Detection of Low Risk Genotypes of Human Papilloma Virus 6 and 11 in Patients with Cervicitis by Real Time PCR Assay

Mushtak T.S. Al-Ouqaili, ***Dean of College of Pharmacy - University of Anbar***
Shaymaa H.M. Al-Kubaisy, ***College of Pharmacy –University of Anbar***
Ahmed M.T.Al-Aethwy, ***College of Science–University of Anbar***
Tamadhir M.H. Al-Alousi ،*Al-Anbar Health Office*

Abstract

Background: The human papillomavirus (HPV) infection is one of the most prevalent sexually transmitted disease. The low risk genotypes of Human Papilloma Virus such as 6, 11, 40, 42, 43, 44, 54, 61, 70, 72 and 81 are mainly associated with benign genital warts and rarely leading to cancer. This study has been undertaken for detection of the occurrence of HPV 6 and 11 in patients with cervicitis depending on Real-Time PCR assay.

Patients and methods: A total of 100 females (80 patients and 20 healthy females); ages (range from19- 62 years) were included and studied during the period from December 2014 to April 2015. They included 80 (80%) patients were diagnosed at private clinic in Ramadi from patients with different cervical lesions and healthy females: (health individuals 20 (20%).Real time-PCR technique was done for DNA extracted from these samples.

Result: The mean range for age showed that patients with cervical lesion from patient with pap smear samples were (31.63 ± 6.12), polyp samples (30.32 ± 8.35), ulcer samples was (31.52 ± 10.70), while the wart samples (30.19 ± 7.38), Leukemia patients have wartic lesion samples was (30.50 ± 2.52). No significant difference between age and location results for the other groups ($P > 0.05$). The purity of DNA from clinical specimen was (1.39 ± 0.23). There was extracted DNA from clinical specimens regarding purity or DNA concentration at which ($P > 0.05$). These results showed that the rate of HPV 6/11 DNA detection in different clinical samples were statistically significant for the patient group ($P = 0.0005$). In addition to that, the distribution of positive and negative HPV 6/11 between patients and control is also significant (P=0.002).

Conclusion: The low risk genotypes of HPV 6 and 11 have a significant role in patients with cervicitis. The prevalence rate of Low-Risk HPV genotype 6 was found to be (17.5%), while that of HPV genotype 11 was (12.5%).

Key words: Human Papilloma virus, cervicitis, RT-PCR.

Corresponding author: **Shaymaa H.M. Al-Kubaisy, College of Pharmacy –University of Anbar**

Introduction:

Cervical cancer is the second most common cancer among women in worldwide after breast cancer[1]. The incidence rate is 500,000 among the new cases annually with a mortality of about 250.000 worldwide [2, 3].

The role of HPV as a human carcinogen was discovered in the late 1970's, when Dr. Harold ZurHausen discovered the causal link between HPV infections and cervical cancer[4]. Recently, cervical cancer remains a significant global health burden which affecting over 500,000 women each

year worldwide[5]. HPV infection remains essentially the main cause of this cancer[6]. HPV types that infect genital tract are divided into two categories Low risk (LR) and high risk (HR) types. The LR types induce only benign genital warts and include HPV6 and 11.

The human papillomavirus (HPV) infection is one of the most prevalent Sexually Transmitted Disease (STD) with the number of cases continuing to grow[7]. Depending on the techniques used for detection and the observed population the prevalence of genital HPV infection in young women has been estimated to range between 20% and 46% in various countries, but some studies suggest that the potential lifetime risk of infection to be around 60% or greater[8]. Since HPV cannot be cultured efficiently and the clinical performance of serological assays is poor, the diagnosis of HPV infection is almost entirely based on molecular tools including polymerase chain reaction (PCR)[9].

Thus, identification of HPV DNA by PCR has been reported by several investigators which documented that this technique is more sensitive than cytology by the identification, diagnosis and in monitoring of the progression of CIN[10].The estimated prevalence of HPV infection in the Hungarian female population is approximately 17.6%; with the highest rates occur in sexually active women among 17-22-year olds [11].The Low risk types HPV6 and 11 are found in 90% of genital warts [12].The genomic DNA of HPVs is organized in three regions. These regions are the upstream regulatory region (URR), the early region and the late region. The early and late regions are both protein-encoding, but URR is non-encoding protein[1]. The cycle is initiated by infectious particles reaching the basal layer of the epithelium, where they bind and enter into cells through small breaks [13, 14]. Receptors involved in viral entry are thought to be heparin sulphate proteoglycans playing a role in initial binding and/or virus uptake [15].

