Original article

Prevalence of human papillomavirus genotype among Moroccan women during a local screening program

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Abstract

Introduction: Many studies have indicated a causal relationship between genital human papillomavirus (HPV) infections and cervical cancer. This study aimed to determine the prevalence and genotypes of six high-risk oncogenic human papillomaviruses in cervical lesions from Moroccan women with normal and abnormal cytology.

Methodology: The study included 938 women from the Children's and Mothers' Pathology Department of Ibn Sina Hospital, Rabat. Cytopathology examination was done by routine PAP smear testing. HPV DNA testing was conducted using DNA amplification by Polymerase Chain Reaction with subsequent typing by hybridization with specific probes for HPV types 16, 18, 31, 33, 35 and 45.

Results: Cytopathology testing showed that only 16.3 % had an abnormal cytology, with a predominance of atypical squamous cell of undetermined significance (ASCUS) cases. The overall HPV prevalence was 15.7%. According to the cytology results, HPV infection was detected in 15.8% of normal and 14.38% of abnormal cases. Specific HPV genotyping showed a predominance of HPV 16 and 18. Double infection (HPV 16 + 18) was found in two cases whereas multiple infections (HPV 16+18+31) were detected in only one case.

Evaluation of the relationship between HPV status and some environmental risk factors, including individual, socio-economic, and hygiene status, showed a significant association between HPV infection and oral contraceptive use.

Conclusion: Based on these data, a combination of cytology and HPV DNA testing allows for identification of patients with a high risk of developing high-grade cervical lesions and improves cervical cancer prevention.

Key words: HPV; cervical cancer; PCR; molecular hybridization; Morocco

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Introduction

Worldwide, cervical cancer is the second most common malignancy in women with nearly half a million new cases diagnosed and 200,000 deaths each year [1]. In Morocco, published statistics showed that cervical cancer represents a serious public health problem and in the absence of a national cancer registry, data are limited to the number of cases registered in some medical centres [2]. The hospital-based cancer registry of the National Institute of Oncology (INO), Rabat, reports more than 500 new cases annually [2].

Clinical and epidemiological studies have identified human papillomavirus (HPV) as the central risk factor for cervical cancer development [3]. HPV belong to the *Papillomaviridae*, and infection with specific mucosal HPV types has been shown to be associated with the development of cervical intraepithelial neoplasia lesions and cancer [4,5].

To date, more than 200 HPV genotypes have been identified [6], but the interest is focused only on genital HPV (40 genotypes) that are associated with precancerous and cancerous lesions of the cervix [7]. HPV genotypes vary in oncogenic potential and are

associated with anatomically and histologically different diseases.

HPV types have been grouped into low-risk and high-risk types based on the frequency of association with invasive cervical cancer. Some HPV types considered low risk, such as 6 and 11, cause benign condylomas, whereas a wider number of subtypes considered as high risk have been shown to be involved in cervical carcinogenesis. Among them, HPV 16 and 18 genotypes, which are frequently found in association with cervical cancer, are considered the most oncogenic types. Other HPVs, 31, 33, 35, 45, 51, 52, 58 and 59, that are also considered as carcinogenic, are less frequent in cervical carcinomas [8,9,10]. When HPV infection occurs, the viral DNA is present in the cell as an episomal plasmid. Persistence of infection favors viral integration in the cell genome which, together with other factors, can progress to high-grade squamous intraepithelial lesions (HSIL) and cancer [11,12]. Usually, there is a long latency period between the time of HPV infection and the appearance of cancer.

Currently, cervical lesion diagnosis is based only on cyto- and histopathology analyses. Cytology diagnosis, especially using the Pap smear screening test, has largely contributed to decreasing mortality by cervical cancer and has been very successful in lowering the rate of cervical cancer in countries with high coverage and good quality control. However, a number of problems with cytology have been demonstrated, including sensitivity and quality sample interpretive errors due to the subjectivity of the reading of slides, leading to a greater number of interpretive errors [12]. The low sensitivity of cytology has major medical, economic, and medico-legal implications [12]. Cuzick *et al.* (2006) provided a meta-analysis with a direct comparison between cytology and HPV DNA testing. In this analysis, sensitivity of cytology was substantially lower than for HPV DNA testing and varied considerably between studies; however, HPV DNA testing is less specific [12].

