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THE ASSOCIATION OF HUMANA PAPILLOMAVIRUS WITH CERVICAL NEOPLASM IN BASRAH

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Abstract

Cervical cytobrush and Pap smears were collected from 103 women attending the outpatient department at Basrah Maternity and children hospital during the period from October 2009 till the end of January 2010. DNA was successfully extracted from 91 cytobrush samples, amplified for the detection of human papilloma viruses (HPVs) using GP5+/GP6+ primers, in addition to typing using type-specific primers for HPV-16 and HPV-18 genotypes.

The overall HPV prevalence was 20.8% with the dominance of genotype 16 (36.6%) over the genotype 18 (10.5%) and the presence of non-16, non-18 genotype(s) in 42.1% of all HPV positive cases. Younger women aged 25 years or less were more infected (26.3%) with the dominance of genotype-16 (21.1%) and among women aged 36-45 years, the non-16/non-18 genotype(s) were the more frequently observed (13.3%). Infection rates were more frequent among women married above 30 years (33.3%) and those with more than one lifetime husband (28.6%). Women's husband with polygamy practice significantly covariate with HPV infections ($P< 0.05$).

Significant association ($P<0.001$) was obvious between infection with any HPV and abnormal cytology. The non-16/non-18 genotype(s) were more involved (28%) whereas genotype-16 was detected more frequent (20%) than genotype-18 (4%). In addition mixed infection of both high risk types (16 and 18) was limited only to women with abnormal Pap smears.

In archival blocks, the presence of HPV- DNA was constant in pre-invasive sequamous cervical lesions as all blocks revealed a positive HPV-16 either alone or in combination with genotype-18.

Introduction

In 1996 the world health association, along with the European Research Organization on genital infection and neoplasia and the National Institute of health consensus conference on cervical cancer, recognized human papillomavirus (HPV) as an important cause of cervical cancer[1].

Genital HPVs are primarily transmitted through sexual contact with infected cervical, vaginal, vulvar, penile or anal epithelium[2]. In utero transmission could be caused either by ascending infection from infected birth canal or hematogenously via the placenta; HPV-DNA has been detected in born by cesarean section[3,4] as well as spontaneously aborted materials[5].

Although most women will acquire an infection with at least one HPV genotype during their lifetime, particular factors have been found to be associated with increased risk of HPV infection[6,7], as young age, sexual behavior and early age of first sexual intercourse. Women who had had two or more sexual partners has double the risk of women who had only one partner and women whose husbands has had extramarital sexual relationships had a 50% higher HPV positivity rates[8].

In order to assess worldwide variability in types of HPV, workers of International Agency for Research on Cancer (IARC) tested random samples of women from[9-11] countries for HPV. The overall crude prevalence of any HPV type was 9%.

Grouping by region showed that HPV prevalence was five times higher in sub-Saharan Africa than Europe, with intermediate rates in Asia and South America. Age standardized prevalence for any HPV was 26% in Africa, 14% in south America, 9% in south east Asia and 5% in Europe[9].

In middle east countries, the prevalence of HPV was 1.8% - 13% in the low risk general population (GP) and 32 – 88% in cervical Inter-epithelial Neoplasm (CIN) (HPV 16 in 10 – 30%) and cervical cancer (CCA) varied from 45% - 78% (HPV 16 in 31 – 67%)[13]. However, the prevalence of HPV in the Extended Middle East and North Africa (EMENA) is around 5–12% in the GP, 30-80% in CIN and 60–90% in CCA with HPV-16 as the dominant type. The GP age range was 30-50 years, while in CIN it was 30-60 years and CCA was 30-70 years. These studies were varied with small number of patients and varied detection methods that difficult to compare[10].

This study aimed on estimation of HPV prevalence in cervical specimens of women from normal GP of Basrah and patients with malignancy or pre-malignancy neoplasm in relation to age, age of marriage and some demographic variables based on molecular method (PCR) compared to histopathological finding (PAP smear) among normal and abnormal women with the identification of HPV genotypes in our area.

