Prevalence of Human Papillomavirus in Women with Abnormal Pap Smears

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Abstract:

Background: Human papillomavirus (HPV) is a widely prevalent sexually transmitted virus. Because HPV is the causative agent of cervical cancer, knowledge of the epidemiology of HPV is critical.
Objective: To study the prevalence of high-risk human papillomavirus (HR-HPV) in cases of abnormal cervical cytology in Kirkuk city.
***Methods*:** In this prospective cross-sectional study, we collected 55 Pap smears samples from women attending Azadi Teaching Hospital and private clinics in Kirkuk over the period from 2012 to 2015. Two specimens were collected from each patient; one for a Pap smear study and the other for PCR assay to detect HPV.
Results: HR-HPV was positive in 58.2% of patients. The HR-HPV positivity was highest in the age group 21-30 years (90.9%) and lowest at the age group ≥ 51 years (25%). The highest positive rate was in the group married at age 21-25 years (76.2%) and this rate decreased with progression of the marriage age until reaching (20%) in the age group married at ≥31 years. The largest group was para 3-4 (58.2%), followed by para 1-2 and nulliparous (29.1% and 9.1%, respectively). The highest positive rate was in the group para 3-4 (65.6%). Majority of the patients (67.3%) had ASC-US on cytological diagnosis, while LSIL and HSIL accounting for (18.2%) and (14.5%) respectively. The prevalence of HR-HPV infection rate increases with increasing the grade of abnormality reaching to (80%) and (87.5%) in cases of LSIL and HSIL respectively, while the positivity for ASC-US was ranking (45.9%), which was statistically significant (P-value=0.029), although the positivity in cases of HSIL was more than in the cases of LSIL the difference was statistically not significant (P-value =0.671).
Conclusion: It is worthy to add HR-HPV screen by PCR to Pap test to increase the sensitivity of primary screening for cervical cancer.
Key words: Abnormal Pap smears, HPV.

Introduction:

Human papillomavirus (HPV) is a widely prevalent sexually transmitted virus. Although the majority of infections are benign and transient, persistent infection is associated with the development of cervical, vulvar, vaginal and anal cancers [(1)].The genital HPV types are divided into two categories, 'high risk' and 'low risk', originally assigned based on whether the HPV type could or could not be found as a solitary isolate in cervical cancer specimens [(2)]. It is now universally accepted that nearly all the invasive cervical cancers and high grade intraepithelial neoplasia and

approximately (45%) of cases of penile cancer are associated with the high-risk HPV (HR-HPV) types which are types 16, 18, 31, 33, 45, 51, 52, 56, 58, 59, 68, 73, and 82. Owing to the strong association, it has been suggested that HR-HPV detection might be used as a tool to identify women at risk for the subsequent development of cervical cancer and knowledge of the epidemiology of HPV is critical [3].

Cancer of the cervix uteri is the second most common cancer among women after breast cancer worldwide, with approximately 500.000 cases of cervical cancer and it is the 5th deadliest cancer in women [4] with about 275.000 deaths worldwide every year. This is due to the fact that the majority of women in the world do not have access to cervical screening, which can prevent up to (75%) of cervical cancers, and although the incidence of cervical cancer is lower in Middle East compared with the rest of the world, most of cases of cervical cancer are detected at a late stage when the disease may have become more advanced [5].

In Iraq which has a population of 10.12 million women ages 15 years and older who are at risk of developing cervical cancer, cervical cancer ranks as the 12th most frequent cancer among women in Iraq and the 10th most frequent cancer among women between 15 and 44 years of age. Current estimates indicate that every year 291 women are diagnosed with cervical cancer and 142 die from the disease. As more than two-thirds of the patients had late diagnosis, a feasible control strategy would be to encourage Iraqi women to seek early detection of Carcinoma In Situ (CIN) [6].

