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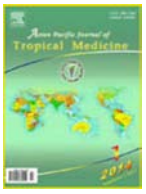

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Identification of human papillomavirus as a preventive strategy for cervical cancer in asymptomatic women in the Peruvian Andes

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ABSTRACT

Objective: To detect the most prevalent human papillomavirus (HPV) genotypes in cervical smear samples of asymptomatic Peruvian women by analyzing the correlation between Papanicolaou (PAP)-stained cervical tests and PCR-sequencing.

Methods: A total of 254 women attending routine gynecological examinations were included in this study. The samples were analyzed by PAP technique and examined under a microscope by a pathologist and classified by the Bethesda system. HPV amplification was done using the primers specific for E1 region and positive specimens were confirmed by direct sequencing.

Results: The prevalence of HPV was investigated in 254 cervical scrape samples by PCR. PAP smear showed that 94.9% cases had normal morphology and 5.1% had an inflammatory pattern; 20.5% were found to be infected with HPV, comprising 20 different genotypes. HPV16 was the most prevalent genotype in correlation with changes in cervical cytology.

Conclusions: Our results suggest the HPV is very frequent even in women with negative PAP, and PCR seems to be the best option to determine the causative agent of HPV infection in endocervical samples. Identification of the HPV genotype in asymptomatic women may allow the implementation of appropriate prophylactic measures which may have a direct impact on the natural history of the disease and the subsequent development of cervical malignancy.

1. Introduction

Cervical cancer is the third in incidence and the fourth in mortality among female cancers worldwide, with a variable prevalence according to the implementation of successful public health screening and treatment programs[1-3]. Recent molecular and epidemiological studies have shown that the human papillomavirus (HPV) is the main causative agent of cervical neoplasm[3]. Currently, genital HPV has been described to be a sexually transmitted disease of epidemiological importance, especially among the sexually active population, affecting a high percentage of persons, both women and men, living in underdeveloped countries[4]. Additionally, mother-to-child vertical transmission in the birth canal in mothers with genital condylomas has been suggested[5].

Infection with one of the few oncogenic HPV types is necessary to cause invasive cervical cancer[6,7]. Currently, more than 120 different HPV types have been isolated and sequenced, of which about 40 are known to infect the

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genital tract, and 18 are classified as carcinogens[8,9]. These later types are classified into two groups according to their oncogenic potential: those of high oncogenic risk including HPV16, 18, 23, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82, and types 6, 11, 40, 42, 43, 44, 54, 61, 69, 70, 72, 81 and 89 regarded as possessing low oncogenic risk[10]. The most frequent HPV types associated with cancer and cervical dysplasia are HPV16, 18, 31, 33 and 45 accounting for 90% of these neoplasms[11].

According to the World Health Organization[12], HPV infection is very common and most men and women are infected at some point of their life. Transmission does not necessarily require penetration but may occur with single-skin contact of the genitals. Most infections disappear (90% of cases) during the two first years. Although other factors involved in cervical oncogenesis are required, prolongation of HPV infection may lead to the development of pre-cancerous lesions and invasive cancers[13].

The highest incidence of cervical cancer has been described in the Caribbean and Latin America with mortality observed mainly in women from 35 to 54 years of age[14]. In Peru, cervical cancer is the most common malignancy (24.9%), being closely related to socioeconomic level[15]. However, there are still no general epidemiological data to indicate the overall prevalence of HPV infection and its association with cervical neoplasia in this country. Traditional screening for HPV infection is crucial, but early detection of most cervical infections can be difficult since HPV is asymptomatic. In Peru, screening programs for HPV involve the traditional Papanicolaou (PAP) protocol. This technique has a high frequency of false negatives (between 15%-50% cases) and false positives (30% cases), and the results should be considered with caution[15,16]. The introduction of molecular techniques in the cervical screening programs would help to improve the detection of pre-cancerous lesions and reduce equivocal results by employing better collection, preparation and testing methods[17]. Technologies such as PCR, which can amplify a specific HPV DNA sequence present in clinical samples and can detect 50-100 integrated virus/cell, is considered the most sensitive HPV detection technique worldwide[18].

