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Genotyping of Human papilloma virus DNA isolated from cervical tissue biopsies of Iraqi women with different cervical abnormalities

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Abstract: *Human papillomaviruses(HPV) are the etiological agents of cervical and other ano-genital malignancies. More than 200 genotypes of HPV have been identified, of these, genotypes 16,18,31,33,45,51,52,53,58 have been classified as "high-risk" (HR-HPV) because they are associated with the malignant progression of cervical tumors and with other genital and head-neck malignancies. This study aimed to evaluate the association between the infection with HR-HPVs and histo-pathological presentations. Material and method: This cross-section and prospective study was conducted during March 2016 to March 2017.The study included 150 fresh cervical tissue biopsy samples collected from hysterectomy done for women with different cervical abnormalities. All samples were diagnosed histo-pathologically and classified into two groups; group of patients whom have cervical abnormalities and control group which includes samples with unremarkable changes taken from women by hysterectomy done for reasons other than cervical abnormalities. DNA was extracted from cervical tissue biopsy samples and the integrity was estimated by amplification of glyceraldehyde phosphate dehydrogenase (GAPDH) gene. Detection of HPV was done by PCR amplification using HPV-specific primer sets of MY09/MY11 gene. DNA samples show positivity for the above two reactions were further amplified for genotyping using specific primer sets for the HR-HPV genotypes (16, 18, 31, 35, 45, 51, 52, and 66). Results: Only 90/150 DNA samples were included in the results of this study. The most important histopathological findings were 6/90(6.7%) patients with squamous cell carcinoma(SCC), as a total, HPV-16 detected in 3/6(50%) patients, HPV-45 detected in 3/6(50%) patients and HPV-18 detected in 2/6 (33.3%) patients. As co-infection with HR-HPV genotypes, 2/6(33.33 %) patients have co-infection with genotypes (HPV16, 18, 45) and only 1(16.66%) patient has a co- infection with two HPV genotypes (HPV 16, 45). Risk estimation for SCC with respect to HPV-16,18,45 were significant (P<0.001).Conclusion: However, only a minority of pre-cancerous lesions progress to cervical cancer,HPV genotyping is an important risk factor for differentiation progression. Testing of HPV DNA should introduced into clinical practice in Iraq with the aim of monitor women whom are at risk of cervical cancer.*

Keywords: *Human Papilloma Virus, genotyping, histopathological findings ,risk factor.*

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I. Introduction

Cervical cancer is one of the most important health problem for all women and its accounts 15% of female cancers and its more common in developing countries than in the developed one [1,2,3].Human Papillomaviruses consider the most etiological agents of cervical cancer and have been found in high percentage of women with high-grade cervical intraepithelial neoplasia (CIN II,III) and cervical carcinoma [4]. It was recognized that all cervical cancers (both the squamous and adenocarcinoma) and their precursor lesions are related to cervical infections with one of the HR-HPV genotypes which numbered as16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68, that mainly diagnosed by molecular methods like polymerase chain reaction(PCR) [5,6]. However, only a minority of pre-cancerous lesions progress to cervical cancer, the HPV type is an important risk factor for differentiation progression [7]. Many molecular methods for HPV DNA testing are available such as polymerase chain reaction method by which amplifying of nucleic acids of this virus are used because of the limited sensitivity of Pap smear test ,insufficient serological tests and difficulty of *In vitro* cultures, make using the PCR technique as routine test more useful in the detection of the virus [8]. Detection of DNA of the virus mainly in latent infections, helpful in detecting precursor lesions and cervical carcinoma [9]. In Iraq, it was indicate that every year, 291 women are diagnosed with cervical cancer and 142

die from the disease [10].This is a study aimed to evaluate the association between the infection with eight HR-HPV and histo-pathological presentations which could be considered very useful in the early diagnosis of cervical abnormalities.

II. Material and methods

1. Patients

In the current cross-section and prospective study,150 fresh cervical tissue biopsy samples were collected from hysterectomy done for women attend to the department of Gynecology of Al-Emamain Medical City, Baghdad, Iraq, from March 2016 to March 2017. Samples were taken by the consultant physician and preserved in the normal saline. Extraction of DNA was done at once if possible or the sample was stored at -20◦C until extraction of DNA was done.

