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MOLECULAR GENETICS STUDY ON HIGH AND INTERMEDIATE RISK GENOTYPES OF HUMAN PAPILLOMAVIRUS AMONG PATIENTS WITH BENIGN AND MALIGNANT CERVICAL LESIONS

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ABSTRACT: Background and Objectives: It is well realized that cervical cancer is one of the most critical diseases in the world. This study has been laid down for molecular detection (qualitatively and quantitatively) and genotyping of high and intermediate risk HPV genotypes in patients with high suspicion of cervical cancer. **Patients and Methods:** A total of 120 females were included in this study. DNA has been extracted automatically using Samaga DNA extraction unit. Real time-PCR technique for detection and genotyping of high and intermediate risk genotypes was performed. **Results:** The genes amplification revealed that only 9(11.3%) out of 80 were positive for high-risk HPV. The genotyping study yielded that the most prevalent genotypes were HPV 16, 18, 39, 33 and 35. Among the result of high-risk genotypes of HPV, the viral load was 6.42 ± 0.71 for clinical status CIN III. Only 11 out of 20(55 %) of FFPE were positive for high-risk HPV. On the other hand, The clinical status of high-risk HPV genotypes from FFPE samples were 4(20%), and 5(25%) for A7 and A9 with clinical status CIN II and viral load was 5.57 ± 0.75 for A7 and 6.28 ± 0.75 for A9. **Conclusion:** The study concluded that the use of Real-Time PCR as a diagnostic and genotyping tool for HPV has the advantage for women who currently have high-grade cervical lesions and at high risk of developing cervical cancer in the future.

INTRODUCTION: Recently, the World Health Organization documented that cervical cancer is the fourth most frequent cancer in women with an estimated 570,000 new cases in 2018 representing 6.6% of all female cancers. The increase of death rate due to cervical cancer in the world could be sharply reduced through a complete approach that includes prevention, early diagnosis, effective screening, and treatment programmes [1].

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It is believed that that virtually all cervical cancers and their precursors are caused by persistent high-risk human papillomavirus (hrHPV) infection [2]. Modern cervical cancer screening increasingly relies on the use of molecular techniques detecting high-risk oncogenic human papillomavirus (hr-HPV) [3]. However; PCR tests usually target only a 100-500-bp region for amplification whereas the HPV genome is 7.9 kb, so, there is a wide choice of which area to amplify.

Most importantly, differences in biological properties and sequence characteristics of different regions mean that the decision of the target region can be crucial to a reliable outcome of a PCR for HPV [3]. The cancer of the cervix is one of the most frequent cancers globally.

Among the world, the incidence rate of cervical cancer is 500.000 among the new cases annually with mortality of about 250.000. [4] It is well documented that there are clear functional differences between α and *β*-HPV oncologies, and also between high- and low-risk viruses. The role of high-risk HPV types in cervical cancer and some tonsillar and oropharyngeal tumors has been well studied most of the knowledge about HPV-dependent oncogenesis originates from the high-risk types HPV 16 and 18. [5]

Also, HPV types that infect genital tract were grouped into low and high-risk genotypes. The low-risk genotypes will induce only benign genital warts and include HPV type 6 and 11. Functional studies on the HPV early gene products showed that E6 and E7 play a key role in carcinogenesis. These two proteins use multiple mechanisms to evade host immune surveillance, allowing viral persistence, and to deregulate cell cycle and apoptosis control, thus facilitating the accumulation of DNA damage and ultimately cellular transformation [6]. Approximately 15 high-risk mucosal HPV types are associated with cervical cancer; HPV16 and HPV18 are the most carcinogenic since they are responsible for about 50% and 20% of all cervical cancers worldwide, respectively. HPV16 and HPV18 are the most studied HPV types because of their high level of carcinogenic activity. Biological studies have highlighted the critical roles in the cellular transformation of the products of two early viral genes, E6 and E79. [7]

The oncogenic properties of these high-risk HPV types lie in the oncoproteins early (E6 and E7) which can bind to and modulate many different gene products, in particular, the tumor suppressors proteins (p53 and pRb). These interactions led to the disturbance of cell cycle control and a deficiency in DNA repair resulting in genomic instability and increased the risk of malignant transformation. Vjekoslav highlighted the biological consequences of papillomavirus targeting of various cellular substrates at diverse anatomical sites in the development of HPV-induced malignancies [8].

