Genetic Profile of Human Papilloma Virus Circulating in Cervical Precancerous-Lesions of Cameroonian Women

By Embolo Enyegue Elisée Libert, Ananga Noah Sidonie, Awalou Halidou, Halmata Mohamadou, Ndipho Tatou Christian Kitchener, Doh Ndeh Gilbert, Banai Thomas, Bell Eric Michel, Essame Oyono Jean Louis & Koanga Mogtomo Martin Luther

Abstract- *Background:* Cervical cancer is the most common cancer after breast cancer worldwide, especially in developing countries. Infection by Human Papillomaviruses (HPV), In Cameroon, there is a paucity of studies focused on its epidemiology. This study aimed at appraising the diversity of HPV genotypes circulating in precancerous cervical lesions observed in Cameroonian women. For this study, a total of 4063 women fulfilled inclusion criteria and they were enrolled. For this sample, only 1947 women presented precancerous cervical lesions. This cross sectional study carried out in three areas of the Cameroon namely Niete, Mokolo and Yaounde.

Methods: Cervical swabs were obtained from each participant and thereafter undergone cytological analysis relied on Pap test techniques. DNA was extracted from positive smears for genotyping of Human papilloma virus using multiplex PCR (polymerase chain reaction) method. Data analysis was done by GraphPad Prism 5 and XLSTAT.

Keywords: human papilloma virus, molecular epidemiology, precancerous lesions; cervical cancer; cameroon.

GJMR-F Classification: WP 840, QZ 20.5

GENETICPROFILEOFHUMANPAPILLOMAVIRUSCIRCULATINGINCERVICALPRECANCEROUSLESIONSOFCAMEROONIANWOMEN

Strictly as per the compliance and regulations of:

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Results: Molecular analysis results obtained, demonstrated that around 75% (1460) of the women with precancerous cervical lesions in the study population presented six (6) different Low-Risk (LR) (HPV 6, HPV 61, HPV 11, HPV 81, HPV 62 and HPV 70) and five (5) different High-Risk (HR) (HPV 45, HPV18, HPV16, HPV16, HPV35 and HPV84) genotypes of HPV (P value < 0.001). In several cases, combinations of genotypes of High-Risk HPV and Low-Risk HPV were detected. However, the highest rate of LSIL (80.8%) was observed in women with genotype 35.

Conclusions: Thus, it is possible to confirm with confidence that, there is genotypic diversity of HPV among Cameroonian women with precancerous cervical lesions.

Keywords: human papilloma virus, molecular epidemiology, precancerous lesions; cervical cancer; cameroon.

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I. INTRODUCTION

Though cervical cancer (CC) is largely preventable, it is still the second most common female cancer internationally and the leading cause of cancer deaths among females in African countries[1]. Despite the considerable success of research results based on visual inspection and cytological analyses, cervical cancer still remains a public health problem in Cameroon. Cervical precancerous lesions are often correlated to Human Papilloma Virus infections[2], human papillomavirus (HPV) is currently the most common pathogen responsible for female cancers[3]. Indeed, HPV are viruses that belong to the Papillomaviridae family, consisting of an icosahedral capsid of 55 nm in diameter and a double-stranded DNA of 8,000 base pairs[2]. HPVs have exclusive tropism for metaplastic cells in the junctions of squamous and glandular epithelia and elicit cytopathogenic effects with transformation of keratinocytes into koilocytes [4]. In Cameroon, very few people work on HPV, so very few studies have been conducted. No data are clear concerning viral genotypes circulating in this country and there is no information on risk factors available. In 2009, a US research team headed by Desruisseau, demonstrated that; oncogenic HPV subtypes 45 and 58 were more prevalent in Cameroon [4]. Several other studies conducted in Africa by other researchers had presented different dominant genotypes. In Ethiopia for example, Human papilloma virus type 16 was the most prevalent genotype identified from the subjects screened[5]. In Angola, The most prevalent HPV genotypes were HPV16, HPV6 and HPV 18[6]. Morocco data presented, high rates of infection with HPV genotypes in sexually active Moroccan women making molecular investigation for HPV16, 18 and 31 essential in clinical approach. However, HPV 33, 35 and 45 are less frequent in this population[7], [8]. In Mozambique, among women with cervical cancer HPVs 16 and HPV 18 were the two most frequently identified genotypes (47.0% and 31.3%, respectively), followed by HPV Types 51, 52, 45, 35, 33 and 31[9]. Studies in south Africa demonstrated high prevalence of HPV and multiple HPV

