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HPV PREVALENCE, VIRAL LOAD AND PHYSICAL STATE OF HIGH-RISK HUMAN PAPILLOMAVIRUSES IN CERVICAL SMEARS OF PATIENTS WITH DIFFERENT GRADES OF CIN AND CERVICAL CANCER

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ABSTRACT

Human papillomavirus (HPV) infection is the most important event in malignant transformation of human cervical epithelium. The objective of this study was to develop a method allowing the detection and genotyping, quantification of HPV DNA to improve future strategies for cervical cancer screening.

DNA was extracted from 47 cervical samples, then was sent to the Laboratory of Cellular and Molecular Biology, CHU Jean Minjoz, Besançon France. HPV were first detected by MY09/11 consensus PCR and then genotyped with INNO-LIPA, viral load and integration status of HPV16 was determined by real time PCR.

The prevalence of HPV infection was estimated as 27.65%, the most common genotypes found were HPV 16 and HPV 52 at 38.46% and 15.38%, respectively.

The increase in viral load and integrated HPV type 16 was significantly associated with CIN2, indicating that viral load and physical condition can potentially be useful triage markers for HPV16-positive women during cervical screening.

Keywords : Human papillomavirus (HPV) ; viral load ; integration status ; real time PCR ; cervical cancer.

INTRODUCTION

Human papillomavirus (HPV) infection is frequent in young women and persistent infection may lead to cervical cancer (Heard *et al.,* 2013).

Among the 170 HPV presently known some have been classified as low risk HPV since they are associated rarely with cancerous lesions of the cervix while other are classified as high risk HPV because their high rate of detection in cervical (De Villiers, 2013).

High risk (HR) HPV infection is the most common sexually transmitted infection throughout the world. However, this infection is mostly transient as the virus is generally cleared within a mean period of 8–10 months (Monnier-Benoit *et al.,* 2006). On other hand, several studies have shown that the persistence of the HR-HPV infection is necessary for the development and progressions of cervical intraepithelial neoplasia (CIN) to CIN 2/3 and/or invasive carcinoma (Saunier *et al.,* 2008).

Determining HPV prevalence and examining the genotype distribution of HPV in premalignant and malignant lesions are important parameters for estimating the impact of screening programs and the efficacy of HPV vaccines (De Jesus *et al*., 2018).

The specific HPV DNA load could be a relevant marker of prevalent and incident precancerous and cancerous lesions of the cervix (Jacquin *et al.,* 2013). Integration of HPV into the host genome is a prerequisite for the development of malignant lesions, resulting from a disruption in the HPV genome through partial loss of the E2 gene (Gradıssimo Oliveira *et al*., 2013).

The objective of the present study was to develop a method allowing the detection, genotyping and quantification of HPV DNA to improve future strategies for cervical cancer screening.

MATERIALS AND METHODS

1- Sample collection

Our retrospective study was performed on samples from women attending private gynecologist. An information sheet was established during the collection including personal information about the patient. Forty seven smears have been collected with cotton swab and conserved in the transport media of the amplification kit SACACE and stored at -20 °C pending the extraction of DNA.

2- DNA extraction

Extraction of viral DNA of HPV was performed with the automated SACACE Biotechnologies (Italy) using the kit « *DNA-Sorb-A* » ; following the protocol supplied by the manufacturer. The concentration assessment of DNA extract was realized by spectrophotometry using the NanoDrop method.

3- PCR MY09/MY11

HPV DNA was tested by a standard polymerase chain reaction (PCR) with consensus primer pair MY09/MY11 (5’-CGT-CCM-ARR-GGA-WAC-TGA-TC-3’and 5’-GCM-CAG-GGW-CAT-AAY-AAT-GG-3’), allowing the production of 450-bp fragments in the HPV L1 open reading frame (Belglaiaa *et al.,* 2015).

4- Human papillomavirus DNA detection and genotyping

The papillomavirus strains were performed using the INNO-Lipa HPV Geno-typing Extra kit (Fujirebio, Courtaboeuf, France), according to the procedure previously described (Hanz *et al.,* 2010). This assay allows the identification of 28 types of HPV including 18 high-risk HPV type and probable high-risk types (HPV-16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82), 7 low–

risk (HPV-6, 11, 40, 43, 44, 54, 70) and 3 unclassified types (HPV-69,71,74). HPV amplimers wich did not hybridize to any specific probe were considered as uncharacterized (HPVX) (De Martel *et al*., 2012).

5- Real-time PCR

DNA amplification and quantification of HPV DNA copies were performed by real-time PCR with an AB 7500 thermocycler (Applied Biosystems, Courtaboeuf, France). QPCR targeting E2 and E6 of HPV and albumin gene, primers and probes described in Table1, the modalities were performed as described previously (Jacquin *et al*., 2013).