The aim of this study was to detect the most prevalent HPV genotypes in cervical smear samples of asymptomatic Peruvian women by analyzing the correlation between PAP-stained cervical tests and PCR and sequencing of the E1 region.

2. Materials and methods

2.1. Patients

A prospective study was designed and coordinated by the Liga Peruana de Lucha Contra Cáncer de Cajamarca and Universidad Peruana de Ciencias Aplicadas (UPC) in Cajamarca, Peru, from October 2010 to May 2012. A total of 254 women between 18 to 65 years old, attending routine gynecological examinations were included in this study. All the women were physically examined and cervical scrape samples were collected and stored for HPV testing. The women selected were asymptomatic with no previous history of HPV infection or any cervical neoplasia and were clinically normal. Exclusion criteria were pregnancy, severe gynecological bleeding and previous hysterectomy. A health worker informed all women about the study and informed consent was obtained from each of the participants included in the study. All the specimens studied were anonymous with coding numbers for analysis.

2.2. Sample collection and preservation

Cervical cell samples were collected with a cytobrush from the ectocervix and endocervix of each woman and samples were preserved in a tube containing phosphate buffered saline (pH 8.6). The samples were then stored at -4 °C until being sent to the Molecular Biology Laboratory at UPC and Instituto de Investigación Nutricional. On receipt of the samples, the cytobrushes were discarded and the tubes were vortexed and centrifuged to pellet the cells, which were resuspended in 1 mL of phosphate buffered saline. Three aliquots of each fresh specimen were stored at -20 °C until testing.

2.3. PAP-stained cervical test

PAP smears were obtained from the endocervix and ectocervix by scraping the squamous columnar cells with a wooden Ayre spatula. Using a cytology brush, cervical scrapings were spread over designated slides for each patient. The slides were fixed with ethanol and colored by the PAP technique. The samples were examined under a microscope by a pathologist and classified by the Bethesda system: atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesion (LSIL) (CIN1) and high-grade squamous intraepithelial lesion (HSIL) (CIN2 and CIN3)[19].

2.4. DNA extraction, amplification of HPV DNA and genotypes by sequencing

Viral genomic DNA was extracted from a total volume of 200 μL of the sample by the guanidinium thiocyanate extraction method[20] and the purified material was re-suspended in a final volume of 30 μL deionized water. Samples were electrophoresed on a 1% agarose gel to check the quality of the DNA.

HPV amplification was done using the primers and conditions described by Lurchachaiwong *et al*[21]. PCR products were analyzed on 2% agarose gel stained with ethidium bromide and bands were detected by UV transillumination (Kodack Logic 1500, USA). Positive specimens were confirmed by direct sequencing serving as the gold standard (Macrogen-Korea).

The HPV genotypes were categorized into three groups: high and low risk, and others[6,7].

2.5. Statistical analysis

Quantitative variables are reported as frequency (number or percentage of cases). Odds ratios (OR) with 95% confidence intervals (*CI*) were used to estimate precision. The χ^2-test (or Fisher's exact test when appropriate) was used to compare the distribution of categorical variables. Differences were considered significant at $P<0.05$. Statistical analyses were performed with SPSS software (Microsoft SPSS-PC+, v.15.0; SPSS, Chicago, IL, USA).

3. Results

A total of 254 women between 18 to 65 years of age were included in this study. The women were from different locations in the province of Cajamarca, Peru.

Regarding PAP smears, 94.9% of the cases had a normal morphology (negative PAP) and 5.1% showed an inflammatory pattern (positive PAP). However, the prevalence of HPV infection using the PCR method was 20.5% (Table 1), with statistically significant differences between the two techniques ($P<0.000\,1$, χ^2-test with Yates continuity correction).

Table 1

Comparison of HPV diagnosis by PAP smear and PCR to amplify the E1 viral gene.