The presence of secondary receptors seems to be required for an efficient HPV infection, such as α6 integrin[16]. The up-regulation of the differentiation-dependent promoter is critical for the onset of late events and directs two set of transcripts; one set terminates at the early poly-A site while the second set terminates at the late poly-A site downstream of L1.

The first group of late transcripts encodes E1^E4, E5, E1 and E2 and the second encodes the capsid proteins L1 and L2. The first protein expressed in the late phase of the viral life cycle is E1^E4. It is detected in the spinous and granular cell layers and has several functions [17, 18]. Virus capsid proteins L1 and L2 are expressed only in cells of the granular layer with viral particle assembly taking place in the cornified layer. Infected cells are scaled off from the epithelial surface and may be transmitted directly to other individuals[18].

Patients and Methods

Patients: A total of 100 females (80 patients and 20 healthy females); ages (range from19- 62 years) were included and studied during the period from December 2014 to April 2015. They included 80 (80%) patients were diagnosed at private clinic in Ramadi from patients with different cervical lesions. healthy females: (health individuals including natural and regular of menstrual cycle, pH 3.8-4.5, no itching, health discharge doesn't have a strong smell and color, no irritation and no infection).

Sample collection, Storage and Transportation Cervical swabs:

A- Excess mucus was removed from the cervical of and surrounding ectocervix used a cotton or polyester swab. This swab was discarded.

B- The sample cervical brush was inserted 1.0-1.5 centimeters into the cervical os until the largest bristles touch the ectocervix has not inserted brush completely into the cervical canal. Rotate brush 3 full turns in a counterclockwise direction, removed from the canal.

C- The brush was inserted into the nuclease-free 2.0 ml tube with 300 µL of transport medium.

D- The brush was vigorously agitated in medium for 15-20 sec.

E- Snap off shaft at scored line, the brush end was leaved inside tube.

The cervical swab was processed immediately in the laboratory by centrifugation at 3500 rpm for 10 minutes and the supernatants stored at – 20°C until analysis at the laboratory ASCo. Learning center, Baghdad, Iraq[19].

DNA Extraction from cervical lesion swabs for detection the Human Papilloma Virus genotypes 6 and 11.

Lysis and washing solutions was warmed up to 60–65°C until disappearance of ice crystals. The required quantity of 1.5 ml polypropylene tubes was prepared included one tube for negative control of extraction.2. To each tube 300 µl of lysis solution was added.3. 100 µl of samples was added to the appropriate tube.4. The controls were prepared as follow:-100 µl of C– (Neg Control provided with the amplification kit) was added to the tube and labeled C neg.5. The tubes were vortexed and incubated for 5 min at 65°C for 7-10 sec and centrifugated. If the sample was not completely dissolved re-centrifuge of the tube for 5 min at a maximum speed (12000-16000 g.).

was occurred and transferred the supernatant into a new tube for DNA extraction.6. Sorbent was vortexed vigorously and 20 µl to each tube was added.7. Vortexing was achieved for 5-7 sec and all tubes were incubated for 3 min at room temperature.

This step was repeated.8. All tubes were centrifuged for 30 sec at 5000g and a micropipette was used with a plugged aerosol barrier tip, carefully removed and discarded supernatant from each tube without disturbing the pellet. Changed tips between the tubes.9. 500 µl of washing solution was added to each tube and vortexed vigorously and centrifuged for 30 sec at 10000g. Removed and discarded supernatant was removed and discarded from each tube.10. Step 9 was repeated and incubated all tubes were incubated with open cap for 5-10 min at 65°C.11. The pellet was re-suspended in 100 µl of DNA- eluent and incubated for 5 min at 65°C and vortexed periodically.12. The tubes were centrifuged, for 1 min at 12000g.13.

The supernatant were contained DNA ready for amplification. If amplification was not performed in the same day of extraction, the processed samples can be stored at 2-8°C for at maximum period of 5 days or frozen at -20°/ -80°C[19, 20].