All cervical tissue samples were diagnosed histopathologically by histopathologists and classified according to the histopathological diagnosis into two groups; group of patients whom have cervical abnormalities such as (cervical dysplasia (CIN I, II, III), squamous cell carcinoma(SCC), atypical squamous cell and chronic cervicitis). The second group was the control group which includes samples with unremarkable changes taken from women by hysterectomy done for reasons other than cervical abnormalities. Data was taken from each patient include (name, age, smoking state, medical history of patient, family history , marital status , using birth control pills and number of children).

From the total of 150 samples, 90 samples were subject to the all tests done in the current study, while 60 samples were excluded because the quality of DNA is low according to the negative results of testing housekeeping gene, glyceraldehyde phosphate dehydrogenase (GAPDH) gene, which used to check the quality of DNA.

This study was approved by the ethical committee of the College of Medicine-Al-Nahrain University, Baghdad ,Iraq..

2. DNA extraction

Extraction of Genomic DNA from the cervical tissue biopsy was done using QIAamp® DNA Mini Kit (Cat. No. 51304, USA),following manufacturer instructions. Concentration and purity of DNA was measured using Nanodrope instrument. Extracted DNA with concentration between (9-500ng/µl) and purity between (1.7-2) was included in the current study, otherwise, the samples were excluded.

3. DNA quality estimation using PCR amplification of GAPDH

PCR amplification of GAPDH was performed in 50µl total volume reaction mixture per one reaction according to Rameshkumar et al[11]. After cycling, the PCR products were electrophoresed in 2% agarose gel. Presence of 240 bp band means positive result (the quality of DNA is good).

4. Detection of the HPV-DNA using PCR

Detection of HPV-DNA was done by using specific primer sets of MY09/MY11. The PCR amplification of MY09/MY11 was performed in 50 µl total volume of reaction mixture per one reaction according to Venceslau et al [12]. After cycling, the PCR products were electrophoresed in 2% agarose gel. The presence of 450 bp band means positive result which indicates detection of the HPV.

5. HPV-DNA genotyping

Samples show positivity for the above two reactions were further amplified for genotyping using specific primer sets for HR-HPV genotypes (16, 18, 31, 35, 45, 51, 52, and 66). PCR amplification for genotyping was performed in 50 µl total volume mixture per one reaction according to Sotlar et al [13]. After cycling, the PCR products were electrophoresed in 2% agarose gel. The presence of band with molecular size as in the table 1 demonstrate the presence of such genotype.

Table 1 The molecular weight of amplified products of each HPV-genotype used in this study [13]

Genotypes	Molecular weight (bp)
HPV 16	457 bp
HPV 18	322 bp
HPV 31	263 bp
HPV 35	358 bp
HPV 45	151 bp
HPV 51	223 bp
HPV 52	229 bp
HPV 66	172 bp

5. Statistical analysis

Data were collected, analyzed and presented using two statistical software programs: statistical package for social sciences (SPSS version 22) and Microsoft Office Excel 2013. Numeric variables were presented as mean, standard deviation and range, whereas categorical variables were presented as number and percentage. One

proportion Z-test was used to study differences in ratios, Fischer exact test was used to study association between categorical variables, Kruskal Wallis test was used to study differences in mean rank among more than two groups, Odds ratio test was used to estimate risk together with etiologic fraction. Sensitivity, specificity, positive predictive value and negative predictive values were estimated according to equations outlined in (Daniel, 2009)[14]. P-value was considered significant at ≤ 0.05 and highly significant at ≤ 0.01.

III. Results

1. Patient demography

The age distribution of patients included in the current study ranged from 25 years to 75 years with a total mean of 49.08 years ± 11. The mean age of control group was 50.21 ± 13 years. There were no significant statistical differences *P*>0.05 between different groups as shown in table 2.

Table 2 Mean of age of patients according to histo-pathological groups

Group	Mean of age	SD	Minimum	Maximum
Negative	50.21	13.42	29	72
Chronic non-specific cervicitis	50.00	8.30	35	60
Squamous hyperplasia	48.25	16.56	35	71
Chronic cystic cervicitis	46.91	11.78	25	75
Endocervical polyp	45.86	8.67	34	56
CIN I	49.71	10.44	25	67
CIN II	54.78	12.89	33	70
SCC	49.50	10.71	37	63
NHL	33.00	---	---	---
Total	49.08	11.40	25	75