Polymerase chain reaction (PCR) is the most powerful tool for the epidemiological investigation of HPV infection, especially in cervical and breast cancer. PCR is now the standard gold test for HPV research. This methodology has overcome the misclassification of HPV status that initially confused the scientific community regarding HPV in cervical and breast cancer [9]. Masahiko and Zhi-Ming [10] documented that there is a crucial role of HPV16 E6^E7 in stabilizing E6 and E7 oncoproteins and evidenced its contribution to oncogenicity in cooperation with E6 and E7, although E6^E7 itself does not independently affect the stability of p53 or pRB.

Low risk-HPV genotypes were also reported to be a cause of cervicitis at which Al-Ouqaili and associates [11] concluded that 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 six was found to be (17.5%), while that of HPV genotype 11 was (12.5%). Detection of HPV DNA by PCR has been documented by several researchers who reported that this technique was more sensitive than cytology by the license, diagnosis and in the monitoring of the progression of CIN [12]. Thus, this study has been laid down for molecular detection the presence of high-risk HPV genotypes qualitatively and quantitatively from clinical samples in patients with high suspicion of cervical cancer depending on Real-Time PCR assay. Also, to perform genotyping high and intermediate risk genotypes of HPV.

PATIENTS AND METHODS: A total of 120 females (100 patients and 20 healthy women); ages (range from 19- 62 years) were studied during the period from December 2014 to April 2015. They included 80 (100%) patients were diagnosed at Private Clinic in Ramadi from patients with different cervical lesions and 20(100%) retrospective samples of formalin-fixed, paraffin embedded tissue (FFPE) from patients with different cervical cancer. Control group: (health individuals including natural and regular of the menstrual cycle with pH 3.8-4.5, and no itching.

Also, health discharge doesn't have a strong smell and color; no infection or irritation was involved in this study. The samples (cervical swabs) were collected and transported. After that, the cervical swab was processed immediately in the laboratory.

DNA Extract from Fixed Formalin Paraffin Embedded Blocks (FFPE): DNA was extracted from formalin fixed paraffin embedded tissue as follow:

Day 1: One ml of xylol was added (pre-incubated at 37 ºC for 1 h) incubated at RT for 30 min. Then, overtaxing for 30 sec was done. After that, spin down for 5 min, 13000 rpm and supernatant were discarded. The step 1, 2 and 3, was repeated and then the pellet was re-suspended in 180 µl buffer ATL. 20 µl proteinase K was added (stock solution 20 mg/mL), and mixed by vortexing. Then, incubation overnight at 56 °C in the water bath was achieved.

Day 2: 200 µl buffer AL added to the sample and mixed thoroughly by vortexing for 15 sec. Then, while RNA-free genomic DNA was required, three µlRNase A added (100 mg/mL) and incubated for 30 min at room temperature. 200 µl of ethanol was cooled (96-100%) was added, and mixed again thoroughly by vortexing for 15 sec and incubated for 5 min at 37 ºC. Then, 1.5 ml tube was centrifuged briefly to remove drops from the inside of the lid. The entire lysate to the QIAamp MinElute column was carefully transferred without wetting the rim, closed the cover, and centrifuged at 6000 × g (8000 rpm) for 1 min. The QIAamp MinElute column was held in a clean 2 ml collection tube and discarded the collection tube containing the flow-through.

In some case, the lysate has not entirely passed through the membrane after centrifuged, centrifuged second time at a higher speed until the QIAamp MinElute column was empty. The QIAamp MinElute column was opened carefully, and 500 µl buffer AW1 added without wetting the rim. After that, the lid was closed and centrifuged at 6000 × g (8000 rpm) for 1 min. The QIAamp MinElute column was also placed in a clean 2 ml collection tube and discarded the collection tube containing the flow-through. The QIAamp MinElute column was carefully opened and 500 µl Buffer AW2 added without wetting the rim. The lid was closed and centrifuged at 6000 × g (8000 rpm) for 1 min. The QIAamp MinElute column was placed in a clean 2 ml collection tube and discarded the collection tube containing the flow-through. The QIAamp MinElute column and the flow-through contact between have been avoided. Carefully, QIAamp MinElute column was opened and add 700 µl ethanol (96 -100%) without wetting the rim. The lid was closed and centrifuged at 6000 × g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through. Contact between the QIAamp MinElute column and the flow-through should be avoided. At full speed (20,000 × g; 14,000 rpm) centrifugation was occurred for 3 min to dry the membrane entirely. This step was necessary, since ethanol carryover into the eluate may interfere with some downstream applications. The QIAamp MinElute column was held in a clean 1.5 ml microcentrifuge tube (not provided) and discarded the collection tube containing the flow-through. The lid of the QIAamp MinElute column was carefully opened and incubated at R.T. for 10 min or at 56 ºC for 3 min. 30 µl from deionized distilled water was added at room temperature for 5 min, centrifuged at full speed (20,000 × g; 14,000 rpm) for 3 min. Step 16 was repeated, and 30 µl from deionized distilled water was added at room temperature for 5 minutes at high speed (20,000 × g; 14,000 rpm) was centrifuged for 3 min. After that, the samples were incubated at -20 until use [13].