Protocol

1. The required quantity of reaction tubes for samples (N) and controls (N+2) were prepared.
2. The mixture was prepared as follow: for 60 samples: PCR- buffer-FRT 30 µl of TaqF DNA Polymerase was added into the tube. The tube was vortexed carefully. This mix was stabled for 3 months at +4°C.
3. Reaction mix was prepared by added for each sample into the new sterile tube 10 µl of PCR-mix-1-FRT and 5 µl of mix PCR- buffer-FRT/ Taq F DNA Polymerase.
4. To each reaction tube was added 15 µl of reaction mix was added and 10 µl of extracted DNA and mixed by a pipette.
5. For each panel 2 controls were prepared:

- Ten µl of DNA-buffer was added to the tube labeled amplification negative control.
- 10 µl of Positive Control Complex C+ was added to the tube labeled amplification positive control.

6. The tubes were inserted in the thermal cycler. The results were interpreted through the presence of crossing of fluorescence curve with the threshold line.

DNA quantitation:

Ten µl of DNA sample was added to 990µl of distilled water, and mixed thoroughly, and then the Optical Density

(OD) was measured in a spectrophotometer at wavelengths of (260 nm and 280 nm). The DNA concentration in the solution was calculated according to the following formula:-DNA concentration (µg/µl) = [OD260 X 100 (dilution factor) X50 µg/ml] /1000.Theoretically, OD260 of one corresponds to approximately (50µg/ml) for double strand DNA. The ratio between the reading at 260 nm and 280nm (OD260/OD280) provides an estimation of the purity of nucleic acid[19].
Pure DNA will give an A260/A280 of 1.8 or higher. Values of A260/A280 of less than 1.8 indicate contamination of the DNA by protein. For good PCR results, DNA is required with an A260/A280 quotient of 1.6 or greater [21,22].

Polymerase chain reaction (PCR):Principle of the assay:
In this novel technique, genomic DNA is first heated and denaturated to form single strands. In the annealing phase, the DNA is cooled, allowing hybridization with primer sequences that flank the region of interest. Then the reaction is heated to an intermediate primer extension temperature, in which DNA polymerase add free bases in the 3'direction along each single strand, starting at the primer. Blunt- ended DNA fragments are formed, and these provide a template for the next cycle of heating and cooling. Repeated cycling produces a large number of DNA fragments bounded on each end by the primer sequence.

Amplification
1-Create a temperature profile on the instrument as follow.

Real-time PCR program of HPV 6/11			
Step	**Temperature °C**	**Time**	**Repeats**
1	95	15 min	1
2	95	5 s	5
	60	20 s	
	72	15 s	
	95	5 s	
3	60	30s fluorescent signal detection	40
	72	15s	

Statistical analysis:
The data of the 100 cases in this study were entered into and analyzed by the statistical package for social science (SPSS) software version 17.Descriptive statistics were presented as mean, standard deviation for age, purity and DNA concentration. Frequencies and percentages for the other categorical variables.

The difference and distribution in age according to methods of contraception, diagnosis and HPV results were tested using student t test. The relation of the HPV with education level, contraception and site was tested by using the Chi square test (X^2). A level of significance (P value) of ≤ 0.05 was considered significant.

Results

Clinical parameters

The study was carried out on 100 clinical specimens obtained from 80 study patients and 20 healthy individuals. The specimens of patients were classified clinically according to experient clinician as represented in Table (1).

The age was arranged from 19-62 years with mean 30.9 ±8.5. The clinical specimens of this study were distributed as follow, 33 (41.25%) ulcer, 16 (20%) warts, 19 (23.75%) polyp .while Pap smear was obtained from 8 (10%) and 4 (5%) Leukemia patients have wartic lesion samples.