- CIN I,II: Cervical Intraepithelial Neoplasia type one and type two; SCC: Squamous Cell Carcinoma; NHL: Non-Hodgkin Lymphoma
- P>0.05 "Kruskal Wallis test"

2. Histo-pathological findings

According to the histopathological examination of 90 samples, these samples were classified to control group (14 samples) and patient groups (76 samples). The most common histopathological type was the chronic cystic cervicitis which shown in (22,24.4%) patients, while the less common group was the Non-Hodgkin lymphoma which shown in (1,1.1%) patient as shown in table 3. The highest frequency of histo-pathological abnormalities was observed in married patients with 100% in CIN II as shown in table 4.Regarding the association between family history and histopathological findings, the positive history of family was significant in SCC group which shown in 4/6 (66.7%) patients, table 5.

Table 3 Classification of patients according to histo-pathological findings

	Groups according to histopathology	Number	%
Control	Negative	14	15.6
Inflammatory and reactive conditions	Chronic non-specific cervicitis	10	11.1
	Squamous hyperplasia	4	4.4
	Chronic cystic cervicitis	22	24.4
	Endo-cervical polyp	7	7.8
Cervical intraepithelial neoplasia	CIN I	17	18.9
	CIN II	9	10
Malignant neoplasm	SCC	6	6.7
	NHL	1	1.1
Total		90	100.0

-CIN I,II: Cervical Intraepithelial Neoplasia type one and type two; SCC: Squamous Cell Carcinoma; NHL: Non-Hodgkin Lymphoma.

Table 4 Marital status according to histopathological groups

Histopathological Group	Married		Unmarried		Total	
	N	%	N	%	N	%
Negative	14	100.0	0	0.0	14	100.0
Chronic non-specific cervicitis	7	70.0	3	30.0	10	100.0

Squamous hyperplasia	2	50.0	2	50.0	4	100.0
Chronic cystic cervicitis	16*	72.7	6	27.3	22	100.0
Endo-cervical polyp	7	100.0	0	0.0	7	100.0
CIN I	15*	88.2	2	11.8	17	100.0
CIN II	9	100.0	0	0.0	9	100.0
SCC	5*	83.3	1	16.7	6	100.0
NHL	1	100.0	0	0.0	1	100.0
Total	76	84.4	14	15.6	90	100.0

-N: Number of cases; CIN I,II: Cervical Intraepithelial Neoplasia type one and type two; SCC: Squamous Cell Carcinoma; NHL: Non-Hodgkin Lymphoma.
-P-value was assessed using one proportion Z test; -* Significant at P≤ 0.05.

Table 5 Family history of subjects included in the study

Group	Family history					
	Positive		Negative		Total	
	n	%	n	%	n	%
Negative	1	7.1	13*	92.9	14	100.0
Chronic non-specific cervicitis	4	40.0	6	60.0	10	100.0
Squamous hyperplasia	2	50.0	2	50.0	4	100.0
Chronic cystic cervicitis	2	9.1	20**	90.9	22	100.0
Endo-cervical polyp	3	42.9	4	57.1	7	100.0
CINI	2	11.8	15**	88.2	17	100.0
CINII	2	22.2	7*	77.8	9	100.0
SCC	4*	66.7	2	33.3	6	100.0
NHL	0	0.0	1	100.0	1	100.0
Total	20	22.2	70	77.8	90	100.0

-N: Number of cases; P-value was assessed using one proportion Z test; * Significant at P≤ 0.05; ** highly significant at P≤0.01.

3. HPV-DNA detection and genotyping

DNA extracted from cervical tissue biopsies samples that show good quality were 90/150 samples, as shown in Fig. 1,A. Positivity for HPV-DNA was detected in 10/90(11.1%) samples using primer sets MY09/MY11 specific for HPV- DNA detection, Fig.1,B. Regarding the association between the positivity for HPV-DNA and histopathological findings, it was shown that the high incidence of the HPV infection was in SCC patients (4/6,66.7%), as shown in table 6.