Ten µl of DNA sample was added to 990 µl of D.W. and mixed thoroughly, and then the optical density (OD) was measured in a spectrophotometer at wavelengths of (260 nm and 280 nm) [14]. The DNA purity and concentration was estimated at [15, 16]. Agarose gel electrophoresis for extracted DNA from fixed formalin paraffin embedded tissues (FFPE) was achieved and a digital picture was made for the evaluation and documentation of the results [13, 15].

Real Time-PCR Protocol: The required quantity of tubes (N + 3 (standards) + 1 (Neg. control) were prepared. Mixture for 40 samples was ready: the tube with PCR-mix-2 buffer 20 µl of Taq F DNA polymerase was added, mixed by pipette. This mixed was stabled for three months at 2-8 °C. For each sample tube 7.0 µl of PCR-mix-1-FRT was added and 8.0 µl of Mix (PCR-mix-2 buffer and Taq F DNA polymerase). Ten µl of the extracted DNA sample was added to the appropriate tube with a reaction mix. (Re-centrifuged all the cells with extracted DNA for 1 min at maximum speed

(12000-16000 g) and take carefully supernatant. The samples were positioned and the concentrations of (was reported in the Quant Data Card) in the Joe (Yellow) / HEX, FAM (Green), Rox (Orange) and Cy5 (Red) channels to generate standard curves. A temperature profile was created on our instrument as follows:

TABLE 1: REAL TIME-PCR PROTOCOL

Real-Time PCR program of high-risk HPV			
Step	**Temperature °C**	**Time**	**Repeats**
1	95	15 min	1
2	95	5 sec	5
	60	20 sec	
	72	15 sec	
	95	5 sec	
3	60	30 sec fluorescent signal detection	40
	72	15 sec	

Statistical Analysis: The data of the 120 female clinical cases in this study were submitted and tested by SPSS (Statistical Package for Social Science) with an updated version. The requested measurements which analyzed were mean, the standard deviation for study variables in addition to percentages and frequencies for the other categorical factors. The t-test was used for checking the statistical differences regarding age and other factors according to methods of contraception, diagnosis and HPV results. On the other hand, the relation of the HPV with education level and contraceptive method was examined using the Chi-square test (X2). The statistical difference (P-value) of less than 0.05 was considered significant.

RESULTS: The study was carried out on 120 clinical specimens obtained from 100 study patients and 20 healthy individuals. The samples of patients were classified clinically according to the experiment clinician as represented in **Table 4**. The age was arranged for 19-62 years with mean 30.9 ± 8.5 and for the fixed formalin paraffin embedded tissues (FFPE) 49.1 ± 8.5 years. The clinical study specimens of this study were divided as follow, 33(41.25%) ulcer, 19(23.75%) polyp, 16(20%) warts while Pap smear was obtained in 8(10%) and 4(5%) leukemia patients have warts lesion samples.

The other part of our study specimens, fixed formalin paraffin-embedded tissues which defined as an experiment histopathologist previously diagnosed retrospective samples as 11(55%) high-grade squamous intraepithelial lesions (HSIL) type, 6(30%) cervical cancer and 3(15%) adeno-carcinoma (ADCA).