Table 1: Clinical classification of study specimens according to the type

Clinical specimens 80(100%)				
Ulcer	**Warts**	**Pap smear***	**Polyp**	**Leukemia ****
33 (41.25%)	**16 (20%)**	**8 (10%)**	**19 (23.75%)**	**4 (5%)**

*Pap smear was previously diagnosed
**Leukemia patients have wartic lesion samples

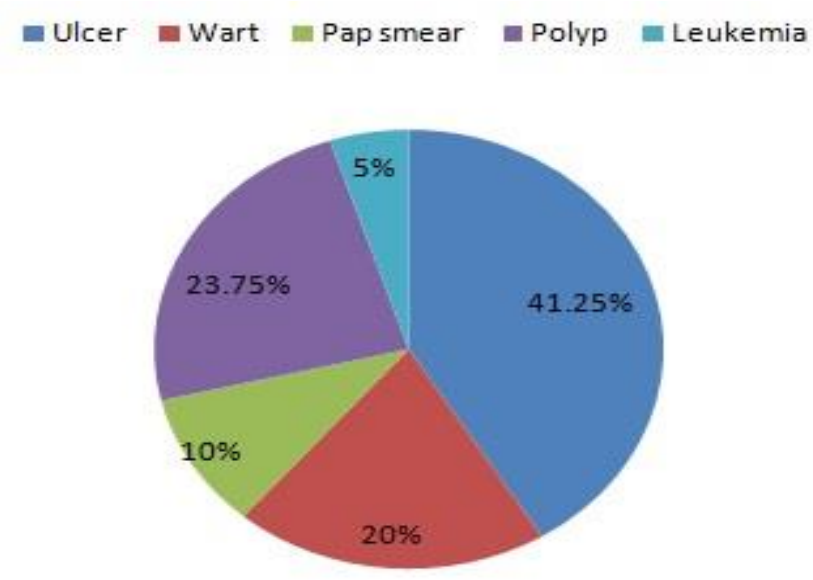

Figure 1: The classification of clinical specimens according the type the cervical lesions

The age parameters according to the prevalence of Human Papilloma virus

The age parameters of the study samples 100 (80 patients with cervical lesions swabs and 20 healthy females) according to the type of samples were reflected in the table 2.

This table showed that the age groups for the patients and distribution into positive and negative results obtained from real time-PCR for low risk Human Papilloma Virus. There are no significant relation between age groups and HPV results in control and patients (P > 0.05).

Table 2: The prevalence of Human Papilloma virus according to the age

Age		Human Papilloma Virus					
		Positive		Negative		Total	
		Count	%	Count	%	Count	%
Cervical lesion swabs	19-28	12	33.3%	24	66.7%	36	45.0%
	29-38	16	50.0%	16	50.0%	32	40.0%
	39-48	3	42.9%	4	57.1%	7	8.8%
	49-58	2	50.0%	2	50.0%	4	5.0%
	>=59	0	.0%	1	100.0%	1	1.2%
	Total	33	41.3%	47	58.8%	80	100.0%

The age parameters according to the type of samples

The mean range for age showed that patients with cervical lesion from patient with pap smear samples were (31.63 ± 6.12), polyp samples (30.32 ± 8.35), ulcer samples was (31.52 ± 10.70), while the wart samples (30.19 ± 7.38), Leukemia patients have wartic lesion samples was (30.50 ± 2.52) in the following table showed the summarized results. No significant difference between age and location results for the other groups (P > 0.05).

Table 3: The mean age for the studied sample according to location

		Age (year)	
		Mean	Standard Deviation
Cervical lesion swabs	Pap smear	31.63	6.12
	Polyp	30.32	8.35
	Ulcer	31.52	10.70
	Wart	30.19	7.38
	Leukemia	30.50	2.52

Molecular detection of HPV results
Genomic DNA extraction

The genomic DNA extraction was carried out on the cervical swabs samples taken from patients and healthy subjects group for obtaining optimum yields of genomic DNA for PCR amplification.

The purity of DNA from clinical specimen was (1.39 ± 0.23). There was extracted DNA from clinical specimens regarding purity or DNA concentration at which (P> 0.05).

Table 4: Purity and concentration of genomic DNA extracted from clinical specimens

No. of sample	Purity (OD.260/OD280)		Concentration of DNA (ng /µl)	
80	Range	Mean ± SD.	range	Mean ± SD.
	0.9-1.77	1.39 ±0.23	50-1390	806.4±257.5

Real Time PCR for detection of Low Risk human papilloma virus (6 and 11) results

The results of Low risk HPV 6/11 revealed that 24 out of 80 (30%) cases from clinical sample give positive results, among the studied samples with different clinical lesion the results showed that the HPV 6 DNA positive cases were found in 2 out of 80 (2.5%) of patients with ulcer, 1 out of 80 (1.25%) of patients with polyp, 1 out of 80 (1.25%) of patients with leukemia and 10 out of 80 (12.5%) of patients with warts.