Result	PAP smear		PCR	
	Cases (N)	Frequency (%)	Cases (N)	Frequency (%)
Positive	13	5.1	52	20.5
Negative	241	94.9	202	79.5
Total	254	100.0	254	100.0

Considering PCR as the "gold standard", the specificity and sensitivity of PAP were 81% and 25%, respectively, with false negative and false positive rates of 75% and 19%, respectively.

The results showed that 52/254 women (20.5%) had HPV infection, related to different HPV genotypes. Twelve different high risk genotypes were observed, with the HPV16 (38.5%) and the HPV39 (9.6%) genotypes being significantly more frequent ($P<0.005$); The remaining 10 high risk genotypes had low frequencies (from 1.9% to 3.8%). Five low risk viruses genotypes were detected with low incidence (1.9% to 3.8%). Finally, three genotypes (HPV34, 71 and 91) were classified as other HPV, of which the most significant ($P<0.005$) was HPV71 with an incidence of 9.6% (Table 2).

Table 2

Distribution of HPV genotypes identified in asymptomatic women.

Viral type		Cases	Frequency	95% *CI*		χ^2-test (*P*)
		(N)	(%)	Low	Upper	
High risk	HPV16	20	38.5	25.31	58.67	<0.000 1
	HPV18	1	1.9	0.05	10.25	0.1100
	HPV31	2	3.8	0.47	13.20	0.0297
	HPV39	5	9.6	3.20	21.00	0.001 1
	HPV51	1	1.9	0.05	10.25	0.1100
	HPV52	2	3.8	0.47	13.20	0.0297
	HPV53	1	1.9	0.05	10.25	0.1100
	HPV56	1	1.9	0.05	10.25	0.1100
	HPV66	2	3.8	0.47	13.20	0.0297
	HPV67	2	3.8	0.47	13.20	0.0297
	HPV68	1	1.9	0.05	10.25	0.1100
	HPV73	1	1.9	0.05	10.25	0.1100
Low risk	HPV6	1	1.9	0.05	10.25	0.1100
	HPV69	2	3.8	0.47	13.20	0.0297
	HPV70	1	1.9	0.05	10.25	0.1100
	HPV44	1	1.9	0.05	10.25	0.1100
	HPV90	1	1.9	0.05	10.25	0.1100
Others	HPV34	1	1.9	0.05	10.25	0.1100
	HPV71	5	9.6	3.20	21.00	0.001 1
	HPV91	1	1.9	0.05	10.25	0.1100
	Total	52	100.0			

We observed an age-related distribution of viral risk genotypes (Figure 1). Thus, only high risk genotypes were found in the 18-24 years of age group, while in women over 55 years only 1 out of 5 HPV viruses detected belonged to this risk group. The women between 25 and 54 years of age showed a large diversity of genotypes, while in the 25-34 years group the highest genotype dispersion was observed. In addition, a progressive increase in the incidence of HPV16 was observed in the 18-24, 25-34 and 35-44 year groups (3.8%, 9.6% and 21.2%, respectively).

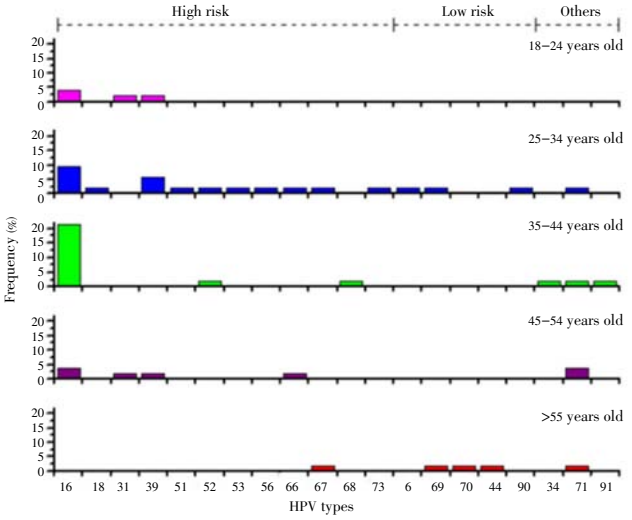

Figure 1. Distribution of HPV genotypes in age groups of the population of asymptomatic women.