Materials and methods

Population and study design: The study consisted two parts, the first is a cross-sectional study conducted in the period extended from the beginning of October 2009 till the end of January 2010 from the patients attending the outpatient department of Basrah Maternity and Children Hospital from whom a cervical cytobrush (for DNA extraction and viral detection) and Pap smear (for cytology) were taken in addition to demographic data regarding age, residence, smoking, parity, contraceptive use following special questionnaire form designed for this study. The criteria for inclusion, the patient should fulfill the followings: Married or previously married, non-pregnant, no heavy vaginal bleeding. By the use of sterilized speculum, cervical cytobrush was introduced into the cervix and rotated for 360 degree 3 minutes and the cytobrush placed into labeled eppendorff tube containing 0.2 ml transport medium, kept in ice pack till immediate processing at the department of microbiology, Basrah medical college. More materials was obtained by wooden Ary's spatula and smeared on labeled slide which was immediately placed in 95% ethyl-alcohol allowed to remain in the fixative for at least 15 minutes before air dry then submitted for Pap staining.

During this period 103 samples were collected and the internal control was successfully amplified in 91 samples[11,12].

The second part of the study was done on archival samples where the DNA was extracted from formalin fixed, paraffin embedded cervical tissue blocks of 12 patients diagnosed as cervical neoplasia or pre-neoplastic lesions that collected in a period from December 2008 till december 2009 which was obtained From Al-Sader Teaching hospital and many private laboratories.

Molecular procedure: DNA extraction from both cervical cytobrush and paraffin embedded tissue were amplified by polymerase chain reaction (PCR) for viral detection and typing using DNA sorb extraction kit of Sacace Biotechnologies (Promega corperation, USA) following the manufacturers instructions. To assess the successfulness of extraction, the samples were subjected to 0.8% agarorse gel electrophoresis (company) following standardized procedure.

A. Detection of HPV using consensus non-degenerate primers (GP5+/GP6+). PCR was used for the detection of HPV DNA from the DNA collected of both cervical cytobrush and paraffin embedded

cervical tissues. Two sets of primers were used for each sample: i) internal control primers to assess the quality of the extracted DNA, Beta globin primer (CH20/PCO4), ii) HPV was detected using consensus non-degenerated primers GP5+/GP6+ (supplied by Alpha-DNA, Canada). The sequence of primers used following sense/anti-sense order which were as the following: Betaglobin gene: CH20, 5'-GAA GAG CCA AGG ACA GGT -3' / PCO4, 5'- CAA CTT CAT CCA CGT TCA CCC-3' and L1-gene of HPV: GP5+, 5'-TTT GTT ACT GTG GTA GAT ACT AC-3'/ GP6+; 5'-GAA AAA TAA ACT GTA AAT CAT ATT-3' The annealing temperature of the primers (GP5+/GP6+) was adjusted to 45C using gradient PCR thermal cycler (Thermal cycler: EO-Esto-Swiss Mari, USA) to give a better yield with multiplex PCR. After amplification of the DNA, the aliquots were subjected to 2% agarose gel electrophoresis and the resulted bands were compared with DNA ladder to determine the exact size of the amplified genes.

B- Typing of the detected HPV using type-specific primers of HPV types 16 and 18: Typing of HPV was directed using the HPV16/18 kit provided by Sacace, Biotechnologies. The target region of the primer in the kit is E6 gene.

Results

Out of the 91 specimens tested with consensus non-degenerate HPV primers, 19(20.8%) cases were positive for HPV-DNA (Table-I). Two peaks of HPV infection was observed; younger women aged 25 years or less had more infection rates (26.3%). The second peak was observed among women aged between 36-45 years (23.3%) and the least prevalence was found among women aged above 45 years.