Although traditional screening for HPV infection relied on the Pap smears, and in spite of the well-known benefits of the Papanicolaou (Pap) smear test, a very small number of women in Iraq are tested annually, largely through opportunistic screening during a regular gynecologic examination. Furthermore in view of the high number of false negative and positives associated with it, and the need for more sensitive methods of detection, especially in women with a high suspicion of HPV infection, cytology screening is no longer sufficient to assess cervical neoplasia. In contrast, identification of HPV DNA by a molecular technique is sensitive in identifying and monitoring the progression of CIN [7, 8].

Data is not yet available on the prevalence of cervical abnormalities and HPV type distribution in the general population of Iraq, a country in which routine Pap smears are generally not done. However, in Western Asia, the region Iraq belongs to, about (2%) of women in the general population are estimated to harbor cervical HPV infection at a given time and 76.4% of invasive cervical cancers are attributed to HPVs 16 and 18, the two vaccine-preventable types, therefore accurate information about HPV prevalence in Iraq is needed to make vaccination recommendations [6].

The aim of this study:

To determine how often high-risk human papillomavirus (HR-HPV) could be detected in a selected group of women presented with abnormal Pap smears.

Materials and Methods:

During a period of two years and five months from October 2012 to March 2015, a prospective cross sectional study involving women attending to gynecological outpatient clinic at Azadi Teaching Hospital and a private clinic suffering from one or more symptoms of

vaginal discharge, pelvic pain, irregular vaginal bleeding, post-coital bleeding and postmenopausal bleeding. Exclusion criteria were: pregnant women, , were in menstrual period, had sexual intercourse during the 24 hour before sample taking, and had used any vaginal medications. Data were collected from the participants about age, residence, menstrual, reproductive and gynecological histories, sexual history and smoking habits, and the use of contraceptives. The cervical smear samples were taken through using non-lubricated, sterilized speculum, cervical cytobrush (Rovers® Cervex-Brush® Rovers Medical Devices B.V. Lekstraat 10 5347 KV Oss, The Netherland) was introduced into the cervix and rotated for 360 degrees, for the Pap test, the slides directly prepared from the brush by spreading the brush on pre-labeled clean slides and wet fixed in (95%) ethanol for routine staining, then the brush was placed into labeled tubes containing transport medium, sealed with the cap stored at -20 °C for subsequent molecular study. The Pap smears were interpreted using the Bethesda system (TBS) 2001[9], the adequacy of smears was determined by the presence of a good number of ecto- and endocervical components, no air dryness and no artifacts. In our analysis an abnormal Pap smear test result was used for "squamous cell abnormalities", including atypical squamous cells of undermined significance (ASC-US), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), and cancer. Fifty five women with abnormal Pap smear results were included in the study, for which the detection of 12 types of HR-HPV DNA (16, 18, 31, 33, 45, 51, 52, 56, 58, 59, 68, 73, and 82) using real time polymerase chain reaction (PCR) in molecular laboratory was done.

DNA extraction:

Briefly, DNA extraction was performed from 200 µL of the cell suspension. Cells were centrifuged at 3,000 rpm for five minutes (min) and the sediment obtained was suspended in 400 µL of a cell lysis solution and centrifuged at 10,000 rpm for five min. This procedure was repeated until a white pellet has been obtained. The pellet was then suspended in 300 µL of cell lysis solution containing SDS (0.5%) and proteinase K to a final concentration of 0.05 ng/mL and incubated at 56 ºC, during one hour and afterwards for 10 min at 95 ºC for denaturation. DNA was then precipitated by addition of ammonium acetate (1.4 mM) and an equal volume of iced isopropanol and centrifugation at 10,000 rpm for 15 min at 4 ºC. The pellet was washed with iced (95%) ethanol and centrifuged at 14,000 rpm for 10 min at 4 ºC. The pellet was dried and suspended in 100 µL of Tris (10 mM) and EDTA (1 mM).