TABLE 2: CLINICAL CLASSIFICATION OF RETROSPECTIVE SAMPLES (FIXED FORMALIN-PARAFFIN EMBEDDED TISSUES)

Retrospective samples of fixed formalin paraffin-embedded tissues	
High-grade squamous intraepithelial lesions (HSIL) type	11(55%)
Cervical cancer	6(30%)
Adenocarcinoma (ADCA)	3(15%)

Regarding the age parameters of the study samples 100 (80 patients with cervical lesions swabs and 20 blocks for fixed formalin paraffin embedded tissue) according to the type of samples, the study age groups for patients were distributed into positive and negative results as obtained from real time-PCR for human papillomavirus. There was no significant relationship between age groups and HPV results in control and patients (P>0.05). The mean for patients age showed that patients with cervical lesion from samples of pap smears were (31.63 ± 6.12), (30.32 ± 8.35) from polyp samples, (31.52 ± 10.70) represent ulcer samples, while the wart samples (30.19 ± 7.38), leukemia patients have warts lesion samples was (30.50 ± 2.52). The mean age ranged for the fixed formalin paraffin blokes were the adenocarcinoma type (ADCA) (50.00 ± 9.64), cervical carcinoma type was (57.17 ± 7.41), and high-grade squamous intraepithelial lesions (HSIL) type was (44.36 ± 5.35) in the following table showed the summarized results. Cervical carcinoma age is statistically different from the age of HSIL cases (P=0.004). No significant difference between age and the results of location for the other groups (P>0.05).

The genomic DNA extraction was achieved from the cervical swabs at which samples had been taken from patients and healthy subjects for obtaining optimum yields of genomic DNA for PCR amplification. The purity of DNA extracted from a clinical specimen was 1.39 ± 0.23 while 1.43 ± 0.23 for those derived from fixed formalin paraffin-embedded tissues sample. There was no significant difference between fixed formalin paraffin-embedded tissues and that extracted DNA from clinical specimens to what regard to purity or DNA concentration at which (P>0.05).

TABLE 3: QUANTITATIVE MEASUREMENTS OF GENOMIC DNA EXTRACTED FROM FIXED FORMALIN PARAFFIN-EMBEDDED TISSUES

No. of sample	Purity (OD.260/OD280)		Concentration of DNA (ng/µl)	
	Range	Mean ± SD.	Range	Mean ± SD.
20	0.97-1.8	1.43 ± 0.23	60-1430	936.75 ± 325.1

TABLE 4: QUANTITATIVE ASSESSMENT OF GENOMIC DNA EXTRACTED FROM CLINICAL SPECIMENS

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

Real-Time PCR for Quantitative Detection of High-Risk Human Papillomavirus (16, 18, 31, 33, 35, 39, 45, 52, 58 and 59): After optimization of real-time PCR HPV reaction conditions, 100 samples were subjected to the same RT-PCR HPV reaction conditions. The results revealed that only 9 out of 80(11.3 %) were positive for high-risk human papillomavirus.

Moreover, among the study samples with different clinical lesions categories, the results showed that RT-PCR HPV high-risk positive cases found in 1 out of 80 (11.12%) of patients with ulcer and leukemia patient for each one for high-risk human papillomavirus 18. Two out 80(22.22%) of patients with polyp for high-risk human papillomavirus 39 and 2 out of 80(22.22 %). Also, one out of 80(11.12%) of patients with ulcer and Pap smear respectively for high-risk human papillomavirus [16].

Further, one out 80(11.12%) of patients with Pap smear for high-risk human papillomavirus 33 and high-risk HPV 35. The study results showed that the rate of high-risk human papillomavirus detection was statistically not significant among the studied samples (P-value=0.09). There was no significant relationship between the clinical sample and HR HPV 7/9 results, P>0.05. The results represented in the following table.

TABLE 5: REAL TIME-HIGH RISK HUMAN PAPILLOMAVIRUS (16, 18, 31, 33, 35, 39, 45, 52, 58 AND 59)

Clinical samples	High risk Human Papillomavirus (16, 18, 31, 33, 35, 39, 45, 52 , 58 and 59)								
Cervical swabs	HR HPV A7				HR HPV A9				HPV-positive cases
	18	39	45	59	16	33	35	58, 31, 52	
	No%	No%	No%	No%	No%	No%	No%	No%	
Ulcer	1(11.1)	0(0.0)	0(0.0)	0(0.0)	2(22.2)	0(0.0)	0(0.0)	0(0.0)	3(33.33)
Warts	0(0.0)	00.0)	00.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0.0
Polyp	0(0.0)	2(22.2	00.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	2(22.2)
Pap smear	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(11.1)	1(11.1)	1(11.1)	0(0.0)	3(33.3)
Leukemia	1(11.1)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(11.12)
Total no.	2(22.2)	2(22.2	0(0.0)	0(0.0)	3(33.3)	1(11.1)	1(11.1)	0(0.0)	9(100)