infection among HIV-positive women compared to HIV-negative women across all ages [10]. Study carry out in Zambia shows that among high-risk (HR) types, HPV 52 (37.2%), 58 (24.1%) and 53 (20.7%) were more common overall than HPV 16 (17.2%) and 18 (13.1%) in women with high-grade squamous intraepithelial lesions or squamous cell carcinoma (SCC) on cytology[11]. All this Human Papilloma Viruses diversity demonstrates the importance of accurately determining HPV genotypes and subtypes that prevail in the sub-Saharan zone, especially in Cameroon.

II. MATERIAL AND METHODS

a) Study sites

This was a cross-sectional study, which took place in hospitals in the selected areas of three region of Cameroon (the South, The Far North and the central regions).

a. District of Niete

Niete is an agro-industrial locality located in southern Cameroon near the Atlantic coast in the Ocean Division and the Southern Region. The commune of Niete has about 40 894 inhabitants in 28 villages. The communal population is composed of 19 137 men, 11 154 women, 5 655 and 4 948 young people aged 5- 16 years and less than 5 years respectively. The work was carried out in three subdivision medical centers, namely the V15 hospital, the V4 hospital and the ADJAP hospital.

b. District of Mokolo

Mokolo is a city located in the Far-North region, near the border with Nigeria. It is the county town of the Mayo-Tsanaga Division. The study was conducted at the District Hospital of Mokolo. The commune of Mokolo is one of the largest municipalities in the Far North with an area of 1650 km^2 for a population estimated at 310,000 inhabitants in 106 villages. The economy of Mokolo is relied mainly on agriculture (rainy season sorghum, dry season sorghum, groundnut, cowpea, soybean, sweet potato, and vegetable and fruit crops), livestock (cattle, goats, sheep, and poultry), small trade and crafts.

c. District of Yaoundé 1

The district of Yaounde III, Department of Mfoundi, Central Region. It covers an area of 5552 hectares for a population estimated at 281 586 inhabitants, ie a density of about 507habitants / km2. Gender related statistics estimate 141,525 for males and 140,011 for females; Leading to a sex ratio of 101.05%. It is managed by a municipal council of 41 members, a communal executive composed of a mayor and four deputies. There are several hospitals and dispensaries, where we collected the data.

b) Study population and sample size

These analyses targeted all women with or without cancer at different stages of development.

i. Selection criteria

a. Inclusion

All Cameroonian women over the age of 18 were eligible. They should not have undergone hysterectomy, they had to be willing to participate in the study, the must have signed the informed consent form, they must be sexually active.

b. Exclusion

All pregnant women;
All women with cervical cancer.

ii. Sampling method

A convenient sampling technique in which potential participants were consecutively recruited at the different sites.

iii. Questionnaire

An investigator administered semi structured questionnaire was used to collect data of each woman through 15-20 minutes' individual interview. The first part of questionnaire focused on sociodemographic information such as age, level of occupation or religion. The second part allowed for collecting obstetric information as well as those related to sex behavior.

iv. Cervix sample collection and visual inspection

After counseling participants were placed in gynecological position on an examination table. A clean, sterile non-lubricated speculum was gradually introduced into their vagina for eye examination of cervix. 2 Samples were obtained by collecting exfoliated cells, from the transformation zone of the cervix using cytobrush and ayre spatula. Thereafter, these cells from ayre spatula were transferred directly onto a slide and fixed using the conventional technique and the cells from cytobrush were used for PCR. The visual inspection with acetic acid was performed according the atlas of cytology, with Lugol's iodine recommendations. Results were classified as: normal cervix, abnormal cervix, and cervix with suspected cancer. The cytological analysis has been performed using the Papanicolau test, and the Bethesda classification system for interpretation of results has been used.

v. Cytological analysis

Pap smears were obtained by collecting exfoliated from the transformation zone of the cervix using ayre spatula. The cells were transferred directly to a slide and fixed using the conventional technique. The Bethesda system was used to interpret results from slides.

c) DNA extraction

The cells collected with cytobrush were used for virus isolation.DNA extraction of fresh cervical cells was made using the QIAGEN extraction kit, which is a

commercially available. Extraction was made according to the manufacturer.

d) *HPV genotyping strategy*

a. *Primers design*

The table in supplementary material shows the newly designed primers that were used throughout this study.

