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Detection and Genotyping of Human Papillomavirus DNA in Cervical Neoplasia of Iraqi Women Using Real Time PCR Technique.

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I. Introduction*:*

Cervical cancer is the second most common cancer in women worldwide, but the commonest in developing countries (Ferlay J et al., 2001), and a compelling body of clinical, epidemiological, molecular, and experimental evidence has established the etiological relationship between some sexually transmitted HPV genotypes and cervical neoplasia throughout the world. Based on the frequency of detection of HPV genotypes from different grades of Cervical Intraepithelial Neoplasia (Grades/CIN I–III), HPV genotypes are subdivided into High-risk HPV types which frequently associated with invasive squamous cell carcinoma (Schiffman et al., 2007), the low-risk types are rarely found in carcinomas and are often associated with premalignant or benign diseases (Gravitt and jamishidi, 2005). In cervical carcinomas, HPV is needed for malignant transformation (Bosch et al., 2002). HPV is mainly transmitted through sexual contact and most people are infected with HPV shortly after the onset of sexual activity (Jawetz et al., 2004).

II. Material and methods

The study involved 731 cervical swabs of Iraqi women referred from middle provinces of Iraq to the Central Public Health Laboratory of Baghdad. Specimens were collected during the period from December 2012 to December 2014, the ages of related cases range from 15-70 years. For each patient there was a case investigation form filled with information by a physician, this form includes personal information, gynecological history, result of pap smear examination and result of histological examination.

Sample collection

Cervical swabs collected according to the following instruction:
After Remove excess mucus from the cervical and surrounding ectocervix, insert the Sampling Cervical Brush (1-1.5)cm into the cervical and rotate brush 3 full turns in a counterclockwise direction, remove from the canal, insert brush into the nuclease-free 2ml tube with 0.5ml of transport medium (Sacace), leaving brush end inside tube. Swabs samples stored at 2–8 °C for no longer than 48 hours, or freeze at –20/80°C.

Material

Applied Biosystem7500/USA used for detection of 12 HPV High Risk types using Real Time PCR kits of Sacace/Italy (Screen and Typing) for 12 HPV high risk types. Method was conducted according to the instructions of manufacturing company leaflet. Workflow in the laboratory was preceded in a unidirectional manner, beginning in the extraction area and moving to the Amplification and Detection Area.

III. Methods

Principle of the screen and genotype assays:

Kit HPV High Risk Screen Real-TM PCR is based on two major processes:
1- Isolation of DNA from specimens: DNA-Sorb-A (Sacace, Italy) kits used for DNA extraction from swabs samples. Procedure carried out according to the manufacture's instruction.
2- Real Time amplification/ principle of the Assay:
For HPV screen Real-TM: Real time amplification of the E1-E2 region of gene HPV (channel Joe) and β-globine gene used as Internal Control (channel Fam). If the swab is not correctly prepared (high quantity of mucous or insufficient quantity of epithelial cells) the Internal Control will not be detected.

For HPV Genotypes 12 Real-TM: the kit is based on multiplex Real Time amplification of 4 PCR tubes for each sample. The kit detects the most widespread and oncogenic 12 genotypes of HPV human papilloma virus. Since the HPV human papilloma virus is an intracellular agent, there is need to monitor the presence of cellular material in the sample, in order to avoid false-negative results. HPV Genotypes 12 Real TM kit also contains the internal control (human β beta-globin gene), which allows to control the presence of cellular material in the sample.

IV. Data Analysis:

In Screen HPV Real Time PCR Kit the experiment considered valid if the Negative Controls haven't any positive fluorescence signal and the standards have positive signals in all channels (Fam, Joe/HEX/Cy3). The result of the sample is considered:

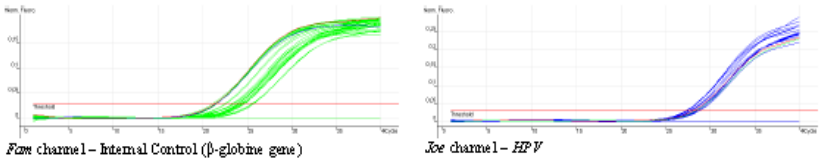
• Invalid in case of absence of any fluorescence signal (positive or internal);
• Negative if signal is present only in the Fam (Green) channel with the conc. of genomic DNA > 5 x 10^3;
• Positive: if in the Joe/HEX/Cy3 channels the fluorescence signal are present (Ct ≤ 33) at least in one of the 2 tubes. In these samples fluorescence signal in the channel Fam can be absent.