Quantitative Detection of High-Risk Human Papillomavirus (16, 18, 31, 33, 35, 39, 45, 52, 58 and 59): The genotypic study for human papillomavirus among the study samples from the cervical lesions showed that the most prevalent genotypes were 16, 18, 39, 33 and 35 (33.3%, 22.2%, 22.2%, 11.1%, and 11.1%) respectively. The viral load was 14(17.5%), 10(12.5%) for the clinical samples in low-risk genotypes of human papillomavirus (6, 11) respectively were with mean ± SD (2.72 ± 1.07) in clinical status cervical cervicitis (CIN I). Moreover, among the result of high-risk genotypes of human papillomavirus (16, 18, 31, 33, 35, 39, 45, 52, 58 and 59) viral load was 6.42 ± 0.71 for clinical status CIN III.

TABLE 6: THE RESULT OF REAL-TIME PCR (VIRAL LOAD) OF HUMAN PAPILLOMAVIRUS AMONG LOW-RISK GENOTYPES (6 AND 11) IN ADDITION TO HIGH-RISK GENOTYPES A7 WHICH INCLUDES (18, 39, 45 AND 59) AND A9 (16, 33, 35, 58, 31 AND 52) GENOTYPES OF DIFFERENT CERVICAL LESIONS

Low-risk genotypes		Clinical status	Viral loadcopies / 10^5 cell	High risk genotypes (16, 18, 31, 33, 35, 39, 45, 52, 58 and 59)		Clinical status	Viral loadcopies /10^5 cell
Genotype 6	Genotype 11	CIN	2.72 ±1.07	A7	A9	CIN	6.42 ± 0.71
14(17.5%)	10(12.5%)	CIN I		4(44.4%)	5(55.5%)	CIN III	

Real-Time PCR for Quantitative Detection of High-Risk Human Papillomavirus (16, 18, 31, 33, 35, 39, 45, 52, 58 and 59) in Fixed-Formalin Paraffin-Embedded Samples: The results revealed that only 11 out of 20(55.0%) were positive for high-risk human papillomavirus and 9 out of 20(45.0%) were negative for high-risk human papillomavirus. Moreover, among the study samples with different cytological categories, the high-risk human papillomavirus DNA positive

cases were found in 2 out of 20(10%) of patients with HSIL for high-risk human papillomavirus. Also, 18 and 1 out of 20(5%) for cervical cancer for high-risk human papillomavirus 18, 1 out of 20(5%) of patients with HSIL for high-risk human papillomavirus 39, 45. Further, 1 out of 20(5%) of patients with HSIL for high-risk human papillomavirus 16, 33, 58 for each one of them, 2 out of 20 (10%) of patients with HSIL for high-risk Human papillomavirus 35. Furthermore, 1 out of 20(5%) of patients with cervical cancer for high-risk Human papillomavirus 16.

No significant difference between EFPE and HR HPV appeared 7/9 results, P>0.05. These results are represented in the following table.

TABLE 7: THE RESULTS OF REAL-TIME PCR FOR HIGH-RISK HUMAN PAPILLOMAVIRUS EXTRACTED FROM FIXED FORMALIN PARAFFIN-EMBEDDED TISSUES

Clinical samples	High Risk HPV (16, 18, 31, 33, 35, 39, 45, 52, 58 and 59)							
FFPE	HR HPV A7			HR HPV A9				No. HR HPV-pos. cases
	HPV18	HPV39	HPV45	HPV16	HPV33	HPV35	HPV58	
	No%	No%	No%	No%	No%	No%	No%	
HSIL grade 2	2(10.0)	1(5.0)	1(5.0)	1(5.0)	1(5.0)	2(10.0)	1(5.0)	9(45%)
Cervical cancer (HSIL grade 3)	1(5.0)	0(0.0)	0(0.0)	1(5.0)	0(0.0)	0(0.0)	0(0.0)	2(10.0%)
ADCA	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Total no.	3(15)	1(5)	1(5)	2(10)	1(5)	2(10)	1(5)	11(55%)

Real-Time PCR for Quantitative Detection of High-Risk Human Papillomavirus (16, 18, 31, 33, 35, 39, 45, 52, 58 and 59) in Fixed Formalin - Paraffin-Embedded Samples: The higher rates observed in HSIL grade 2, 9(45.0%) in comparison with those observed in cervical cancer cases with HSIL grad 3, 2(10%). The prevalence the HPV genotypes in study in this type of sample (FFPE) showed that 3(15.0%) for HPV genotype 18, 2 (10.0%) for HPV genotypes 16 and 35, while one (5.0%) for HPV genotypes 39, 45, 33 and 58. The clinical status of the high risk of HPV genotypes obtained from FFPE samples were 4(20%) and 5(25%) for A7 and A9 with clinical status CIN II and viral load 5.57 ± 0.75 and 6.28 ± 0.75 respectively. The clinical status CIN III were 1(5.0%) for A7, and A9 with viral load mean ± SD (6 and five copies/105 cellular DNA) respectively.