The beta globin gene was used to check the quality of the reaction.

DNA sequences of the HPV genotypes targeted obtained from Genbank. The primers were modeled for each type of HPV which the literature showed a high prevalence in Cameroon, as presented above. With the Primer 3 online program (https://primer3.ut.ee/), we were able to generate the primers for 6 low risk genotypes (LR), (6, 11, 61, 62, 70 and 81) and 6 high risk genotypes (HR) (16, 18, 35, 45, 58 and 84)

b. *Mix and PCR*

The master mix contains pre-optimized concentrations of HotStarTaq DNA Polymerase and MgCl2, plus dNTP, and a PCR buffer that contains the new MP factor. The use of a master-mix format reduces the time and handling for the reaction configuration and increases the reproducibility by eliminating many possibilities.

The following conditions were used for PCR amplification on a Thermal Cycler (Applied Biosystems): Denaturation for 3 minutes at 91°C, followed by 42 cycles of 27 seconds at 94°C, 45 seconds at 50°C, and 10 minutes at 64°C, and a final elongation step of 5 minutes at 65°C. After amplification, the reaction mixture was transfer for electrophoresis to 8°C.

Analyzing of the PCR product was done using agarose gel electrophoresis, which separates DNA products on the basis of size and charge, it allows for the determination of the presence and the size of the PCR product. Visualization was done using transilluminator.

c. *Control quality*

The β-globin was a quality control gene for PCR amplification used to show the smooth progress of the PCR. The β-globin (-) represents the samples whose β-globin internal control gene could not be amplified. In our study, 5.16% of the samples were β-globin (-); and as a result were they were declared unsatisfactory. β-globin (+) represents samples whose β-globin internal control gene has been successfully amplified which accounted for 94.84%.

e) *Ethical statement*

The study was carried out in conformance with the guidelines for human experimental models in clinical research as stated by the Cameroon Ministry of Public Health and the Helsinki declaration. To do so, ethical clearance was issued by the National Ethics Committee of Cameroun with registration number 2014/08/485/CE/CNERSH/SP. likewise, administrative clearance was issued by regional delegations. The aim and objectives of the study were explained to each woman in the language they understood best (English or French), and their questions were answered. Only women who signed an informed consent form for their participation were enrolled. Participation in the study was strictly voluntary and women were free to decline answering any question or totally withdraw if they so wished at any time.

f) *Statistical analysis*

Data were keyed and verified for consistency into Excel spreadsheet and thereafter analyzed with Graph Pad Prism version 6. Independence Chi-square test and one-way ANOVA, were used to compare results. Qualitative variables were presented as percentage with confidence interval at 95% in tables and graphics. XLSTAT 2015 software was used to perform principal component analysis (PCA) in order to establish correlations between $\geq$ 2 quantitative variables. Significance was set at P$\leq$0.05.

III. RESULTS

a) *Characterization of sample collected*

The table 2 below presents some patients with atypical profiles who were selected for molecular analysis and cytology and PCR results. The aim was to clarify the involvement of HPV among lesions observed. Most of cervix cell alterations were represented by ASCUS (43.2%). Out of 1836 β-globin positive samples, HPV genotypes were found in 1484 samples giving a HPV prevalence of 76.2%. Low Risk HPV genotypes were identified in 75.51% of all positive samples. 297 cases of multiple infections were recorded in the study and were mainly represented by co-infection with genotypes of low risk. This table presents characterization of women for molecular analysis. We selected 1947 women with positive uterine cell alteration according to VIA/VILI or Pap Test. Out of 4063 women fulfilled inclusion criteria. 1947 presented uterine cell alterations according to VIA/VILI or Pap test and were therefore included in this study. The prevalence of ASCUS (61.8%) (P value < 0.001), LSIL (43.2%) (P value < 0.001), and HSIL (59.4%)(P value < 0.001), were higher in women from Yaounde than in their counterparts from, Niete and Mokolo. In addition, ASCH lesions were more frequent in Mokolo (40.6%) as summarized in table below.