In Typing 12 HPV Real TM PCR Kit there were specified table includes the dyes in each mix with HPV genotypes.

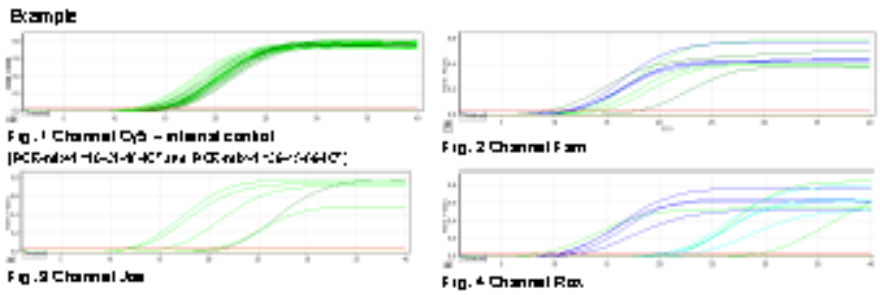
**** Vague details of interpretation of results

V. Results:

The following figure shows the positive and negative results of HPV RT PCR screening and confirmatory results (figure 1).



a



b-

Figure 1: Positive and negative results of Real Time PCR (a- Results of HPV Screen RT PCR b- Results of genotyping RT PCR)

Highest percentage of HPV infection were found among women aged (15 to 40) years and lowest prevalence in old age women (Table-1).

Table 1: Distribution of HPV infection among Iraqi women according to age groups.

#	Age group	Negative	Positive	Total	%
1.	15-29	145	23	168	13.7
2.	30-39	245	26	271	11.9
3.	40-49	208	11	219	5.02
4.	50-59	55	6	61	9.8
5.	60-70	12	0	12	0.0
6.	Total	665	66	731	9
Statistic analysis: T -Test = 0.041422203 < 0.05 , Significant difference					

Regarding to the prevalence of HPV among provinces, they were (12.5%), (11.8%), (10.5%), (5.1%), (3.7%) and (1.7%) in Kerbala, Babylon, Baghdad, Basrah, Diwaniya and Diyala subsequently (Table-2). However, the statistical analysis showed that there was no difference in the prevalence of human papilloma virus among studied provinces.

Table 2: Distribution of HPV infection among Provinces of Iraq

#	Province	Negative	Positive	Total	%
1.	BAGHDAD	399	47	446	10.5
2.	BABYLON	75	10	85	11.8
3.	DIYALA	59	1	60	1.7
4.	KERBALA	14	2	16	12.5
5.	BASRAH	92	5	97	5.1
6.	DIWANIYA	26	1	27	3.7
	TOTAL	665	66	731	9
T -Test = 0.111078 > 0.05 , No Significant difference					

The test results of RT-PCR genotyping in (Figure-2) showed that HPV16 is predominant type (25%) among Iraqi women, followed by HPV59 (18%), HPV56 (13%), HPV18 (12%), HPV35 (9%), HPV31 (8%), HPV39 (6%), HPV45 (5%), HPV52 (3%) and the lowest type is HPV66 (1%).