Real-Time Polymerase Chain Reaction (RT-PCR):

For differential determination of DNA of high carcinogenic human papillomavirus (HPV) types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59, all samples were submitted to a PCR reaction using Realine HPV high risk kit (Bioron, Germany). The PCR reaction consisted of 0.05 mM of each dNTP; 1 UI of Taq polymerase; 1.5 mM of $MgCl_2$; 0.5 pmol/µL of each primer and 1.0 µL of the extracted DNA. RT-PCR conditions comprised fifty amplification cycles, performed on ECO Illumina RT-PCR machine (California, USA), of 50ºC for 2 minutes, 95ºC for 2 minutes, denaturation for 10 seconds at 94 ºC,

annealing for 1.0 minute at 60 ºC and extension for 1.0 minute at 72 ºC.

All samples were then searched for HPV high risk infection by RT-PCR. The viral type was considered not determined (HPV-X) in HPV positive samples when neither DNA-HPV high risk 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 subtypes were amplified, since only these HPV types were investigated in the present study.

Statistical analysis:

Data were analyzed using the statistical package for social sciences (SPSS, version 19); Chi-square test was used for comparing proportions. A P-value of ≤ 0.05 was considered statistically significant.

Results:

During the study period 55 patients with abnormal Pap smears were tested for HR-HP DNA. The mean age of the studied cases was 32.7 years with the age range between 15 and 52 years. All women were married. No one was smoker and all had one partner. Forty nine patients (89%) were premenopausal while six patients (11%) were menopausal.

High risk-HPV was positive in 32 patients (58.2%). The HR-HPV positivity was highest in the age group 21-30 years (90.9%) and lowest at the age group ≥ 51 years (25%), while the HR-HPV positivity for age groups ≤20, 31-40 and 41-50 were (83.3%,51.9% and 28.6% respectively), as shown in (table 1).

Regarding marital age, the highest HR-HPV positive rate was in the group married at age 21-25 years (76.2%) and this rate decreased with progression of the age at marriage until reaching (20%) in the age group married at ≥31 years, as shown in (table 2).

In respect to the parity of the women included in the study, the largest group was those who were para 3-4, 32 cases (58.2%), having the highest positive rate for HR-HPV, 21 cases (65.6%) followed by para 1-2, 16 cases (29.1%), with a positivity rate of (62.5%)10 cases, while 5 of the women were nulliparous and none of them was positive. There was highly significant difference in the positivity rate between parous and nulliparous women (P-value=0.0056), but there was no statistically significant difference in the positivity rate in relation to the number of parity (P-value=0.894), this is shown in (table 3).

Among all the 55 patients with abnormal Pap smears, majority of the patients (67.3%) had ASC-US on cytological diagnosis, while LSIL and HSIL accounting for (18.2%) and (14.5%) respectively.

(Table 4) show distribution of HR-HPV positivity with regard to grading of cervical abnormality, the prevalence of HR-HPV infection rate increases with increasing the grade of abnormality reaching to (80%) and (87.5%) in cases of LSIL and HSIL respectively, while the positivity for ASC-US was ranking (45.9%), which was significantly less (P-value=0.029), although the positivity in cases of HSIL was more than in the cases of LSIL the difference was statistically not significant (P-value =0.671).

Table (1): The age distribution of the patients in correlation to the HR-HPV prevalence.

Age group (years)	HR-HPV				Total	
	Positive		Negative			
	No.	%	No.	%	No.	%
≤20	5	83.3	1	16.7	6	100
21-30	10	90.9	1	9.1	11	100
31-40	14	51.9	13	48.1	27	100
41-50	2	28.6	5	71.4	7	100
≥51	1	25	3	75	4	100
Total	32	58.2	23	41.8	55	100

Table (2): The marital age in relation to HR-HPV prevalence.

Age at marriage (years)	HR-HPV				Total	
	Positive		Negative			
	No.	%	No.	%	No.	%
≤20	7	53.8	6	46.2	13	100
21-25	16	76.2	5	23.8	21	100
26-30	8	50	8	50	16	100
≥31	1	20	4	80	5	100
Total	32	58.2	23	41.8	55	100

Table (3): The parity in relation to HR-HPV prevalence.