HPV DNA testing for cancer-associated HPV DNA is now accepted as a viable and valid option in the treatment of women with equivocal cytological findings and, in recent years, there has been an increasing interest in the use of HPV DNA testing in cervical samples from asymptomatic women without cytological abnormalities [13,14].

This study was conducted to determine the prevalence of the most oncogenic HPV in the Moroccan population and to evaluate the simultaneous screening of cervical lesions with Pap smear and HPV DNA testing to establish the contribution of HPV DNA testing to cervical cancer management in Morocco. Also, some risk factors associated with cervical cancer development were evaluated using the hospital case control database.

Methodology

Specimens

A total of 938 uterine cervix samples were collected from women visiting the family planning service at the maternity department of Centre Hospitalier Universitaire (CHU) Ibn Sina, Rabat, between October 2006 and October 2007. Women who had undergone a hysterectomy or who were pregnant were excluded. For each woman, a questionnaire was completed including age, marital status, socio-economical status, parity, contraceptive practice and the presence of any gynaecological symptoms.

The characteristics of the study population are reported in Table I. The mean age of women was 42.5 with extreme ages at 17 and 80. More than 90% lived in an urban area, most frequently in Rabat. All of them were married and had been pregnant. The majority of the participants reported one lifetime sexual partner. A history of contraceptive use was reported by about 47.9% of women, including 19.2% using an intrauterine device and 28.7% using an oral contraceptive. Two women reported family neoplasia history (breast and digestive tracts) and more than 39% of participating women had never had a Pap test for cervical lesion diagnosis.

Scraped cervical cells were collected by an experienced gynaecologist or pathologist from each woman using a cervix brush. Cell suspensions, containing ectocervical and endocervical cells, were smeared one slide for Pap testing. The remaining scraped cervical cells were transferred to sterile vials containing 5 ml phosphate buffered saline (PBS) for HPV DNA testing.

Pathology

Cells were fixed with alcohol or with lacquer and coloured using the Papanicolaou method. Histological assessment was performed by pathologists in the anatomy pathology department at the Children's and Mothers' Pathology Department, CHU Ibn Sina, Rabat. The results of the Pap smears were classified according to the Bethesda System 2001 [9,10,15,16].

HPV detection and typing

DNA extraction: DNA was extracted from cervical cells. Cells were lysed in the digestion buffer (Tris-HCl 10 mM pH 8.0, EDTA 10 mM, NaCl 150 mM and SDS 2%) containing proteinase K (0.1mg/ml) (Promega, Charbonnieres - France). DNA isolation was performed with phenol-chloroform extraction and ethanol precipitation. DNA was then resuspended in sterile distilled water, and stored at -20°C until use [17].

DNA amplification: All the primers and probes used in this study were purchased from Operon (Paris, France). The first aliquot of a given extract was screened with a *β-globin* Polymerase chain reaction (PCR) using PC03 and GH20 primers that allow the amplification of a 123 base pair (bp) fragment [18]. This first amplification was used to check the DNA quality and competence. A second aliquot was used for HPV DNA detection and typing. DNA was amplified by the polymerase chain reaction (PCR) using consensus primers: MY 09 and MY 11 that allow the amplification of 450-bp target sequences of the L1 region that is highly conserved on a broad spectrum of HPVs [19].

Amplification reaction was performed in total volume of 50 µl. The amplification mixture contained 50 pmol of each consensus primer, 200 µM of each dNTP (dATP, dCTP, dGTP and dTTP), 0.625 units *Taq* DNA polymerase (Amersham, England) and 5 µl of DNA sample in 1x *Taq* polymerase buffer. The mixture was first denatured at 94°C for seven minutes. Then 35 cycles of PCR were performed, in the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA), with denaturation at 94°C for 30 seconds, primer annealing for one minute at 56°C and primer extension for one minute 30 seconds at 72°C. At the end of the last cycle, the mixture was incubated at 72°C for seven minutes. For every reaction, a negative control in which the DNA template was omitted from the amplification mixture and a positive control were included.