Concentrations of HPV DNA were expressed as copies of HPV genome in 50 ng of cellular DNA. Ratios of E2 to E6 of less than 1 indicated the presence of both integrated and episomal forms. The integrated E6 was calculated by subtracting the copy numbers of E2 (episomal). The ratio of E2 to integrated E6 represents the amount of the episomal form in relation to the integrated form (Ho *et al.,* 2011).

Table1. Sequences of primers and probes used for viral load and E2/E6 ratio determination (Saunier *et al.,* 2008).

Target	Primer or probe	Sequence
E6 HPV16	Forward primer	5'-GAG-AAC-TGC-AAT-GTT-TCA-GGA-CC-3'
	Reverse primer	5'-TGT-ATA-GTT-GTT-TGC-AGC-TCT-GTG-C-3'
	Probe	3'-BHQ-1-CAG-GAG-CGA-CCC-AGA-AAG-TTA-CCA-CAG-TT-FAM-5'
E2 HPV16	Forward primer	5'- AAC-GAA-GTA-TCC-TCT-CCT-GAA-ATT-ATT-AG-3'
	Reverse primer	5'-CCA-AGG-CGA-CGG-CTT-TG-3'
	Probe	3'-BHQ-1-CAC-CCC-GCC-GCG-ACC-CAT-A-FAM-5'
Albumin		
	Forward primer	5'-GCT-GTC-ATC-TCT-TGT-GGG-CTG-T-3'
	Reverse primer	5'-ACT-CAT-GGG-AGC-TGC-TGG-TTC-3'
	Probe	3'-BHQ-1-GGA-GAG-ATT-TGT-GTG-GGC-ATG-ACA-GG-FAM-5'

FAM, 6-carboxyfluorescein; **BHQ-1**, black hole quencher 1.

RESULTS

1- Patient characteristics

Table2. Characteristics of the enrolled women

Mean age (min-max)	47 (25–78)
Mean age of first sexual intercourse	23.6 (14–44)
Marital status (n=47) **- Married** **- Widow**	 45 (95.8 %) 2 (4.2%)
Number of sexual partners (n=47) **- One** **- ≥ 2 sexual partners**	 46 (97.9%) 1 (2.1 %)
Previous abortions (n=47) **- Yes** **- No**	 17 (36.1%) 30 (63.8 %)
Oral contraceptives (n=47) **- Yes** **- No**	 34 (72.34%) 13 (27.65%)
Passive Smoking status (n=47) **- Yes** **- No**	 6 (12.8%) 41 (87.2%)

A HPV infection was observed in 27.65% (13/47) of patients. Characteristics of patients are detailed in table 2. Mean age was 47 years and mean age of first sexual intercourse was 23.6. Most of these women were married and had only one sexual partner ; never had an abortion performed ; did not smoke and used oral contraceptives.

2- HR -HPV prevalence according to cytology

Table3. Prevalence of HPV Infection in Women with Normal and Abnormal Cytology

HPV genotypes	(%)	NILM(%)	LGSIL (%)	HSIL (%)	ASCUS (%)
HPV16	(38.46**%**)	-	-	(40**%**)	(60**%**)
HPV 31	(7.69**%**)	-	-	(100**%**)	

HPV 52	(15.38**%**)	-	-	-	(100**%)**
HPV 45	(7.69**%**)	-	-	-	(100**%)**
HPV 16/18	(7.69**%**)	-	-	-	(100**%)**
HPV 16/67	(7.69**%**)	-	-	(100**%)**	-
HPV 31/35/52	(7.69**%**)	-	-	(100**%)**	-
HPV X	(7.69**%**)	-	-	-	(100**%)**
total	(100%)				

NILM : negative for intraepithelial lesion or malignancy ; **LGSIL** : low grade squamous intraepithelial lesions ; **HSIL** : high-grade squamous intraepithelial lesions ; **ASCUS** : atypical squamous cells of undetermined significance.

The results of the cytological examinations revealed abnormalities in 100% (13/13) of HPV infected women. Cytological abnormalities include (61.53%) atypical squamous cells of undetermined significance (ASC-US) an (38.47%) high-grade squamous intraepithelial lesions (HSIL).

HR HPV DNA was detected in 28% (12/47) of cases but one sample (2.1%) could not be genotyped because they did not match any of the specific probes of the assay (HPVX). As HR HPV genotypes are observed in 92.30% (12/13) of HPV infections, HPV 16 are observed in (38.46%) of infections, HPV 52 (15.38%), HPV 31 (7.69%), HPV 45 (7.69%), HPV 16/18 (7.69%), HPV 16/67 (7.69%) and HPV31/35/52 (7.69%).