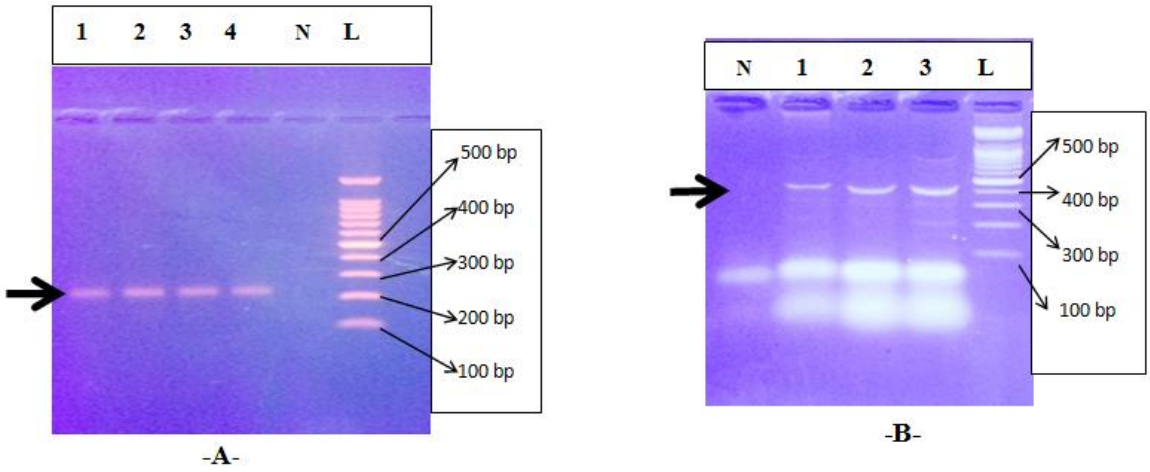

Figure 1 (A) Identification of DNA quality using specific primer for GAPDH gene. Lane(1,2,3,4): DNA of patients with good quality (240bp).Lane N: no template control. Lane L:DNA ladder (100bp).Electrophoresis was done in 2% agarose gel at (5V/cm) for 60 minutes. (B)Detection of HPV-DNA using specific primer for MY09/MY11 gene. Lane(1,2,3 and 4): patients positive for HPV-DNA (450 bp). Lane N: no template control. Lane L:DNA ladder (100bp).Electrophoresis was done in 2% agarose gel at (5V/cm) for 60 minutes.

Table 6 MY09/MY11 expression according to histo-pathological groups

Histo-pathological groups	My09/MY11					
	Positive		Negative		Total	
	N	%	N	%	N	%
Negative	0	0.0	14	100.0	14	100.0
Chronic non-specific cervicitis	2	20.0	8*	80.0	10	100.0
Squamous hyperplasia	0	0.0	4	100.0	4	100.0
Chronic cystic cervicitis	1	4.5	21**	95.5	22	100.0
Endo-cervical polyp	1	14.3	6*	85.7	7	100.0
CIN I	1	5.9	16**	100.0	17	100.0
CIN II	1	11.1	8*	88.9	9	100.0
SCC	4	66.7	2	33.3	6	100.0
NHL	0	0.0	1	100.0	1	100.0
Total	10	11.1	80	88.9	90	100.0

-N: Number of cases; P-value was assessed using one proportion Z test; * Significant at P≤ 0.05; ** highly significant at P≤0.01.

Regarding HPV-DNA genotypes, the most detected genotype was HPV-16 which was shown in 9/90 (10%) patients, Figure 2. One DNA samples was positive for HPV-DNA but was negative for the genotype sets used in the present study (HPV16,18,31,35, 45, 51, 52, 66) as shown in the table 7.

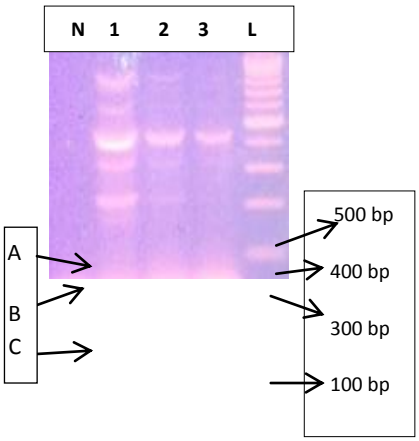

Figure 2: Amplified products of HPV-DNA genotypes. Lane 1: patients positive for HPV-16,18,45(A=457 bp,B=322 bp, C=151 bp, respectively). Lane 2,3: Two patients positive for HPV-16(A=457 bp). Lane N: No template control. Lane L: DNA ladder (100bp).Electrophoresis was done in 2% agarose gel at (5V/cm) for 60 minutes.