HPV 11 DNA positive cases were found in 4 out of 80 (5%) cases positive of patients with ulcer, 2 out of 80 (2.5%) of patients with polyp and 4 out of 80 (5%) of patients with warts, it was not detected in control group.

These results showed that the rate of HPV 6/11 DNA detection in different clinical samples were statistically significant for the patient group (P = 0.0005). In addition to that, the distribution of positive and negative HPV 6/11 between patients and control is also significant (P=0.002). The results are summarized in following table.

Table 5: The prevalence of Low risk Human Papilloma virus 6/11

Clinical samples	HPV 6 DNA positive	HPV 11 DNA +ve	HPV6/ 11 DNA -ve	Total no. of study group
	No (%)	No (%)	No (%)	No (%)
Ulcer	2(2.5%)	4(5%)	27(33.75%)	33(41.25%)
Polyp	1(1.25%)	2(2.5%)	16(20%)	19(23.75%)
Leukemia patient	1(1.25%)	0.0	3(3.75%)	4(5%)
Pap smear	0.0	0.0	8(10%)	8(10%)
Warts	10(12.5)	4(5%)	2(2.5%)	16(20%)
Total no. of patients	14 (17.5%)	10 (12.5%)	56 (70%)	80 (100%)
Controls	0(0.0%)	0(0.0%)	20(100%)	20(100%)

Real Time PCR for detection of Low-Risk human papilloma virus (6 and 11) analysis

The results of this study in Low-risk HPV 6/11 revealed that 24 out of 80 (30%) cases from clinical sample gave positive results, among the study samples with different clinical lesion 14 (17.5%) for LR-HPV 6 and 10 (12.5%) for LR - HPV genotype 11.

Discussion:

The prevalence of Human Papillomavirus (HPV) has not been well studied in Iraq especially in Anbar Governorate. The present study is the pioneer study, which utilized Real-Time Polymerase Chain Reaction (PCR) technique as molecular tool for detection of low -risk genotype; Human Papillomavirus (HPV).The results revealed that the increased rate of HPV infection from 12 out of 36 (33.3%) among study group with younger age group 19-28 years. while, the high raised rate of HPV infection about 16 out of 32 (50%) among another group (older age 29 - 38 years), this elevated rate also observed at age ranges (39 - 48 years) at HPV infection about 3 out of 7 (42.9%), 2 out of 4 (50.0%) at age group 49-58 years and no reported results for HPV infection at age group > = 59 (0.0%).Regarding the increased rate of HPV infection in correlation to age group. Carestiato and coworkers, 2006were revealed in their study that highest rate was among the age group of 21-30 years[23] whereas, On the other hand, Eslami and co-workers, 2008 documented that the highest rate was among cases with the age range of 35 - 44 years[24].Chan and colleagues, (2009)were also observe that the second minor peak of HPV prevalence in women aged 46-55. This dissimilarity represents the variation in incidence and prevalence of genital HPV infections in the different geographical area. In addition to several other factors such as study design, the sensitivity of virus detection test, virus subtypes studied and sexual behaviors. Superior to these aforementioned factors, adopting and the effectiveness of cervical cancer screening programs for the diagnosis and treatment of women with HPV-induced cervical lesions[25].The highest rates occur in sexually active women among 18-28 year-olds [7]. The estimated prevalence of HPV infection in the Hungarian female population is approximately 17.6%; with the highest rates occur in sexually active women among 17-22 year - olds [11].The result obtained from present study showed that high rate of HPV infection at age group 29-38 and an older age group 49-58 years. Most of the studies suggested that above 35 years of age HPV infection might represent viral persistence, probably caused by oncogenic HPV and induced the risk of cervical cancer development[26]**.** In other study by Dunne, et al., (2007), Human Papilloma Virus prevalence was 24.5 % among females ages 14-19 years, 44.8% among women ages 20-24 years, 27.4% among women ages 25-29 years, 27.5% among women ages 30-39 years, 25.2% among women ages 40- 49 years, and 19.6% among women ages between 50 to 59 years[8]. There was a statistically significance trend for increasing HPV prevalence with each year of age from 14 to 24 years P value < 0.001 followed by a gradual decline in prevalence through 59 years (P = 0.06). Al-Shabanah and coworker, 2013[26], find that the patient's mean age was 50 ± 11 years (range, 25-78 years). The patients' age distribution was analyzed and the prevalence of HPV genotypes was detected among them. There was no significant difference (P > 0.5) observed in HPV infection among patients with age < 45.0 year compared with more than 45 years old, in which 15/35 (42.8 %) of the patients with age <

