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Relation of HPV genotyping by PCR to cervical Pap smear results in women with genital warts

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ABSTRACT:

Background Papillomavirus is genera of the family Papovaviridae. The papilloma virus yield in their host benign skin tumors. The greatest common genital lesion produced by HPV infection is the benign genital wart (condylomata acuminata). Types HPV-6 and -11 are the main types associated with these lesions. This wart is distributed throughout the female genital tract on the cervix, vaginal wall, and vulva and perianal region. The importance of genital warts is its relation to the risk factors of cervical carcinoma, so perfect genotyping is essential in the management of these patients. In addition, the finding of premalignant lesions of the cervix is also essential in the management and this can be done easily by cervical pap smears. **Aim:** HPV genotype in patients with genital warts by PCR technique and the identification of the relation of genital warts with pap smear results in women from Nineveh Governorate.

Materials and methods: the current study a cross sectional was performed over 12 months period from June 2013 through June 2014, with a total of 551 cervical/Pap smears test were collected of women seeking gynecological advice for different complaints attending the major three hospitals in Mosul city, the ages between 11 and 76 years, with a mean age of 38.4 years. Chi-square test of independence; were designed for two catigoral variables. P-values ≤ 0.05 were taken into account statistically significant all over data analysis. The other part of the work including the HPV DNA tests which were applied by the PCR technique (by HPV Direct Flow Chip for in vitro detection, Master Diagnostica Kit, is aimed at simultaneous screening and genotyping of 36 HPV genotypes, High risk-HPV and low risk-HPV by PCR, after that reverse dot blot automatic hybridization, based on DNA-Flow Technology (e-BRID System)

Results: The cervical pap smears results of the women were classified according to Bethesda system into two groups. The largest No. of the results was negative intraepithelial or malignancy (NILM), 452/551 (82.03%), while the cervical epithelial abnormalities (EA) in the other group was 99/551(17.97%) women. The highest rate of cases with cervical EA cytological results was found in women with non genital warts (93.94%). The rate of cervical EA in women with genital warts compared to no warts, were 6.06%. The infection of HPV was representing 17.96% same as the percentage of cervical EA results. There were three categories of genotypes for HPV in cervical EA: low risk-HPV, high risk-HPV, and mixed infection (low and high risk-HPV). All cases had history of genital warts in the present study were positive for HPV100% (6/6). Different HPV genotypes found in the present study, five distinct HPV genotypes were detected in genital warts which were HPV-6, 11, 16, 18, 31. The predominant type in genital warts to HPV-18 3/6.

Conclusion: From present results it can be concluded that; 1- the rate of HPV infection in Nineveh women suffering from genital warts were 6/6 (100%). 2- the most predominant HPV genotypes commonly related with genital warts were the high risk HPV predominantly genotype HPV-18. 3- the relation between genital warts with pap smear results were non- significant p=0.5.

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Introduction

Papillomavirus is "genera of the family Papovaviridae". The papilloma virus (Latin: papilla=nipple; oma=tumor) yield in their host benign skin tumors (papilloma) [1].

HPV is a DNA virus, double strand that can affect basal epithelial cells of all human squamous epithelia. The genome of HPV encodes for 9 viral proteins; the certain early ('E')- region encodes for regulatory, transforming and replication viral proteins. The viral capsid proteins encodes for at late ('L')-region which are only expressed in highly differentiated epithelial cells, a high number of HPV copies resulting in superficial epithelial layers. E6 and E7 viral proteins are known "cancer promoting genes"[2].

At current, 170 HPV genotypes have been categorized in accordance with their biological niche, oncogenic potential and phylogenetic position [3, 4]. Human papilloma viruses (HPVs) genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56,58, 59, 68, 73 and 82 are thought oncogenic or high-risk genotypes (HR-HPV), with genotypes 26, 53, and 66 being possibly oncogenic[5].