Table 7 Genotype expression according to histo-pathological factors

Groups	Total	16		18		45		X	
		N	%	N	%	N	%	N	%
Negative	14	0	0.00	0	0.00	0	0.00	0	0.00
Chronic non-specific cervicitis	10	2	20.00	0	0.00	1	10.00	0	0.00
Squamous hyperplasia	4	0	0.00	0	0.00	0	0.00	0	0.00
Chronic cystic cervicitis	22	1	4.6	0	0.00	0	0.00	0	0.00
Endocervical polyp	7	1	14.3	0	0.00	0	0.00	0	0.00
CIN I	17	1	5.9	0	0.00	0	0.00	0	0.00
CIN II	9	1	11.11	0	0.00	0	0.00	0	0.00
SCC	6	3	50.00	2	33.33	3	50.00	1	16.67
NHL	1	0	0.00	0	0.00	0	0.00	0	0.00
Total	90	9	10.0	2	2.22	4	4.44	1	1.11

X: genotype not included in the present study

4.Risk estimation for squamous cervical carcinoma with respect to HPV-DNA genotypes

Squamous cell carcinoma was identified in 6/90(6.7%) patients, as a total, HPV-16 detected in 3/6(50%) patients, HPV-45 detected in 3/6(50%) patients, while HPV-18 detected in 2/6 (33.3%) patients as shown in table 8. As co-infection with these HR-HPV genotypes, 2/6(33.33 %) patients have co-infection with genotypes (HPV16,18,45) while only 1/6(16.66%) patient has a co- infection with HPV-16, 45.

Table 8 Risk estimation for squamous cervical carcinoma with respect to genotype

Genotype	SCC (study group = 6)	Others (control group= 84)	P* value	OR	95 %CI		EF
					Lower	Upper	
16	3 (50%)	6 (7.1%)	<0.001	13.00	2.14	78.88	0.31
18	2 (33.3%)	0 (0.0%)	<0.001	95.00†	---	----	---
45	3 (50%)	1 (1.2%)	<0.001	83.00	6.55	1051.86	0.74

SCC: Squamous cell carcinoma;* Fischer exact test; OR: Odds ratio; CI: Confidence Interval; EF: Etiologic Fraction; †: Approximate Odds Ratio.

IV. Discussion

Infection with HPV is the main causative agent of cervical premalignant and malignant lesions and cervical lesions remain the significant public health problem worldwide [15,16]. The important role of HR-HPV genotypes infection in the development of cervical lesions has been clearly demonstrated [17]. Prevention of high risk group transmission is difficult, therefore screening for HPV infection and cytological analysis are the most important ways in order to defenses against cervical lesions and are widely practiced in the developed world [18-20].

Little is known about the data of Iraqi patients regarding infections with HPV-genotypes. In the current study, the mean age of patients with cervical abnormalities was 49.08 ± 11; this result was in consistence to results of Lena, 2011 and Shirish et al 2014 [21, 22].

The histopathological findings results from current study demonstrated that the most common type of cervical carcinoma was squamous cell carcinoma. A similar trend to this result was reported in another studies [23-27]. In this study, (10,11.1%) of samples tested were positive for HPV infection. Three different HPV genotypes were detected in 9/10 samplis, including HPV-16, 45, 18. In line with previous studies, HPV-16 was the most frequent genotype detected [16, 28-29].

In the current study the HPV-DNA presentation was not found to be significantly associated with other histopathological parameters, and this result likewise the results of Lee et al 2004,Hassan et al 2015[30-31].

Regarding to the incidence rate of infection with multiple HPV genotypes, (3/10,30%) patients whom HPV-positive have co-infection with more than one genotype included in this study(16,18,45 and 16,45). Previous studies have indicated a different prevalence of co-infection with HPV genotypes, varying from 10% to 80% [32-34]. These variations may be due to differences in geographic characteristics, sexual behavior, and HPV detecting methods . The results of the current study were shown an association between HPV genotypes co-infection and an increased risk for squamous cell carcinoma and this result correlate with some reports that shown a correlation between an elevated risk of cervical carcinogenesis and HPV genotypes co-infection [35-36], while the findings of other studies support the lack of this association [37-38].

In conclusion, high risk HPV 16, 18, 45 infections seems to be common in cervical cancer tissues suggesting that HPV might play an important role in the pathogenesis of cervical abnormalities and the progression of these abnormalities into cervical carcinoma.

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Conflicts of interest
There are no conflicts of interest

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