3- DNA load and physical state of HPV according to histological lesion grade

Patient N°	**HPV lods (copies/10^3 cells)**	**physical state**	**Histological lesion grade**
1	325036	Integrated	CIN 2
2	1177	Integrated	CIN 2
3	432	Integrated	Cancer
4	1063	Episomal	CIN2
5	5213	Mixed	Cancer

CIN: cervical intraepithelial neoplasia.

The results of the physical state and the copy numbers of HPV16 summarized in Table 4. The viral load per 50 ng of DNA from different samples varied over the very wide range (4×10^2 to 3×10^5 copies).

DISCUSSION

The present study reports for the first time the HPV infections in Constantine, Eastern Algeria. The prevalence is estimated to be 27.65%. It was lower to those of other authors: 41.5 % according to Ouédraogo *et al*., 2015 ; and higher than those of Luquain *et al*., 2015 (22.7%). Our prevalence probably related by the mean age of the patients in our study which was higher than that of the study populations of the different authors.

All studies agree that it is particularly important in young women in early sexual activity and decreases thereafter as age increases due to both clearance and acquisition of immunity (Ouédraogo *et al*., 2015). This could be explained by the fact that the majority of the women in our study had one sexual partner. Indeed, it has been reported that having many sexual partners is associated with a high prevalence of HPV infection (Zohoncon *et al*., 2013).

The most prevalent HR-HPV subtypes were HR-HPV16 (38.46**%**) and HR-HP52 (15.38**%**). HPV16 was the most common genotype and HPV52 and 31 were the second most common genotypes in Africa and Europe, respectively (Ardhaoui *et al.*, 2016).

In several studies, HPV 16 is the leading type but HPV 18 does not come right in the second place. In Tunisia, HPV 16 is followed by HPV 58 (Guettiti *et al.*, 2014). HPV 16 is followed by HPV 18 in Algeria (Hammouda *et al*., 2010) but is followed by HPV 31 and 51 in Egypt Shaltout *et al*., 2014), in Morocco the most prevalent types were HPV 53 followed by HPV 16 and 35 (Souho *et al*., 2016). In this study, we quantified HPV 16 E6 and E2 DNA copy numbers by real time PCR to analyse the load and physical state of the HPV 16 genome. Most studies on viral load and HPV integration focused only on type 16 (Manawapat-Klopfer *et al*., 2018 ; Shukla *et al*., 2014) and very few examined other types (Takehara *et al*., 2011 ; Cheung *et al*., 2009). This is because sample size is a

major obstacle to accurate evaluation of the role of type-specific viral load except in the case of HPV 16. In the report by Kim *et al* (2019) the viral loads were different from type to type in lesions with the same pathologic grade. Similarly, in our study, the viral loads were different from type to type.

Several studies have reported the association of a high viral load with the risk for cervical cancer and precursors. The majority use HCII to measure viral load, and while some find viral load to be positively associated with increased risk for prevalent or incident disease others do not (Andersson *et al.,* 2005). The highest viral load values were observed for patients with CIN 2. However, there decrease in viral load value for women with cervical cancer. Briolat *et al* (2007) found that the average of HPV 16 copies per 100 cells were not significantly different in the CIN1, 2 and 3 cases. However, there was a slight decrease in viral load value for women with cervical cancer. Low viral load in cancer lesions could be linked to the integration of the HPV genome into the host cell genome, in other hand, Lowe *et al* (2011) also reported that the viral load declines in response to therapy.

Integration was considered a critical event in carcinogenesis of cervical cancer as it results in loss of the episomal type (Pett *et al*., 2007). In contrast, some studies demonstrated the presence of integrated HPV 16 only in 28% to 67% of subjects (Woodman *et al*., 2007), the integrated from of HPV 16 was 60% in our results. Our results showed that a prevalence of integrated form is higher in CIN2 (66.7%), Briolat *et al* (2007) found a higher prevalence was in CIN 3 (100%), integration of viral DNA frequently occurs in HSIL and CC, and these lesions may often contain episomal and integrated HPV-DNA at the same time (Abreu *et al*., 2012). The frequency of samples with integrated HPV16 genome in cancer cases in our study was 50% similar to that found by Shukla *et al* (2014) (50%).

CONCLUSION

Our study has some limitations. First, the majority of cervical samples were collected from women attending routine gynaecological visits, since the number of women positive for HPV infection was indeed low, also the high mean age of the patients**.** Our preparatory study is an excellent opportunity

to start a study in the future. Further evaluation of the determination of HR HPV physical status as a predictive biomarker must be addressed.

In conclusion the challenge remains to propose a future strategies in order to reduce significantly the incidence of cervical cancer.

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