Table 1: Cytology and PCR results

		Effective	Percentage	95% CI	P value
Cytology Diagnose					
	ASCUS	841	43.2	35.3-51.7	
	ASCH	653	33.5	26.1-40.4	
	LSIL	319	16.4	9.4-23.6	< 0.001
	HSIL	134	6.9	2.6-15.1	
β-globine Presence					
	β-globine+	1836	94.2	82.4-97.3	< 0.001
	β-globine-	111	5.8	3.2-13.6	
HPV Statut					
	HPV+	1484	76.2	70.1-82.5	
	HPV-	256	13.1	7.4-17.9	
	Unsatisfactory smears	207	10.6	4.6-18.3	< 0.001
HPV Genotypes according to classes					
	LR-HPV	1138	75.5	71.2 – 78.1	
	HR-HPV	346	23.4	17.3 – 29.5	< 0.005
Multiple Infections					
	LR-HPV + HR-HPV	91	31	25.1 – 36.8	NS
	LR-HPV	144	48.2	45.7 – 54.4	
	HR-HPV	62	20.6	14.7- 24.8	

Study sites	ASCUS N (%) (n=1357)	ASCH N (%) (n=1439)	LSIL N (%) (n=957)	HSIL N (%) (n=310)
NYETE	229 (16.8)	321 (22.3)	314 (32.8)	65 (20.9)
MOKOLO	288 (21.2)	586 (40.7)	231 (24.1)	63 (20.3)
YAOUNDE	840 (61.9)	532 (36.9)	412 (43)	182 (58.7)
P value	< 0.001			

NS: Non Significant
ANOVA: One way was the test used
LR-HPV: Low Risk Human Papilloma Virus
HR-HPV: High Risk Human Papilloma Virus
ASCUS: Atypical Squamous Cells Undetermined Significance
ASCH: Atypical Squamous Cell not exclude HSIL
LSIL: Low Grade Squamous Intraepithelial Lesion
HSIL: High Grade Squamous Intraepithelial Lesion

b) Frequency of High Risk genotypes (HR-HPV) identified

Table 2 below present different cervical lesions and HR-HPV genotypes identified. The prevalence of low risk genotypes was higher than of high risk ones in ASCUS, ASCH and LSIL lesions. Conversely, high risk genotypes were more frequently associated with HSIL lesions than Low risk genotypes (20%, versus 6.1%) as presented in table 2. However, the difference was not statistically significant (P value=0.0847). ASCUS (60%); was more representative among women with Genotype 16 no patient with LSIL had the HR 16 genotype. The HR 18 genotype was identified in among all women presenting lesions, with the most prevalence for HSIL (30%). HPV 35 was identified mostly among women with LSIL (80%). The final general analysis shows a high frequency of HR 45 genotypes among all the women selected; in fact, HPV 45 was more representative among women who had presented ASCUS (54.5%).

Table 2: HR-HPV genotypes and precancerous cervical lesions

HR Genotypes		HPV 16 (n=54)	HPV 18 (n=100)	HPV 35 (n=52)	HPV 45 (n=105)	HPV 58 (n=0)	HPV 84 (n=35)
		N(%)	N(%)	N(%)	N(%)	N(%)	N(%)
Precancerous cervical Lesions	ASCUS	32 (59.3)	20 (20)	0(0)	55 (52.4)	0 (0)	11(31.4)
	ASCH	12 (22.2)	28 (28)	0(0)	10 (9.5)	0(0)	9 (25.7)
	LSIL	0(0)	24 (24)	42 (80.8)	23 (21.0)	0(0)	10 (28.6)
	HSIL	10 (18.5)	28 (28)	10 (19.2)	17 (17.1)	0(0)	5 (14.3)
	Total	54 (100)	100 (100)	52 (100)	105 (100)	0(0)	35 (100)
	P value			< 0.001			