Parity	No.	%	HR-HPV			
			Positive		Negative	
			No.	%	No.	%
Nulliparous	5	9.1	0	0.0	5	100
Para 1-2	16	29.1	10	62.5	6	37.5
Para 3-4	32	58.2	21	65.6	11	34.4
Para ≥5	2	3.6	1	50.0	1	50.0
Total	55	100	32	58.2	23	41.8

Correlation between parous & nulliparous: P-value=0.0056.
Correlation between parous patients: P-value=0.894

Table (4): The results of pap smears abnormality in correlation to HR-HPV prevalence.

Grading of abnormality	No.	%	HR-HPV			
			Positive		Negative	
			No.	%	No.	%
ASC-US	37	67.3	17	45.9	20	54.1
LSIL	10	18.2	8	80.0	2	20.0
HSIL	8	14.5	7	87.5	1	12.5
Total	55	100	32	58.2	23	41.8

Correlation of ASC-CS & (LSIL, HSIL): P-value= 0.029
Correlation of LSIL & HSIL: P-value= 0.671

Table (5): The rate of positivity for HR-HPV in different national and international studies.

Country		Rate of HR-HPV Positivity in abnormal pap smears	Method of DNA assay
Iraq	Present study	58.2%	PCR
	Erbil (Ismail et al) [10]	67.6%	Immunohistochemistry
	Baghdad (Salih et al) [11]	40%	PCR
	Baghdad(Alwan et al) [12]	49%	Hybrid capture II
	Baghdad(Fadhil et al) [13]	50.5%	PCR
	Basrah (Fahad et al) [14]	60%	PCR
Saudi Arabia [15]		53%	PCR
Iran [16]		60%	PCR
Turkey [17]		78%	PCR
Taiwan [18]		68.4%	Hybrid capture II
Equador [19]		76.5%	PCR
Cameroon [20]		83.8%	PCR
China [21]		35.1%	Hybrid capture II
Korea [22]		65.4%	PCR
Canada [23]		84.2%	PCR
Guatemala [24]		82.6%	PCR

Discussion:

HPV screening is not practiced routinely in Iraq and voluntary or opportunistic screening is not societally the norm. The finding of this study may not reflect HPV screening results in general population of Iraq, but show the results of a selected group (women with abnormal Pap smear) that underwent HPV testing. It is important to determine the prevalence of an important infection such as HPV that can remain dormant for years and will not become symptomatic until serious pathologies has developed.

Among our studied sample the HR-HPV positive patients could have any (one or more) of the 12 types of HR-HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) and the overall prevalence of HR-HPV infection in abnormal Pap smears was (58.2%), for comparison of the present study with the others, (table 5) summarize the rate of positivity for HR-HPV in abnormal pap smears in different national and international studies.

The variations among the results of these studies could be a reflection to the sample size, type of population studied with respect to risk factors for exposure to HPV, the sampling method preparation or smears results interpretation and the sensitivity of the chosen assay.

In general the Muslim cultures especially in the Middle East countries had low infectivity for HPV, taking in consideration certain socio-cultural life differences, some traditional and religious habits of practicing male circumcision, prohibition of extramarital sex and moral standards making Muslim women less susceptible to HPV infection. In Iraq's culture, the premarital sex is rare and it is even forbidden by the law. The partners of all the patients included in our study were circumcised. In Spain, Castellsague *et al*, noted that circumcision decreased not only the risk of HPV contamination and transmission but also the risk of cervical cancer in women partners [25]. Similarly, Hernandez et al. [26] and Auvert et al. [27] reported that HPV prevalence is higher among uncircumcised men and that circumcision is a preventive measure against cervical cancer.

Snijders *et al*. [28] recommend that to accurately study HPV epidemiology, impact of HPV infection and monitor the efficacy of HPV vaccination programmes; the test with the highest result sensitivity possible must be used. The shift toward the highly sensitive PCR assays has affected findings in recent epidemiological studies, showing that HPV infections are more widespread than had previously been recognized [29].