HPV genotype-16 was the most prevalent type in the younger age group (21.1%). It was not detected in women aged 26-35 years and appears again in age group of 36-45 years (6.7%) and to a lesser extent at the last age group of women aged above 45 years (5.3%). Genotype -18 was less prevalent than type-16 and mixed infection with both 16 and 18 was observed 2 age groups (26-35 and 36-45 years). The non-16/non-18 genotype(s) was more frequent in the second peak of HPV infection (13.3%) of women aged 36-45 years.

Table-II shows the presence of HPV in relation to selected marital factors. HPV was more frequently detected among women who were still married at the time of the study (21.9%) compared to 11.1% among those separated from their husbands. Women married at age above 30 years carried the highest prevalence of HPV infections (33.3%), followed by those who married at younger age less than 20 years (21%). Women with more than one lifetime husband were more infected with HPV (28.6%) compared to those who had single lifetime husband (20.2%). There were a significant differences between women whose husbands had other wives (38.1%) compared to 15.7% observed among women whose husbands had single marriage ($P < 0.05$).

The detection of HPV and its types in relation to the results of cervical smears cytology is presented in table-III. HPV infections was significantly detected in abnormal cervical cytology (60%) compared to 6.5% of those with normal cervical smears cytology ($P < 0.001$). HPV type 16 and the types other than 16/18 were the commonest in the cases with abnormal cytological finding accounted for 20% and 28% respectively. Type 18 was also detected either alone or forming combined infection with type 16 and 18 which was accounted for 4% and 8% respectively. Type 16 was the most common type in cases with normal cytology (3.2%), type 18 was also detected as a single infection without combination with type 16 forming 1.6% of the total normal cytology. Similarly,

other types presented also in 1.6% of normal cytological smears.

Table-IV shows the distribution of HPV and its types in the archival paraffin embedded blocks of cervical malignant and pre-malignant lesions. HPV-DNA was detected in all the extracted DNA from the blocks of cervical neoplastic and preneoplastic lesions (100%) with the predominance of combined infection of both high risk types 16 and 18 in the invasive lesions (60%). Type 16 was also detected frequently in both types of lesions (50% of preinvasive and 40% of invasive lesions) whereas, type 18 was not detected alone in either histopathological types.

Discussion

HPV is among the most important viruses in the causation of human cancers and large number of epidemiological, biological and clinical studies were directed to know the nature of this infection and its outcome.

The estimated prevalence of HPV is widely variable geographically with many factors affecting the results of each study; the behavioral profile of the studied population and the method used for detection are the main[13]. The overall prevalence of HPV among women involved in this study was 20.8%, nearly similar to that reported in a Turkish study (23%)[14] and Morocco (20.5%)[15] using the same primers utilized in our study. The used of different primers may explain the wide differences in the obtained results in some Arabic countries as Tunisia (14%)[16]. In addition to the size of sample involved in the study which can affect the prevalence as in Lebanon (4.9%)[17] in a study involved 1026 women using both GP5+/GP6+ and MY09/MY11 primers. The The difference in sample collection might also reflect different results as in a Chines study where their sample from women hospitalized for delivery using type-specific L1 primers showed a pre-valence of 53.3%[18].

The dominance of HPV genotype-16 (7.7%) over genotype-18(2.2%) seen in this work was in agreement with many other studies worldwide as in Algeria HPV-16 forms 6.2% compared to 0.7% for HPV-1819 and 5.3% for HPV-16 versus 1.1% for the genotype-18 in Brazil[19-20]. On the other hand, HPV-16 was also dominant over genotype-18 in Saudi Arabia[21] as it forms nearly twice of our figure (13.3%) compared to HPV-18 (3.3%).