TABLE 8: VIRAL LOAD OF HIGH-RISK GENOTYPES (A7 AND A9) OF HUMAN PAPILLOMAVIRUS OBTAINED FROM FIXED FORMALIN-PARAFFIN EMBEDDED SAMPLES AND DISTRIBUTION ACCORDING TO CLINICAL STATUS (CIN)

High-risk genotypes (16, 18, 31, 33, 35, 39, 45, 52, 58 and 59)	Status of cases CIN II	Viral load mean ± SD Copies/10^5 cell	Status of cases CIN III	Viral load mean ± SD (Log10) HPV DNA Copies/10^5 cellular DNA
A7 (18,39,45,59)	4(20%)	5.57 ± 0.75	1(5.0%)	6
A9 (16,33,35,58,31,52)	5(25%)	6.28 ± 0.75	1(5.0%)	5

DISCUSSION: The incidence and distribution of HPV have not been well investigated in Iraq especially in Anbar Governorate on the level of molecular genetics. The present study was a pioneer study which utilized the RT-PCR technique as a molecular tool for detection of high-risk genotype HPV. In addition to the detecting of high-risk genotype HPV in fixed formalin-paraffin embedded (FFPE) tissues for patients diagnosed with cervical cancer previously. The results showed that the elevation rate of HPV infection from 12 out of 36(33.3%) among the study group with younger age group 19-28 years. The high rate of HPV infection about 16 out of 32(50%) among another group (older age 29-38 years). The increased rate of HPV infection was also observed at age ranges from (39-48 years). Also, three out of seven (42.9%), two out of four (50.0%) at age group 49-58 years. The most striking result is that no HPV infection at age group ≥ 59 (0.0%) was observed. In fixed formalin paraffin-embedded tissues, one case from 2(50%) at age group 29-38, 6 out of 9(66.7%) at age group 39-48, 3 out of 6 (50.0%) at age group 49-58, while at the age group ≥ 59 show one case out of 3(33.3%) was recorded. Most of the studies suggested that women in ages more than 35 years, HPV infection might represent viral persistence, mostly caused by oncogenic HPV and induced the risk of cervical cancer development.

In another study by Dunne and associates [17], HPV prevalence was 24.5% among females with ages 14-19 years, 44.8% in women ages 20-24 years, 27.4% in the ages of 25-29 years, 27.5% among ages 30-39 years while 25.2% was with women ages 40-49 years. Also, 9.6% of women aged 50 to 59 years of age. There was a statistically significant 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 incidence through 59 years (P=0.06).

The mean age and standard deviation for the fixed formalin- paraffin blokes (50.00 ± 9.64) for adenocarcinoma (ADCA), (57.17 ± 7.41) for cervical carcinoma typed and (44.36 ± 5.35) was type High grade squamous intraepithelial lesions (HSIL). These results were in agreement with the findings of other researchers who stated that patients with cervical malignant lesions were in the majority in the age range of 50-65 years [18]. Moreover, it has been suggested that the reason for this post-menopausal peak incidence of cervical cancer may be related to reactivation of latent HPV infection acquired at earlier life and progress with gradual loss of type-specific immunity with aging or to a sudden loss of hormonal influences during postmenopausal years [19].

The study result revealed that the purity of DNA which was extracted from cervical lesion was ranged from 0.9-1.77 and the mean 1.39 ± 0.23. Further, in DNA derived from paraffin-embedded tissues, the purity was varied from (0.97-1.8) with mean of 1.43 ± 0.23. The differences in DNA concentration and purity had been checked spectro-photometrically according to tissue origin. The OD260/OD280 ratio values satisfied tissue-specific structural complexity can explain those suggested by Green and Sambrook 13≥1.8, the variability in DNA quality and purity [5].