ASCUS: Atypical Squamous Cells Undetermined Significance
ASCH: Atypical Squamous Cell not exclude HSIL
LSIL: Low Grade Squamous Intraepithelial Lesion
HSIL: High Grade Squamous Intraepithelial Lesion
HR: High Risk
HPV: Human Papilloma Virus

c) *Prevalence of Human papilloma virus infection High risk among Cameroonian women*

Figure 1 below presented different high risk virus identified during the study. HPV 45 and HPV 18 were the most represented with 30.3% and 28.9% respectively.
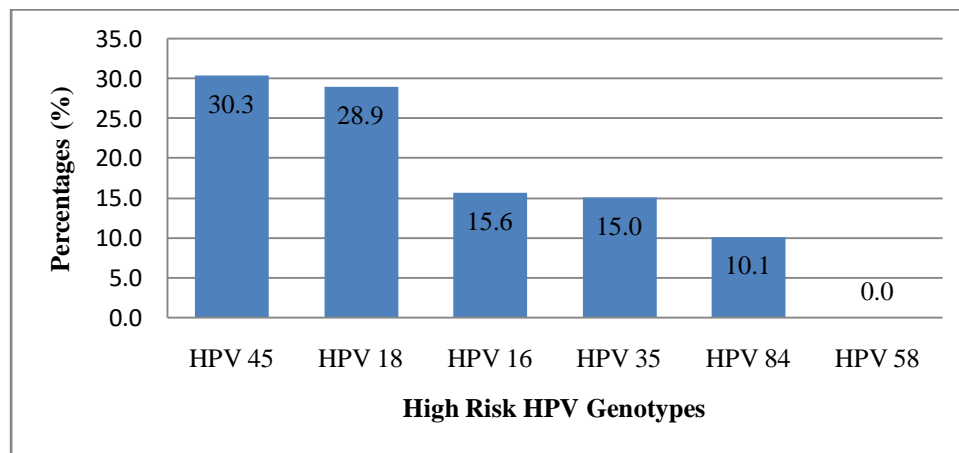

Figure 1: Prevalence of different HR-HPV genotypes targeted in the study population

d) *Frequency of Low Risk genotypes (HR-HPV) identified*

Table3 below presented cervical lesion according to HPV genotype. The distribution of all low risk HPV genotypes targeted in this was significantly unbalanced as presented in Table 5 (P-value<0.0001).

Table 3: LR-HPV genotypes and precancerous cervical lesions

LR Genotypes		HPV 6 (n=294)	HPV 11 (n=190)	HPV 61 (n=218)	HPV 62 (n=138)	HPV 70 (n=139)	HPV 81 (n=159)
		N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Precancerous cervical Lesions	ASCUS	110 (37.4)	71 (37.4)	90 (41.3)	37 (26.8)	80 (57.6)	28 (17.6)
	ASCH	91 (31)	25 (13.2)	50 (22.9)	26 (18.8)	52 (37.4)	69 (43.4)
	LSIL	83 (28.2)	52 (27.4)	78 (35.8)	75 (54.3)	0 (0)	53 (33.3)
	HSIL	10 (3.4)	42 (22.1)	0 (0)	0 (0)	7 (5)	9 (5.7)
	Total	294 (100)	190 (100)	218 (100)	138 (100)	139 (100)	159 (100)
	P value			< 0.001			

e) Prevalence of Human papilloma virus infection Low risk among Cameroonian women

