genotypes.

Our approach was consistent with that used by other investigators who utilized several commercially available kits based on real-time PCR for the detection of HPV infections and their clinical utility (13). In this study, Real-time PCR is carried out in a thermal cycler with the capacity to illuminate each sample with a beam of light of at least one specified wavelength and detect the fluorescence emitted by the excited fluorophore. The thermal cycler is also able to rapidly heat and chill samples, thereby taking advantage of the physicochemical properties of the nucleic acids and DNA polymerase. Then the reaction is run in a real-time PCR instrument, and after each cycle, the intensity of fluorescence is measured with a detector; the dye only fluoresces (such as dsDNA dyes - SYBR Green) will bind to all dsDNA PCR products. In accordance with a previous trial, we utilized that method which had the advantage of merely needing a pair of primers to carry out the amplification. That cost effective method is more sensitivity and highly accurate for real time PCR than conventional PCR (14).

Conclusions

In this study an effective method has been used for detection of HPV genotypes yielding higher sensitivity by using global diagnostics kit (Sacace, Italy).

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