The present study clearly demonstrates the presence of peaks of HPV infection in relation to age. The first at age of 25 years or less and the second was observed in women at age ranging from 36–45 years. In general, it is well observed worldwide that the prevalence estimates of HPV infections are age dependant with three pattern of distributions[13]. Age-specific prevalence is high in the young age women then decline sharply and reach very low levels at older ages[13]. In Turkey there were significant differences with respect to HPV positivity between women under 30 years old and women older than 30 years (34% versus 20%, p=0.005)[14]. In Japan, HPV infections peaked among women of 20-29 years (20.4%)[21,22] and the same pattern was observed in many other countries as China[23], Tunisia[16], USA[24] and Europe[25]. On the other hand in India[26] and Africa (Nigeria)[27] the prevalence of HPV never falls substantially and were high in all age groups. The third pattern that mostly show slight similarity to our results where the age curve of HPV infection tend to rise in middle age[21]. In contrast to Latin America studies[28,29], the second HPV infect rise at 36-45 years was followed by a drop in the prevalence at older ages. Therefore, the second peak might be made by those married above 30 years and other possibility is the re-infection that probably the result of a second marriage is usually around this range of age.

While it's difficult to know for how long a woman has been infected, factors such as older age, high risk of HPV types and presence of multiple genotypes infections are the indicators of persistent infection that is regarded as the main determinant for malignant transformation of cervix[27]. In the current study, the high risk HPV genotype-16 was the dominant in young age group (25 years or less) whereas the non-16/18 genotype(s) alone were the dominant in the second HPV infection peak. The predominance of high risk types in early age groups was seen in many other studies as in Japan[22] and Chile[30]. As the second peak in our study is mainly attributed to the untyped HPV, the probability of those unrecognized types to be part of low risk HPVs can be suggested. However, this needs further investigations to prove it.

The early age of the first sexual practice is consistently associated with HPV infections[8]. Women married prior 20 years of age carry higher infection rate than those married between 21-30 years. This is in agreement with many other studies[30,31]. However, the highest prevalence in this study was among women their marriage was above 30 years. Other factors might play a role in this presentation as women married after 30 years could be an additional wife for a man with polygamy practice.

One of the most important variables that increase a women risk of HPV infection is the sexual activity of her partner, as women whose husband had extramarital sexual relationships had a 50% higher HPV positivity rates[32,33]. In this study the number of husband other wives appears to be the only marital factor that covariates significantly with the prevalence of HPV. This factor also played a significant role in the prediction of HPV infection in Italy[34].

The prevalence of HPV among women with normal Pap smears was beside the lower limit of the estimated range (5-12%) in general population with normal cytology as in EMENA region[10]. Some similarities was observed in many other studies as in Korea(8.5%)[35] and the overall prevalence in Asia(8.7%)[9]. However, the use of less sensitive techniques (in situ hybridization) might explain the low prevalence reported in an Egyptian study (2.6%)[36].

Accumulating evidences indicate a significant association between HPV and the development of cervical intraepithelial neoplasia (CIN) as 90%of this lesions is attributed to the infection with high risk HPV[37]; in addition, infections with high risk HPV is associated with 250 fold increased risk of high grade cervical intraepithelial neoplasia[38]. It is clear from the current study findings, the prevalence of HPV in women with abnormal cytology was significantly higher than that shown in women with normal Pap smears. This also in agreement with international believe worldwide[27,36,39]. HPV-16 genotype was the predominant among normal cytology women in the present study as it forms half of HPV-DNA positive cases. The predominance of HPV-16 was in agreement with many other studies in the region[14,39,40].

Worldwide it is estimated that both genotypes 16 and 18 are associated with 50% of high grade cervical abnormalities and 30% of low grade cervical abnormalities[41]. The presence of unrecognized type(s) in a good percentage (28%) among abnormal Pap smears was obvious in this study, but it is not clear whether this rank is formed by single or multiple viral, genotypes, and if so, the real presentation of each genotype also remain questionable and needs further evaluation to determine their position compared with the genotype-16 which form a high percentage of all abnormal Pap smears (20%). However, dual infection with both high risk genotypes 16 and 18 was limited to cases with abnormal cytology forming 8% of the total abnormal Pap smears women. Although the feature of mixed infection is

still doubtful since the possibility of presence of non-16/18 genotype(s) in association with other genotyped groups of infection is not well clarified in this work regardless this point, this mixed infection was nearly equal to the result seen in Nigeria (8.8%)[27].