The highest communal genital lesion produced by HPV infection is the benign genital wart (condylomata acuminata). The HPV genotypes, HPV-6 and -11, are the major genotypes associated with these lesions, which are infrequent malignant change has only been recognized in patients with an immune deficiency. The warts are distributed all over the female genital tract on the cervix, vaginal wall, and vulva and perianal area. The premalignant changes accompanying with HPV can arise on the same places as for warts, though malignant change is most often observed at the cervix. The premalignant changes of the cervix are called "intraepithelial neoplasia" and are categorized, in accordance with the Bethesda system [6], as "low-grade squamous intraepithelial lesions (LSILs) or high-grade squamous intraepithelial lesions (HSILs)". Genotypes HPV-6 and -11 are found in LSILs, whereas the oncogenic types, HPV-16 and -18, and are discovered in all grades and in cancerous disease. All types of squamous epithelium may be infected by HPV. Warts develop around 3 months (6weeks-2 years) after inoculation [7]. Infect the basal cells of the stratum germintivum with replication and viral assembly taking place as basal cells mature and move to surface. Virions are shed along with dead keratinocytes. Warts and condylomata are associated with proliferation of epidermal layers resulting in acanthosis and hyper keratosis. Some infected cells may develop characteristics perinuclear vaculation- koilocytosis. Excessive proliferation of the basal layer is premalignant feature. The presence of external genital warts should prompt the

consideration of cervical HPV, cervical intraepithelial neoplasia (CIN). Genital warts may spontaneously remit (approximately 10% of cases over 4 months) [7].

The detection of cervical epithelial cells changes can be diagnosed microscopically by Papanicolaou (Pap) conventional cervical smears staining. This systems the basis of cervical screening programs for recognition of women at risk of disease advancement[8]. Bethesda system is now recommended internationally for proper classification and cytological diagnosis of intraepithelial changes and cervical carcinoma (2001). Women who have not had regular Pap smears at risk of invasive cervical cancers. Molecular HPV detection provides a different method to screening and management of the patients, and specific high risk genotype which is regarded as the highest cause of cervical cancer [9].

Furthermore, there was no well, known documented study concerned with HPV infection in Nineveh / Iraq women with genital warts and the role of HPV and its types in genital warts is less clear and has not been well studied. So, the aim is to study the genotypes of HPV in patients with genital warts by PCR technique and to identify the relation of genital warts with pap smear results in women from Nineveh Governorate.

Materials and Methods

1. Patients Selection

A cross sectional study was performed over 12 months period from June 2013 through June 2014, 551 cases were collected from women at ages between 11 and 76 years with a mean age of 38.4 years. From these samples, 99 cases with positive intraepithelial changes detected by Pap smears were selected for HPV DNA tests.

Samples were collected from patients seeking gynecological advice for different complaints attending the major three hospitals in Mosul.

For all of the cases two samples were taken one for Pap smear and the other for viral diagnosis, put in deep freezer, then any abnormal case by Pap smear is selected for HPV DNA testing. All samples were taken in the Gynecology Clinical Units in Gynecological Mosul Hospitals with the aid of the Cytology and Pap Smear Units in these Hospitals.

2. Inclusion criteria:

Women presented with different complaints including genital warts were included in the study.

Every women included in the study were filling the standard questionnaire for sample collection and cervical pap smear reporting paper. The data included women's

background and relevant risk factors such as general characteristics of women, lifestyle, marital status, and menstrual and obstetric history. Also the present of genital warts for women or husbands or each of them (Appendix II) were included in the questionnaire.

3. Exclusion criteria:

Pregnant women were excluded because they are not cooperative, refused the test because of fear of possible abortion, also menstruating women at time of attendance were excluded, because of the artifact and the confusion caused by the red blood cells in the pap smear.

4. Conventional Pap smear cytology:

Specimen Type

Conventional Pap (CP) was used in the study for cytological assessment of the cervix for the diagnosis of malignancy and premalignant dysplastic changes1990s[10].

Exfoliate cytology, or the Pap test the method available for early detection of cervical cancer which is advised worldwide for quantity screening, as the usefulness in the detection of premalignant lesions, and cost effectiveness. It is a reliable and convenient procedure widely used for the collection of exfoliated cervical cells for cytology testing [11, 12, 13, 14].

The Pap test was often done by a senior gynecologist, with the aid of the speculum and the use of a wooden Ayer's spatula by gentle scraping of the cervix for cells collection from the endocervix and the endo-ectocervical junction by rotating the end of the spatula at the junction of the cervix, then was directly smeared onto a microscopic glass slide, and immediately immersed into a jar containing 95% ethyl alcohol for fixation (for 30 minutes).