Using the PAP method we detected 13 samples presenting tissue lesions, 9 being associated with the presence of HPV (Table 3). A positive correlation (Pearson coefficient, r=0.9934) between the uterine tissue lesion and the presence of HPV in tissue, was found, with a relationship between the two variables as shown in Table 3 (P=0.4724, χ^2-test). Regarding the association between lesions and specific genotypes, it was found that the ASCUS lesion was partially related to the HPV66 genotype, while LSIL was related to the HPV16 genotype. HSIL was the most frequent lesion among PAP positive samples being related to several HPV genotypes (HPV16, 71, 91 and 39).

Table 3

Presence of HPV genotypes in PAP positive cervical lesions.

Lesion	PAP smear N (%)	PCR N (%)	Viral genotype
ASCUS	2 (15.38)	1 (11.11)	HPV66
LSIL (CIN 1)	4 (30.77)	2 (22.22)	HPV16
HSIL (CIN 2-CIN 3)	7 (53.85)	3 (33.33)	HPV16
		1 (11.11)	HPV71
		1 (11.11)	HPV91
		1 (11.11)	HPV39
Total	13 (100.00)	9 (100.00)	

Regarding the risk factors analyzed the most relevant were the number of sexual partners. Thus, the percentage of HPV+ women belonging to each group increased from 8.1% (women with 1 sexual partner) to 40.9% (women with >3 sexual parterns), with the OR progressively increasing (0.71, 0.94 and 3.04) according to the increase in the number of partners: 1, 2, and >3, respectively (Table 4).

Table 4

Association between the presence of HPV and risk factors in the population of asymptomatic women.

Group	Frequency N (%)			OR	95% CI		Z-test
	Total (n=254)	HPV positive (n=52)	HPV negative (n=202)		Lower	Upper	(P)
Age range							
18-24	24 (9.4)	4 (7.7)	20 (9.9)	0.76	0.25	2.32	0.0229
25-34	77 (30.3)	20 (38.5)	57 (28.2)	1.59	0.84	3.01	0.9986
35-44	79 (31.1)	16 (30.8)	63 (31.2)	0.98	0.51	1.90	0.9599
45-54	47 (18.5)	7 (13.5)	40 (19.8)	0.63	0.26	1.50	0.1442
>55	27 (10.6)	5 (9.6)	22 (10.9)	0.87	0.31	2.42	0.0459
Age of first coitus							
<17	80 (31.5)	24 (46.2)	56 (12.4)	2.23	1.19	4.18	0.9943
18-24	116 (45.6)	23 (44.2)	93 (46.0)	0.93	0.50	1.72	0.9905
25-34	21 (8.3)	2 (3.8)	19 (9.1)	0.39	0.09	1.71	0.0590
>35	2 (0.8)	0 (0.0)	2 (1.0)	–	–	–	–
No data	35 (13.8)	3 (5.8)	32 (15.8)	–	–	–	–
Number of sexual partners since firts coitus							
1 partner	144 (56.6)	26 (50.0)	118 (58.4)	0.71	0.39	1.31	0.9971
2 partners	55 (21.6)	13 (25.0)	42 (20.8)	0.94	0.46	1.92	0.5000
>3 partners	22 (8.7)	9 (17.3)	12 (5.9)	3.04	1.22	7.58	0.1975
No data	33 (13.0)	4 (7.7)	29 (14.4)	–	–	–	–
Parity							
0	17 (6.7)	4 (7.7)	13 (6.4)	1.21	0.38	3.88	0.0054
1	43 (16.9)	8 (15.4)	35 (17.3)	0.87	0.38	2.00	0.6667
2	50 (19.7)	6 (11.5)	44 (21.8)	0.47	0.19	1.17	0.1437
3	44 (17.3)	15 (28.8)	29 (14.4)	2.42	1.18	4.96	1.0000
4	22 (8.7)	6 (11.5)	16 (7.9)	1.52	0.56	4.09	0.1429
>5	40 (15.7)	7 (13.5)	33 (16.3)	0.80	0.33	1.92	0.3744
No data	38 (15.0)	6 (11.5)	32 (15.8)	–	–	–	–

No clear relationship was observed between age or parity with HPV infection. Thus, despite 38.5% (20 cases) of the positive HPV cases belonging to women between 25 and 34 years of age, these cases represented 26% of the women belonging to this age group, being a similar percentage to that detected in the remaining groups.