Despite the limited number of paraffin embedded tissue involved in this small supportive histopathogy based study, it is clear that the presence of HPV –DNA was constant among pre-invasive and invasive sequamous carcinoma blocks of women from our locality. This is regarded high if compared with more extended study conducted in Baghdad that used in situ hybridization for HPV detection where the HPV-DNA was detected in 25% and 22% of the invasive and preinvasive lesions respectively[42]. The wide difference between this study and our study might be the reflect of the use of different diagnostic methods. Generally estimated prevalence of HPV in cervical cancer patients in EMENA region ranged from 60-90%[10]. Although HPV-16 was the commonest type detected alone or in combination with genotype-18 in all archival samples, dual infection with both oncogenic types (16 and 18) seems to play an important role in the cervical malignant transformation since more than half of malignant cases were associated with such mixed infections, in addition their combined presence was also limited to the cases with abnormal cytology. The dominance of type-16 in this study was in agreement with of Baghdad as type-16 form 58.3% of HPV positive invasive sequamous cancer[42]. This trend was also the dominant in India[43] and Pakistan[44].

In conclusion, HPV infection is prevalent among women in our locality with dominance of HPV-16. Early age and women married after age of 30 years are at higher risk of acquiring HPV infections. Early and polygamy sexual practices play a role in the increased rates of HPV infections and there were a strong association between HPV infection and abnormal cytology with the constant presence of HPV-DNA in invasive sequamous cervical cell carcinoms, and the dual infection with both high risk HPV-genotypes (16 and 18) seems to play an important role in the cervical malignant transformation.

Table I: Distribution of HPV and its genotypes in relation to patient's age groups

Age groups	HPV positive	HPV positive cases			
(Years)	N/T (%)	Genotype 16 N (%)	Genotype 18 N (%)	Genotypes 16&18 N (%)	Non-16/18 genotype(s) N (%)
<25	5/19 (26.3)	4 (21.1)	0	0	1 (5.2)
26-35	4/23 (17.4)	0	1 (4.3)	1 (4.3)	2 (8.8)
36-45	7/30 (23.3)	2 (6.7)	0	1 (3.3)	4 (13.3)
>45	3/19 (15.8)	1 (5.3)	1 (5.3)	0	1 (5.3)
Total	19/91(20.8)	7/19	2/19	2/19	8/19

Table II: The presence of HPV-DNA in relation to different marital factors

Factor	Character	HPV	P-value
		+ve/T (%)	
Marital Status	Married	18/82 (22)	
	Widow/divorce	1/19 (11.1)	NS
Age at	< 20	12/57 (21.1)	
Marriage (Years)	21-30	5/28 (17.9)	NS
	>31	2/6 (33.3)	
Number Of lifetime	1	17/84 (20.2)	
Husbands	more than 1	2/7 (28.6)	NS
Number of Husband's	0	11/70 (15.7)	
Other wives	>1	8 /21 (38.1)	P= 0.027
Total		19/91(20.8)	

Table III: Occurrence of HPV genotypes in relation to results of Cervical smears cytology

Results of Cytology	HPV +/T(%)	H P V genotypes			
		16	18	16&18	Non16/non18
Normal Pap	4/62	2	1	0	1
Smears	(6.3)	(3.3)	(1.6)	(0)	(1.6)
Abnormal	15/25	5	1	2	7
Pap smears	(60.1)	(20)	(4)	(8)	(28)
Total	19/87	7	2	2	8

Table IV: Genotypes of HPV in relation to the histopathology of cervical smears

Histopathology	HPV – genotypes				Total
	16	18	16&18	Non 16&18	
Preinvasive	1	0	1	0	2
Carcinoma	(50)	(0)	(50)	(0)	
Invasive sequamous					
Cell carcinoma	4	0	6	0	
	(40)	(0)	(60)	(0)	10
Total	5	0	7	0	12
	(41.7)	(0)	(58.3)	(0)	

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