At the cytopathology department, the slides were stained by pap stain for cytological diagnosis and all cases were categorized in consistent with the Bethesda system and the report was designed and the results were classified into 4 groups according to Bethesda system(2001) ASC-US, ASC-H, LSIL, and HSIL for the premalignant lesions and SCC for any case of malignancy[15].

5. HPV DNA tests:

The other smear was obtained at the same time by using a swab for viral investigation, which was immediately inserted into a virus transport media VTM(∑VIROCULT) specimen collection and transport system (www.mwe.com. UK). These specimens were stored at deep freezer, until the time of the test. These samples are used for the detection of HPV DNA by PCR method (Appendix IV).

The principle of the test (by HPV Direct Flow Chip for *in vitro* detection, Master Diagnostica Kit, Granada, Spain) is intended for simultaneous screening and genotyping of "36 HPV types, High risk-HPV (16, 18, 26, 31, 33, 35, 39, 45, 51, 52,

53, 56, 58, 59, 66, 68, 73 and 82) and low risk-HPV (6, 11, 40, 42, 43, 44, 54, 55, 61, 62, 67, 69, 70, 71, 72, 81, 84, and 89) by PCR, followed by reverse dot blot automatic hybridization, based on DNA-Flow Technology (e-BRID System)".

This kit is based on the amplification of human papilloma virus L1 consensus region by PCR and hybridization to specific DNA probes immobilized onto a nylon membrane. The DNA-Flow based automatic hybridization platform allows the binding of the amplified DNA to the complementary capture probes in a three-dimensional porous environment, which enables a very fast coupling between the PCR product and its specific probe. Biotinilated PCR products are hybridized with specific probes and the hybridization signals developed by colorimetric immunoenzymatic reaction (streptavidin – alkaline phosphatase and NBT-BCIP chromogen). The substrate –chromogen reaction generates a dark-purple precipitate in the position where the specific probe has hybridized with the PCR amplicon and this signal is automatically captured and analyzed (e-BRID System). This technology has a very high sensitivity for HPV detection and it can be performed in a very short time comparing to other systems reducing total processing time from hours to minutes.

Figure I: e-BRIDSYSTEM

- Automated
- Maximum15 samples
- Processing1 -15 samples in
- 30-90 minutes (hybridization)
- Samples bar-code identification

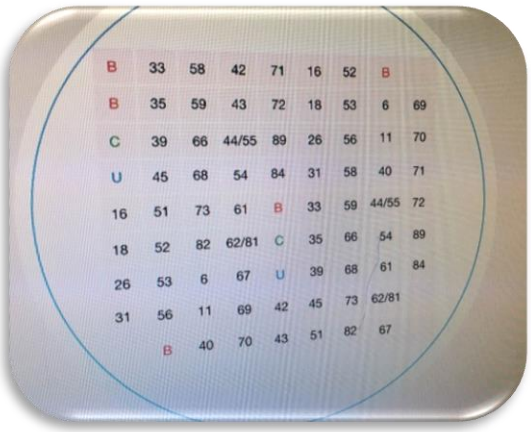

Figure II Genotyping of HPV.

B-Hybridization control

C-DNA quality control.

U-universal control (human beta globin gene).

Numbers- indicate the HPV genotypes

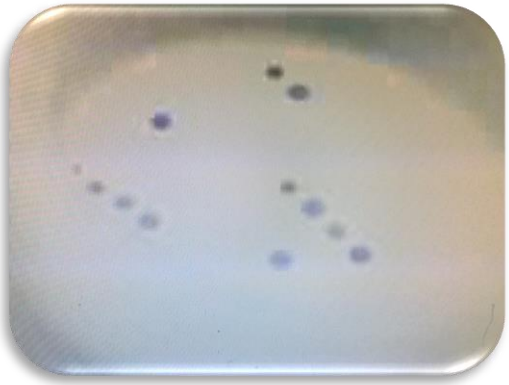

Figure III: Results of reverse dot -blot.

The substrate –chromogen reaction generates a dark-purple precipitate in the position where the specific probe has hybridized with the PCR amplicon**.**

6. Statistical analysis of the data .

Categorization of the data and coding achieved via Microsoft Excel-2007. "The descriptive and analytic statistics was carried out by using Minitab version 16.2 software statistical program". The descriptive statistics comprise frequencies and percentages for catigoral variables. Chi-square test of independence; were used for two catigoral variables. P-values ≤ 0.05 were regard as statistically significant all over data analysis.