Hybridisation analysis of PCR products: Aliquots of 10 µl of the PCR product were analysed by electrophoresis through 1% agarose gel. The 50 bp ladder molecular-weight marker (Amersham, England) was included for detection of the DNA size of the amplification product.

PCR products were transferred onto positively charged nitrocellulose membranes (Hybond N+, Amersham, England) and fixed by incubation at 80°C for two hours. HPV-specific probes were biotin labelled and hybridized under stringent conditions to amplicons on the membrane as described previously [17]. The probes used in this study were MY14 (CATACACCTCCAGCACCTAA), WD74 (GGATGCTGCACCGGCTGA), WD126 (CAAAAGCCCAAGGAAGATC), MY16 (CACACAAGTAACTAGTGACAG), MY115 (CTGCTGTGTCTTCTAGTGACAG) and MY70 (TAGTGGACACTACCCGCAG), which are specific to HPV 16, 18, 31, 33, 35 and 45 respectively. Membranes were then washed under normal and stringent conditions and specific hybrids were revealed using the biotin luminescence detection kit according to the manufacturer's instructions (Biolabs, England). Finally, membranes were exposed for 30 minutes on X-ray film (Hyperfilm ECL, Amersham, England).

Table 1 – Characteristics of 938 participating women in the cytology and HPV testing project

Characteristics		N	%
Age Mean age: 42,5(17-80; 9,5)			
	< 29	90	9.6
	≥ 30	848	90.4
Residence			
	Urban	845	90.1
	Rural	93	9.9
Sexual partner			
	Single	912	97.2
	Multiple	26	2.8
Parity			
	Less than 3 children	401	42.7
	Three children or more	537	57.3
Family Income[a]			
	0-2 minimum salaries per month	258	27.5
	> 2 minimum salaries per month	680	72.5
Abortion		302	32.2
Contraception		449	47.9
	Intra-uterine device(IUD)	180	19.2
	Oral contraceptive(OC)	269	28.7
Previous cytology diagnosis with pap test			
	Never	366	39
	≥ 1	572	61

a: The minimum salary is about 120$

Statistical analysis: A data bank including demographic and behavioural factors was generated and analyzed using the statistical software package SPSS (SPSS, Inc., Chicago, IL, USA). Crude and adjusted odds ratio (OR) tests for trends with 95% confidence intervals (95% CI) and chi-square tests were used when appropriate. The significance level for the tests (p) was set at 0.05.

Results

The cytological results of the 938 swabs showed that 83.7 % had normal cytology (785/938). Among the 153 abnormal cytology cases, 60 were classified as non-specific inflammations; 46 cases were atypical squamous cell of undetermined significance (ASCUS); and 16 cases were atypical squamous cell not excluding high-grade lesion (ASCH), whereas low-grade squamous intra-epithelial lesions (LSIL) and high-grade squamous intra-epithelial lesions (HSIL) were found respectively in 24 cases and 7 cases (Table 2). In this cohort, no cytology indicating cervical cancer was observed.

The presence of amplifiable DNA, using primers for a fragment of *β-globin* gene, was confirmed in all cases and all DNA samples were adequate for further analyses. Successful amplification of HPV-positive DNA preparations with MY11/MY09 primers yielded a DNA fragment of 450 pb corresponding to the PCR products of HPV L1 gene.

Using PCR amplification combined with molecular probing, the presence of HPV DNA in 15.6% of cases (146/938) was revealed. The distributions of viral genotypes in the 146 HPV positive cases are reported in Table 2. Molecular typing showed single and multiple infections with a predominance of HPV 16 and 18. HPV 16 and 18, alone or in coinfections, were present in 15.75% (23/146) and 10.96% (16/146) of cases respectively, whereas the others types had a low prevalence.

In the normal group, HPV DNA was detected in 15.8% of cases (124/785). Molecular analysis showed that there was no conifection. HPV 16 and 18, the most predominant genotypes, were present respectively in 13.71% (17/124) and 8.87% (11/124) of HPV-positive cases.

In the abnormal group, HPV DNA was found in 14.38% of cases (22/153). Viral distribution showed the presence of HPV 16, 18, 31 and 33. Two cases were double infected with HPV 16 and 18 and only one case was infected by HPV 16, 18 and 31. No specimen harboured the other genotypes, HPV 35 and 45. Among the 146 HPV positive cases, 97 were untyped and hence were not related to other HPV genotypes.