World Health Organization [20] mentioned that care must be taken to prevent sample-to-sample contamination during the extraction and processing of samples. Also, at least one water blank was extracted with each batch of samples. For high-throughput extractions, these extraction controls should be interspersed. The DNA concentration was detected by optical density measurement in a spectrophotometer using 50 µg/mL as 1 OD260.

The purity ratio should be in the range of 1.7-1.9 and should not exceed two as this indicates contamination of the preparation of RNA or low molecular weight nucleic acids that lead to overestimation of DNA concentration [21]. The result of this study in fixed formalin paraffin embedded tissue extraction of DNA may be because formalin is known to set protein in tissue samples including all proteins on each cellular level which makes the reach for the DNA difficult and incomplete [13]. Also, it is unsuitable for molecular techniques a slow degradation of DNA occurs with time.

The genotyping distribution of human papilloma-virus among the study samples from the cervical lesions shows that the most prevalent HPV genotypes were as follow: 16, 18, 39, 33 and 35 (33.3%, 22.2%, 22.2%, 11.1%, and 11.1%) respectively. Weizhi and colleagues [22] reported that the majority common high-risk types HPVs are 16, 18, 31, 33, 35, 51, and 52 which include being related through CIN II, CIN III, and invasive cervical cancer. Our study results show the high rates of 9(45.0%) in cervical cancer cases than in case with HSIL grad 3, 2(10.0%) in cervical cancer cases than in cases with cervical cancer (HSIL grade 2), the prevalence the HPV genotypes in our study in this type of sample (FFPE) showed that 3(15.0%) for HPV genotype 18, 2(10.0%) for HPV genotypes 16 and 35, while one (5.0%) for HPV genotypes 39, 45, 33 and 58.

The extracted DNA from formalin-fixed and paraffin embedded (FFPE) tissue remain a real challenge, despite numerous attempts to develop a more effective method. Polymerase chain reaction (PCR) success rates with DNA extracted using current methods remain low [23]. Jin and associates [24] focused a highlight on the genotypes 16, 58, 52 and 18 and its role in the development of CIN and SCC in Longnan females. They were focused on a fully aware of regional differences in HPV genotype distribution which are the tasks for cervical cancer control and prevention. They were documented that HPV 16 was the most frequently identified type among CIN II/III tissues. This result is in agreement with results of previous studies. The significantly uneven distribution of HPV 16 prevalence between CIN grades supports the contention that CIN II/III lesions can arise de novo and/or that HPV 16-infected CIN I may rapidly

progress to high degree. Ahmed [25] reported that HPV 16 and 18, HPV 16 was identified in 57% of the positives were comparable or in agreement with those stated before. Ibrahima and associates [26] concluded that HPV 16 and 18 were identified in 137/166 (82.5%) cervical cancer biopsy samples by PCR. Co-infection with both HPV 16 and 18 was significantly more frequent in women over 50 years of age than in younger women (63.0% *vs.* 37.0%). 44% of study participants said they would be willing to vaccinate their child with the HPV vaccine. Accordingly, HPV16 is the most common HPV type associated with cervical carcinoma in Central Sudan [25].

Practically, formalin fixation may cause extensive DNA damage, including cross-linking and fragmentation. Consequently, it has been documented that successful amplification of HPV genotypes from archival FFPE specimens is inversely correlated to the length of the amplicons of the PCR method and that specimen age may contribute to degradation. Also, differences in sample processing and DNA extraction of FFPE materials may reflect the apparent discrepancies noted in the performance of specific genotyping methods for FFPE specimens [27]. Several types of research conclude that the use of robust extraction methods can improve the performance of nucleic acid tests when applied to fixed specimen types [28]. Regarding the result of the high-risk genotype, the result of the two cervical lesions and FFPE was variables and reflected that there is no significant difference. Statistically, this may be attributed to the fact that the mechanism X2 parameter will delete a square labeled zero.

CONCLUSION: The study concluded that the performing of Real-Time PCR technique as a diagnostic tool and for genotyping of HPV in cervical lesions and sample from FFPE blocks has the advantage for women who currently have high-grade cervical lesions and identifying women who are at high risk of developing cervical cancer in the future. Further, the HR-HPV type 16 was the most predominant type in cervical lesions for different kinds followed by 18, 39, 33 and 35 confirming geographical variation in HPV genotype distribution. The HR-HPV type 18 was the most predominant type in FFPE blocks followed by 16, 35, 39, 45, 33 and 58.

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