Results

1. The Presence of Genital Warts in the Study Women.

The genital warts were found in 22 (3.99%) women seen by physical examination and/or history, and only 9 (1.63%) husbands who had history of genital warts…Table I

Table I: The presence of genital warts by history and/or physical examination in the study Women.

Genital warts	No.	%
No warts	520	94.37
Wife warts*	22	3.99
Husband warts	9	1.63
Total	**551**	**100.00**

*** The husband of two ladies has warts too.**

2. The Relationship between the Genital Warts and Pap smear Results

The highest rate of cases with cervical EA cytological results was found in women with no genital warts (93.94%). The husband of one woman had genital warts (1.01%) of cases with cervical EA cytological results. Women with genital warts represented 3.76% of cases with NILM and 5.05% of cases with cervical EA

cytological results. The results were statistically non-significant…Table II.

Table II: The relationship between the genital warts and Pap smears results.

Items	NILM		EA cervical		Total		P-value*
	No.	%	No.	%	No.	%	
No warts**	427	94.47	93	93.94	520	94.37	---
Wife warts	17	3.76	5	5.05	22	3.99	0.563
Husband warts	8	1.77	1	1.01	9	1.63	0.598
Total	**452**	**100.00**	**99**	**100.00**	**551**	**100.00**	---

*** Chi-square test was used, d.f = 1.**

**** Reference group.**

3. The HPV genotypes distribution in cases with genital warts

Different genotypes of HPV were detected in the present study, five distinct HPV genotypes in genital warts cases which is HPV-6, 11, 16, 18, 31.The distribution of these genotypes were 4/6 with high risk-HPV and 2/6 with mixed infection (low and high risk-HPV), and the predominant type in genital warts to HPV-18 were 3/6…Figure I

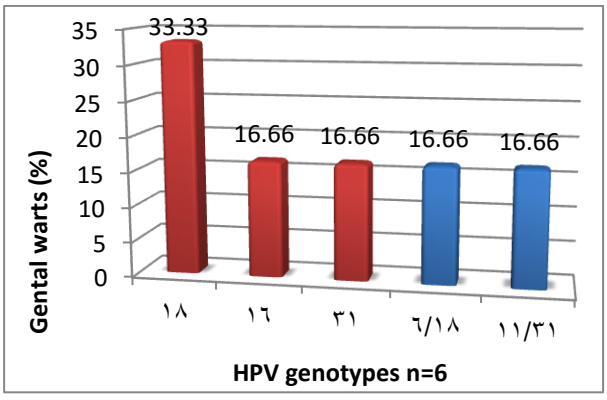

Figure 1: Distribution of HPV genotypes in genital warts cases

Discussion

1. The relationship between the genital warts and Pap smears results

The present study revealed that the rate of cervical EA in women with genital warts compared to no warts, were 6.06%: 5.05% women with cervical EA had genital warts, one women (1.01%) with cervical EA had husband with genital warts. All cases had history of genital warts in the present study were positive for HPV100% (6/6). The prevalence of HPV in genital warts in Egypt was 60% [16]. Concerning the prevalence of HPV and its genotypes in genital warts cases is estimated 7-10% in the Europe and 1% in America[17]. The rate variation may be due to racial differences, high exposure to the virus, or to the relatively small number of patients involved in present study.

2. Distribution of HPV genotypes in genital warts cases

Five distinct HPV genotypes were found in genital warts cases in the present study, which were HPV-6, 11, 16, 18, 31.The distribution of these genotypes 4/6 with HR-HPV and 2/6 with mixed infection, and the predominant type in genital warts to HPV-18 3/6. A study in Egypt were found HPV-6 the predominant genotype[17]. Other study on three patients with cancer found that one patient of three positive for HPV type 18[18].

CONCLUSION: From present results we can conclude that the rate of HPV infection in Nineveh women suffering from genital warts were 6/6 (100%). 2- the highest prevalent of HPV genotypes commonly related with genital warts were the high risk HPV predominantly genotype HPV-18. 3- the relation between genital warts with pap smear results were not significant p=0.5.

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