Figure 2 below present distribution of HPV. On the six low risk HPV genotypes targeted, HPV 6 and HPV 61 were the most represented with 25.8% and 19.2% respectively.
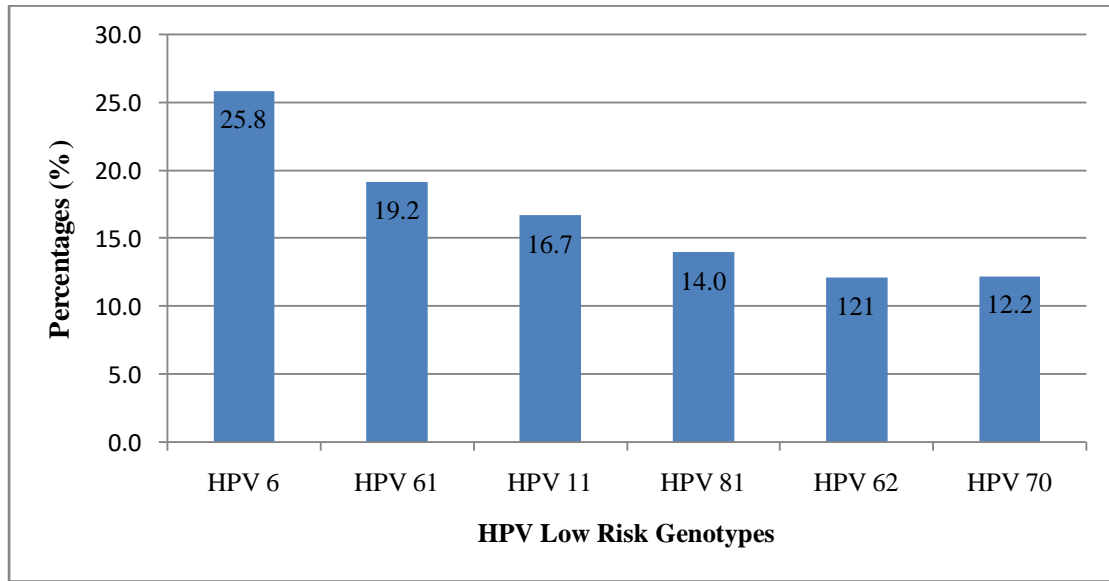
Figure 2: Frequency of distribution of LR-HPV genotypes targeted in the study population

f) Genotype and cervical lesions general association

This figure3 present association between HPV genotypes and precancerous lesions; according to this PCA (principal component analysis) above, many HPV infected are related to specific cervical cells lesions. Appearance of ASCUS is most correlated with HPV genotypes 84 (P value < 0.001). HSIL and LSIL could be respectively associated with HPV genotype 45 and genotype 16 significantly (P value < 0.001).
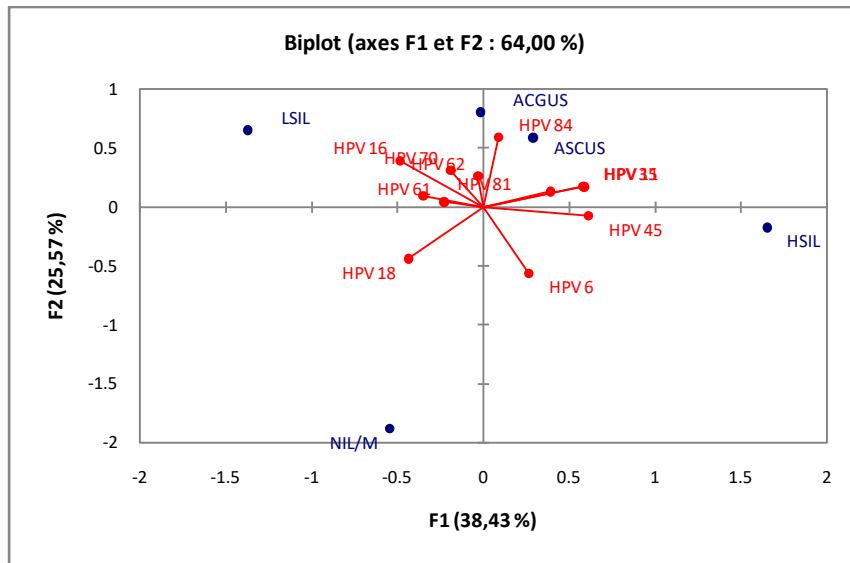

Figure 3: Principal component analysis (PCA) of HPV genotype and precancerous cervical lesions