According to the cytological diagnosis, the viral genotype distribution showed the absence of the six high-risk HPV in the NSI group (Table 2). Moreover, stratification of abnormal cytology showed that HPV 16, alone or in combination with other HPV genotypes (18 and 31), was found mainly in advanced lesions. Interestingly, the triple infection with HPV16, 18 and 31 was found in the HSIL case. Double infection with HPV 16 and 18 were found in two cases; one was LSIL and the other corresponded to an ASCUS.

The evaluation of the relationship between HPV infection and some environmental risk factors is reported in Table 3. Statistical analysis showed that the use of oral contraceptives showed a significant association (p = 0.0206). The other risk factors did not show any significant association in the population study. On the other hand, among the 912 women declaring a single life sexual partner, 141 were HPV positive (15.39%).

Table 2: Distribution of HPV genotypes in cervical swabs according to the cytological results

	N	HPV+ Cases	HPV 16	HPV 18	HPV 16+18	HPV 31	HPV 33	HPV 35	HPV 45	HPV16+18+31	Unknown
Normal	785	124	17	11		4	1	3	2		86
Abnormal											
NSI	60	11									11
SCUS	46	3			1	1	1				
ASCH	16	1		1							
LSIL	24	4	3		1						
HSIL	7	3		1		1				1	
Total	**938**	**146**	**20**	**13**	**2**	**6**	**2**	**3**	**2**	**1**	**97**

NSI: Non Specific Inflammation; ASCUS: Atypical Squamous Cell of Undetermined Significance;
ASCH: Atypical Squamous Cell not excluding high grade lesion; LSIL: Low grade Squamous Intra epithelial Lesions; HSIL: High grade Squamous Intra epithelial lesions

Table 3: Correlation between HPV infection and some environmental risk factors

Factor		N	HPV positive cases	Percentage	OR (95% CI)	*p*
	< 30 years	90	12	13.33	1.23 (0.65-2.33)	0.6443
	≥ 30 years	848	134	15.80		
Family income	≥ 240 $	680	114	16.76	1.18 (0.77-1.81)	0.1226
	< 240 $	258	32	12.40		
Parity	<3 children	401	61	15.21	1.05 (0.37-1.49)	0.8665
	≥3 children	537	85	15.83		
Sexual partner	Single	912	141	15.39	1.05 (0.35-3.13)	0.7959
	Multiple	26	5	19.23		
Abortion	No	636	94	14.78	1.23 (0.91-1.69)	0.3861
	Yes	302	52	17.22		
OC	No	669	92	13.75	1.36 (0.96-1.91)	0.0206
	Yes	269	54	20.07		
IUD	No	758	112	14.78	0.91 (0.75-1.09)	0.2103
	Yes	180	34	18.89		

OC: Oral contraceptive; IUD: Intra-uterine device; $: US dollar; OR: Odds Ratio; CI: confidence intervals

Discussion

Cervical cancer is the most common malignancy among females in developing countries, with the highest prevalence in Latin America, Asia and Africa [20]. In Morocco, cervical cancer is the second most frequent female cancer after breast cancer and represents a major public health problem. The diagnosis is usually made in advanced stages, and mortality is high.

HPV plays a central role in the pathogenesis of cervical cancer and this viral infection is considered to be a necessary, though not always sufficient, cause. The development of human cervical cancer without the involvement of a specific HPV is exceptional.

Recently, prophylactics and therapeutic vaccination has shown promising results for preventing HPV infection as well as for the development of cervical neoplasia. However, development of effective vaccines would require a comprehensive study of the HPV genotypes in different regions of the world [21].

In this study, the prevalence of HPV DNA by PCR with the MY09/MY11 primers was 15.9% and is in concordance with the overall world distribution of HPV in asymptomatic women. A global epidemiologic study conducted by the International Agency for Research on Cancer showed that HPV prevalence is very different between countries. Lower prevalence was found in Europe (10.5%)\ and intermediate prevalence was found in South America (14.3%), whereas the highest prevalence was found in Africa (25.6%) [22]. Moreover, similar results were found in other countries such as India (18.8%) [23], Venezuela (15.6%) [24] and Algeria (12.5%) [25].