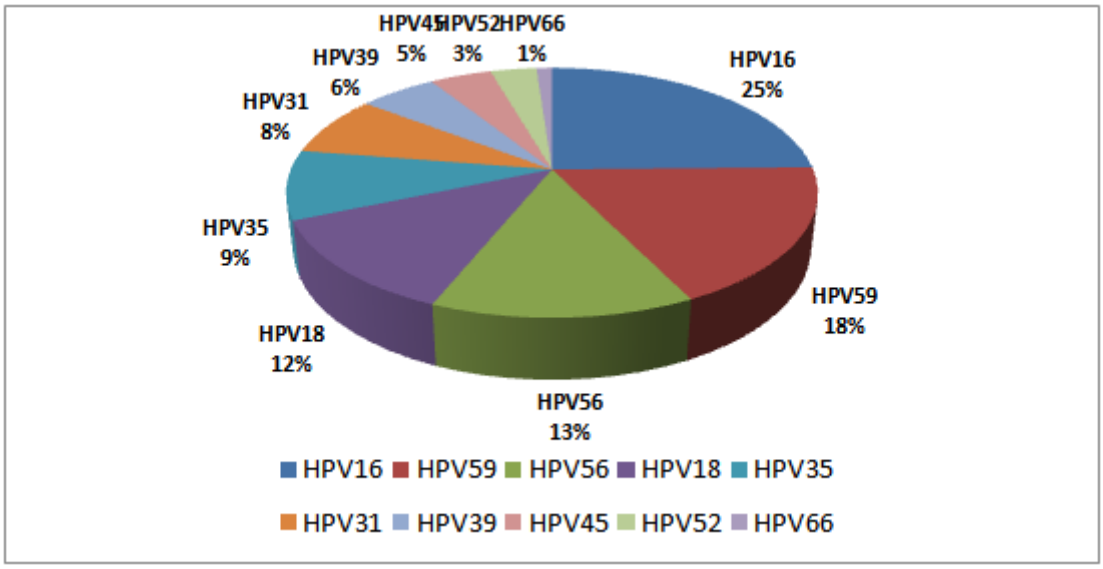


Figure 3: percentages of single and mixed HPV high risk genotypes.

The following table (Table-3) appeared that no significant differences (p>0.05) were found when compare HPV results according to histological and pap smear (Table-3).

Table 3: Distribution of HPV infection among Iraqi women according to histological examination and pap smear

Histological examination				Pap smear test			
Type	**Negative**	**Positive**	**Total**	**Type**	**Negative**	**Positive**	**Total**
AGUS	1	0	1	ASCUS	59	6	65
ASCUS	1	1	2	HSIL	11	3	14
HSIL	4	1	5	LSIL	97	4	101
LSIL	22	2	24	SCC	11	3	14
SCC	1	0	1	Other	163	17	180
Other	31	4	35	No report	324	33	357
No report	605	58	663				
t test	**0.13040791**			**t test**	**0.07988637**		

The relationship between HPV infection with many parameters (smoking history, inter-menstrual bleeding history, post coital bleeding, vaginal discharge, and using different contraceptive methods) were studied and no significant difference (p>0.05) found accordingly (Table-4).

Table 4: Distribution of HPV infection among Iraqi women according to different parameters

#	Parameter	Subtypes	Negative	Positive	Total	T-Test
1	Smoking history.	Smoker	138	15	153	0.97359586
		Non smoker	527	51	578	
2	inter-Menstrual Bleeding history	Bleeding	169	13	182	0.2560622
		No bleeding	433	46	479	
3	Post coital bleeding	Bleeding	195	19	214	0.22064188

		No bleeding	416	41	457	
4	Vaginal discharge	Yes	506	48	554	0.33686702
		No	136	16	152	
7	Contraceptive methods.	Condom	14	0	14	0.06673962
		Injectable	36	7	43	
		IUCD	94	5	99	
		more than one	16	2	18	
		Pill	153	8	161	
		Tubal ligation	2	0	2	
		Without any	285	35	320	
		Unknown	65	9	74	

VI. Discussion:

HPV infections usually clear up without any intervention within a few months after acquisition, and about 90% clear within 2 years. A small proportion of infections with certain types of HPV can persist and progress to cancer. Cervical cancer is by far the most common HPV-related disease. Nearly all cases of cervical cancer can be attributable to HPV infection (jawetz et al, 2004).