45 years old and 27/65 (41.5%) were positive for HPV.It was clear that the cervical lesion from patient with Pap smear samples positive have mild dysplasia and moderate dysplasia, polyp, ulcer and leukemia patients. If the pap result is "ASC-US," then an HPV-DNA test may be done in the lab to see whether HPV is causing this borderline "normal-abnormal" pap result[27].Warts (benign skin papilloma) are common, benign skin tumors. Malignant genital warts contained HPV-6 or -11 DNA five of 27 cervical cancer biopsies were examined contained HPV-11. Similar findings were made by other groups. While, this evidence was suggested a role for HPV in cervical cancer, the low-risk HPV types (mainly 6 and 11) may cause clinically apparent, benign genital warts, the most recognizable signs of genital HPV infection in less than 10% of all infections .According to the results obtained by our study, the purity of extracted DNA from cervical lesion swabs was ranged from (0.9-1.77) with mean 1.39 ± 0.23. Spectrophotometer measurements revealed that the differences in DNA concentration and purity according to the origin of tissue. The OD260/OD280 ratio values satisfied those suggested by Sambrook, et al., (2004), ≥ 1.8, the variability in DNA quality and purity can be explained by tissue -specific structural complexity[19].The DNA concentration was determined by optical density measurement in a spectrophotometer using 50µg/mL as 1 OD260. The 260/280 ratio should be in the range of 1.7-1.9 and should not exceed 2 as this indicates contamination of the preparation of RNA or low molecular weight nucleic acids that lead too overestimation of DNA concentration[19].An advantage of the solution is to allow samples to be stored for longer periods of time without compromising DNA integrity. It also produced good DNA suitable for HPV detection and identification. The method is simple, fast and generates DNA of high molecular weight. Another advantage is that no griding is necessary to obtain sufficient DNA [19, 20].

The results of this study in Low-risk HPV 6/11 revealed that 24 out of 80 (30%) cases from clinical sample gave positive results, among the study samples with different clinical lesion 14 (17.5%) for LR-HPV 6 and 10 (12.5%) for LR -HPV genotype 11. Al-Shabanah and co-worker, (2013) reported that the prevalence of low-risk types 6 were 4/8 (50%) and 4/8 (50%) for HPV 11[26].Genital warts are the most frequent benign tumors in the anogenital region of both males and females. Human papillomaviruses are etiologically associated with the development of virtually all Genital warts. HPV-6 and HPV-11 are the most commonly detected Human papillomavirus genotypes but at least 20 other alpha-HPV genotypes have occasionally been found in Genital warts tissue specimens [28]**.** HPV types are non-oncogenic, or low-risk HPV types, such as HPV 6 or 11, can be caused that (1) benign or low-grade abnormalities of cervical cells, (2) anogenital warts, and (3) a disease of the respiratory tract called recurrent respiratory papillomatosis (RRP**)** [(29,30)]**.** Dunne and associates, 2007reported the prevalence for Low-Risk HPV 6 1.3% while HPV11 was 0.1%[8].The study concluded thatthe relationship between the prevalence of HPV and age factor was highest among the age group of prevalence the LR-HPV (6& 11) genotypes between cervical lesion samples was 29-38 and 49-58 years. Also, the prevalence rate of Low-Risk HPV genotype 6 was found to be (17.5%), while the rate of infection by LR HPV genotype 11 was (12.5%) in the study patients different cervical lesions and it was 0.0% in the control group. Further, the prevalence of LR-HPV genotypes 6/11 was 12.5% in the study patient with warts lesion while the other rates of prevalence for LR-HPV genotypes with different cervical lesions was low such as polyp was 1.25%, ulcer 2.5% , leukemia patients1.25%, and pap smear 0.0%.

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