In the present study, the use of the hybridization method showed that HPV 16 and 18 are the most predominant genotypes in 13.71% and 8.87% respectively of HPV-positive cases. Worldwide, HPV 16 and 18 are the major genotypes found in cervical specimens and are mainly associated with the development of cervical lesions and cancer.

This study focused on HPV 16, 18, 31, 33, 35 and 45. In fact, Munoz *et al*. [7] have shown that theses types are the most frequent genotypes associated with the development of cervical precancerous lesions and cancer. Thus the hybridization method showed that HPV 16 was the predominant infectious type with 15.75% of HPV-DNA positive simples. Worldwide, HPV 16 is the most common type followed by HPV 18 [8,21].

Interestingly, in the present study, multiple HPV infections were observed in only 2% (3/146) of the HPV-positive specimens. A previous study on Moroccan cervical cancer specimens found multiple infections in 35.4% of specimens [26]. Co-infection with multiple HPV types is a common finding of

many molecular epidemiologic studies of cervical cancer. Some HPV types might interact or act synergistically to induce lesion development or progression [27]. Indeed, the risk of high-grade lesions and of invasive cervical cancer seems to be considerably increased among women with multiple-type infections compared with those harbouring a single HPV type [28]. Herrero *et al*. (2005) have shown that the risk of cervical lesions strongly increases when HPV 16 and other types are not present alone [29]. Moreover, Fife *et al*. (2001) suggested that some HPV types might cooperate with HPV 16 to produce dysplasia or cancer [30]. This finding provides evidence that women with multiple infections must be closely monitored to prevent evolution to cervical cancer.

Among the 146 HPV-positive cases, 97 were untyped. These HPVs could correspond to other high-risk HPVs or belong to low-risk HPVs that are usually related to benign lesions.

It is widely accepted that in addition to HPV infection, other cofactors could have a great role in the development of cervical lesions. These factors may be classified into two groups: 1. environmental or exogenous cofactors, including the use of oral contraceptives (OCs), tobacco smoking, diet, cervical trauma, and co-infection with human immunodeficiency virus (HIV) and other sexually transmitted agents; 2. host cofactors, including endogenous hormones, genetic factors such as human leukocyte antigen, and other host factors related to the host's immune response [31]. In the current study, due to limited data, only risk factors related to age, socio-economic level, sexual activity, oral contraception, and family situation were examined.

All the women harbouring HR HPV were more than 30 years old. In this group, 80% were aged between 45 and 55 years. There is evidence that this age group is more exposed to persistent HR HPV. Previous studies have shown that the peak prevalence of invasive cancer occurs at approximately 40-50 years old [7]. Considering the normal and abnormal cytology results, the overall prevalence of HPV was not statistically significant. However, a similar study conducted by Ghaffari *et al*. [21] had shown that HPV infection was significantly associated with cervical lesions in asymptomatic women. This observation could be related to the study populations. Indeed, the results of the current study showed a low frequency of ASC-US/ASC-H, L-SIL and H-SIL based on cytology.

In this study, the only statistically significant association was found between HPV infection and oral contraceptive use. However, no statistically significant association between HPV infection and the other risk factors after adjustment for age group, parity, family income, number of sexual partners, abortion episodes and use of intrauterine device were found by either unvaried and multivariate logistic regression models. Worldwide, different studies have shown that these risk factors are closely related to the development of cervical lesions and cancer [31,32,33,34]. Thus, environmental and endogenous cofactors will not influence HPV infection but could be implicated in the lesion progression and cancer development.

According to the findings in the current study, high rates of infection with HPV genotypes in sexually active Moroccan women make molecular investigation for HPV16, 18 and 31 essential in clinical approach. However, HPV 33, 35 and 45 are less frequent in this population.

In Morocco, the diagnosis of cervical lesions rests exclusively on the cytology-based screening that offers substantial protection, although current coverage is low. The introduction of HPV DNA testing in cervical cancer management will greatly benefit early stage HPV detection and help prevent development of cervical lesions and cancer. This may offer a significant opportunity for the Moroccan National Program against cervical cancer to control this devastating disease and save many lives.

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