In this study there were no significant difference were found when compared HPV positive results among some provinces in Iraq (Table-1), it may be due to low sample size in each area. A large number of cases required to evaluate the occurrence of this virus all over the country.

The prevalence of HPV among Iraqi women was very low (9%) and these finding were consist with other studies in Iraq (Ashna J. Faik et. al. ,2015) (12.38%) and with other studies in our region . Islamic nature and morally committed of Iraq community may explain the limited incidence of HPV and all other sexual transmitted diseases when compared with developed countries.

The prevalence of HPV among different age in the world vary depending on economic, moral, social and religious situation, the age of women at sexual initiation, the lifetime number of partners of women and of their male sexual partners. In this study, high percentages of HPV infection found in women of childbearing age (15-39 years) and these finding are compatible with most other populations investigated (Ho GYF et al, 1998 and Ciaran BJ Woodman et al, 2001). The point prevalence observed and it's country-dependent and are strongly related to the dominant sexual behavior patterns. In some populations, a second mode in HPV prevalence is observed in older women (**Bosch FX et al, 1995)**.

Regarding to viral genotypes, there are, at present, no data to suggest that the risk of developing cervical cancer is significantly different for each of the different HR-HPV types (Chichareon S et al, 1998 and Chaouki N et al.,1998). In our study the percentages of HPV genomes were (25%, 18%, 13%, 12%, 9%, 8%, 6%, 5%, 3%, and 1%) in (HPV16, 59, 56, 18, 35, 31, 39, 45, 52, and 66) respectively. These results were identical to several studies in Iraq, which confirmed that the virus type 16 is the predominant type in the country (Anfal M., 2016). There is another study in Iraq showed that the HPV33 (18%) are the predominant type in Iraq (Ashna J. et al, 2015), also (Ali S.HM., 2001) appeared the that HPV16 (58.3%) is the first predominant type in Iraq followed with HPV31/33 (25%) and HPV18 (16.7%). The difference in HPV genotypes prevalence may be due to samples size, geographic difference, using different techniques and different primer pairs. Also , may be some genotypes of HPV virus is widespread at that time, and over the years may other HPV genotypes is circulated around the country and dominate in particular regions.

Although there is no significant difference were found when compared the results of cellular and histological differences for women with HPV human papillomavirus infection in this study, but many of the studies referring to the importance of a cellular and histological examination as a means to predict cervical cancer. The long time interval (> 15 years in some studies) between HPV infection and cervical neoplasia at the level of HSIL or more advanced lesions. The risk of cervical cancer is specifically related to persistent HPV infections and these can be recognized years before clinical symptoms. HR-HPV is present in at least 50% of ASCUS specimens, 80% of LSIL and 90-95% of HSIL and invasive cancer cases (Koutsky LA et al , 1992).

Retrospective studies of women with cervical cancer show that the same HR-HPV type can be isolated in the preceding abnormal and normal smears for up to 15 years. From these studies, it follows that the time to develop cervical carcinoma after a cervical HR-HPV infection in a woman with a morphologically normal smear is at least 10 years and probably longer (Zielinski GD et al., 2001 & Bosch FX et al, 2001).

Early first sexual intercourse, multiple sexual partners, cigarette smoking, using hormonal contraceptives are several risk factors for HPV persistence and development of cervical cancer. In this study no significant difference ($P>0.05$) found when compared all these factors with the presence of HPV (Muñoz et al, 2002).

Case-control studies also provide evidence on the impact of other risk factors in the promotion of HPV infections to cervical cancer. Consistent associations have been described between long-term use of hormonal contraceptives and cervical cancer among women chronically exposed to HPV.

The high mortality rate from cervical cancer globally could be reduced by effective screening and treatment program and from these finding we recommended the followings:

- Women who are sexually active should be screened for abnormal cervical cells and pre-cancerous lesions, starting from 30 years of age.
- Education about safe sexual practices, including delayed start of sexual activity;
- Introduction of HPV vaccine as part of a national public health strategy that includes a comprehensive approach to prevention and control of cervical cancer.

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