IV. DISCUSSION

The aim of this study was to characterize Human Papilloma Virus genotypes and their prevalence among Cameroonian women living with precancerous cervical lesions. For this purpose, additional analyses were carried out in samples of patients with lesions (ASCUS to HSIL). Out of 4063 patients with ASCUS, ASCH, LSIL and HSIL we selected, 1947samples were PCR positive. Various lesions were identified and were classified according to the Bethesda classification system. 76% of the samples analyzed were positive for at least one genotype of Human Papilloma Virus. However, remaining samples were negative after PCR. This finding is consistent with previous studies [6], [11]. Indeed, HPV presence on these samples presents his

part of contribution during carcinogenesis. Multiples infections with HR and LR-HPV accounted for 31.03% of all mixed infections[13]. Low Risk HPV genotype 6 was the most prevalent in Cameroon, previous studies presented similar finding[14].HSIL precancerous lesions were found in 20% of women carrying High Risk HPV; almost identical to those found by Desruisseau and collaborators in 2009 [3]. Assessment of HPV infection according to lesions shows that, more women with cervical lesions were also infected by HPV subtypes; this situation can be explain by capacities of lesions to allow HPV penetration on the cervical cell. Results obtained in Iran showed almost the same results [15]. This frequency of lesions is not unique and china study has presented similar data[16]. The HR HPV 18 genotype was identified among women presenting LSIL and HSIL; many studies identified the HPV 18 as the most characterized on HSIL samples [15], this may be due to the dynamic of the virus to modify his polymorphism according to the environment. The final global analysis shows a high frequency of HR genotypes 45 (31.42%), followed directly by the genotype 18 (28.57%). HPV 45 and HPV 18 are two HPV genotypes which belong to HPV High Risk Group; their most prevalence could be attributed to interact with host cell domain and lead to degradation of PDZ-domain-containing proteins through its Carboxy-terminal motif [2].

Among women with low-risk genotypes, the highest frequency was observed among women with indeterminate lesions ASCUS and ASCH (63.9%) as in other studies [16] ; this observation present the capacity of the virus to destroy the cervical tissue and to induce dysplasia, as shown Muentes et al.,[18]. However, a study by Castle et al showed that the most prevalent HPV types in the vaginal samples were HPV71 (4.0%), HPV61 (3, 7%) and HPV58 (3.4%) [19]. No patient with genotype 11 had LSIL; this result presents genotype 11 not so implicated in the development of LSIL in this Country, may be because the genotype 11 is identify as low risk and less dangerous. The final general analysis shows a high frequency of LR 6 genotypes (25.4%), followed directly by genotype 61 (18.42%). Genotype 16 and other genotypes have a considerable frequency of occurrence in the study population. The highest frequency of HPV types found in this study were HPV45 and HPV18, HPV6 do not differ too much from those described in Africa[19], in Ethiopia[20], Morocco[7], Uganda [21], Algeria [8].However, the higher frequency of HPV6 found here is different from previous reports in other African countries that found the most widespread type of HPV, HPV58 in Botswana [22], HPV45 in Cameroon [3], VPH53 in Gabon[23], HPV35 in Burkina Faso [24], HPV2 in Kenya [25]. Multiple infections cases were identified. The HPV-LR 11 genotype was found in two cases of co-infection, as in many studies [26]. Other HPV-LR genotypes were also observed in co-infection cases such as HPV-LR 61 / VPH-HR 84 (11.11%); HPV-LR 62 / HPV-HR 58 (11.11%); HPV-LR 81 / HPV-HR (11.11%). Cuschieri and collabator demonstrated that the prevalence of multiple HPV infections is often high 43.3% and the most prevalent type of HPV multiple infection was only HR-HPV types, with 23.3% and a frequency of multiple infections LR-HPV of 0.8% [18].

V. Conclusion

The aim of this study was to characterize HPV genotypes circulating among Cameroonian women and to identify precancerous cervical lesions involved. Results presented that, Cameroon contain a big diversity of HPV. Global results concerning assessment of prevalence of Low Risk-HPV and High Risk, presented that Human Papilloma Viruses are present in Cameroon with various genotypes, it is important to take into consideration these genotypes during the implementation of prophylactic strategy.

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