In our study (41.8%) of the women with abnormal cytological changes in Pap smear were negative for HR-HPV DNA assay results. This observation may be explained by the presence of either other microorganism like herpes virus, parasites and fungi which induce these cellular changes. In addition women positive to other HR-HPV types not included in the probes of our test may have been missed as a negative in our real-time probes, or the tested samples were with very low number of virus DNA copies. At the same time this discordance may be the explanation of erroneous false positive diagnosis of abnormal cytological results.

The present study clearly demonstrates the presence of peak of HR-HPV infection in relation to age, with

increasing the prevalence of HR-HPV infection with advancing age until reaches the peak in the age group (21-30) years then decline after that.

In general, it is well observed worldwide that the prevalence estimates of HPV infections are age dependant and shows three patterns of distributions. The first pattern that mostly show similarity to our results where the Age-specific prevalence is high in the young age women then decline and reach low levels at older ages, that is the picture in some countries like Turkey, in which there were significant differences with respect to HPV positivity between women under 30 years old and women older than 30 years (34% versus 20%) [30]. Also in Japan, HPV infections peaked among women of 20-29 years (20.4%) [31] and the same pattern was observed in many other countries such as China [32], Tunisia [33], USA [34] and Europe [35]. On the other hand in India [36] and Nigeria [37] the prevalence of HPV was high in all age groups and never falls substantially with no peaks at any age. In the third pattern the age curve of HPV infection had two peaks as it tends to rise for the second time in middle age (36-45 years), followed by a drop in the prevalence at older ages which is the case in Latin America. Second lifetime peak of HPV infections may made by those married above 30 years or infections acquired in the earlier years that were now re-emerging with older age and waning immunity [38].

In this study (67.6%) (23/32) of women with positive HR-HPV were in the group who were married prior 25 years of age. The early age of the first sexual practice is consistently associated with higher HPV infections [39], due to long duration of sexual activity, and may be explained by the fact that at younger age the immature mucosal immune system will give higher chance for HPV infection to occur. In addition most cervical cancer arises at the squamocolumnar junction between the columnar epithelium of the endocervix and the squamous epithelium of the ectocervix. At this site there are continuous metaplastic changes, and risk of HPV infection coincides with the greatest metaplastic activity which occurs at puberty and first pregnancy and declines at older ages [40]. However this association of having the first sexual intercourse at an early age with an increased risk of HPV infection was reported by some authors,[41,42] while other studies showed this association to be insignificant [43] or inverse [44].

With respect to parity, our results showed an increase risk of HR-HPV infection with the increasing parity, and it was significantly lower in nulliparous, this may be due to hormonal factors related to pregnancies or explained by pregnancy and labor associated repeated cervical damage and repair making it vulnerable to infection. Similar results shown by others [45].

Accumulating evidences indicate a significant association between HPV and the development of (CIN) as (90%) of these lesions are attributed to the infection with HR-HPV [46]; in addition, infections with HR-HPV is associated with 250 fold increased risk of high grade cervical intraepithelial neoplasia [47].

Our results showed a strong association between HR-HPV type infections with cervical epithelial abnormalities especially high grade lesions. The highest percentage of positive results for HR-HPV was found in patients with HSIL (87.5%) and LSIL (80%)

compared to patients with ASCU-S (45.9%) which is statistically significant (P-value=0.029). Therefore, HPV DNA testing could be useful in supporting results of cytology, predicting the severity and improving the sensitivity of the test. These results in agreement with other study [43].

Unfortunately, our study is limited by the fact that it covers only the women with abnormal Pap smears and had no opportunity to test the women with normal Pap smears and limited sample size due to financial restraints.

Conclusion:

The results of this study provide further evidence for the role of HPV in cervical carcinogenesis. This is a pilot study for bigger studies to test the effectiveness of adding HR-HPV screen to Pap test to increase the sensitivity of primary screening for cervical cancer.

The presence of this rate of HPV infection in sexually active Iraqi women make a molecular investigation for HR-HPV essential for clinical approach in patients with proven epithelial abnormality in their Pap smears and also to those with borderline reports.

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