4. Discussion

HPV infection is a common sexually transmitted disease which is usually benign, but its persistence over time and continuous exposure to the virus may lead to the development of cervical cancer[22-24]. This cancer is very common among women and is the leading cause of death in economically active women especially in low- and middle-income countries[4]. In Peru, cervical cancer is first among all the female malignancies and is mainly associated with HPV infection. However, there are no studies showing prevalence of the HPV in the asymptomatic population. Thus, early diagnosis and prevention is relevant to diminish its mortality by this disease[25].

The present results showed a remarkably high prevalence of HPV infection in an Andean population in Northern Peru. In this population, as in the rest of Latin America, only PAP is used as the screening method. However, the present data demonstrate that this technique has a low sensitivity. On the other hand, using the PCR method showed a higher prevalence (46.2%) of HPV infection in young asymptomatic women of 25-44 years of age, which is higher than that reported in other Latin American countries, such as Mexico (14.5%), Costa Rica (16.0%) and Colombia (14.8%)[26-28]. Although few studies on prevalence of HPV in asymptomatic women has reported a high prevalence of HPV detected by PCR[29], in our population an infrequent high prevalence of HPV among asymptomatic women is present. One explanation for this higher prevalence may be related to an historical low interchange of population.

The prevalence of HPV diagnosed using the PAP method was 5.1%. Among these samples, different cervical tissue lesions were detected using the Bethesda system. The high percentage of false negatives obtained by the PAP method indicates that this is not a good screening method for early detection of HPV infection; Minimal morphological changes can be so subtle that they can easily go unnoticed. The low sensitivity of the PAP method highlights the need for a rapid, affordable, accurate and sensitive method to detect and differentiate HPV genotypes which is essential to identify high risk patients, who are otherwise found to have a normal cytology by PAP smear.

In general, high risk genotypes are closely related to the development of cervical cancer[7,8]. Wang *et al.* reported that HPV16, 18 and 45 genotypes are more likely to be integrated

into the human genome than other HPV genotypes, thereby facilitating early tumors developments[30]. In addition, the relation between HPV16 and 18 infections and the risk of developing high-grade cervical lesions has been described[2,31,32]. In the present study, a high frequency of HPV16 (38.5%) was observed, in accordance with that described in other Peruvian areas[33]. This fact, together with the above mentioned relevance of HPV16 again highlights the need to introduce more adequate diagnostic methods. The present results also support the introduction of HPV vaccination in Peru[34]. Regarding prevalence of other genotypes strong differences were found. Thus Montano *et al.* also showed a high prevalence of other 4 HPV genotypes HPV6, 51, 53, 62 (22.7%, 20.5%, 18.2% and 15.9% respectively) [33], while in our study accounting for 1.9% each, except HPV62, which was not detected. These differences which may be explained by the aforementioned low interchange of population highlights the need to dispose of a more complete scenario of the current distribution of HPV genotypes, including that of rural areas.

The use of PCR showed a frequency of HPV of 20.5%, in the population analyzed, supporting the routine use of PCR to better determine the presence of HPV infection in endocervical samples prior to lesion development. Nonetheless, PAP screening rates are reportedly low in Peru, around of 31% in urban areas and lower in rural areas[35], and thus in order to achieve correct implementation of screening for HPV, population awareness of the severe consequence of this disease is necessary.

The age at which the first sexual relationship has taken place has a direct relationship with HPV infection. In this study, a prevalence of 30.0% and 19.8% was observed in women who had had their first intercourse when younger than 17 years and in the age group of 18-24 years, respectively. This prevalence is higher than that reported in a study of Chilean women (Santiago de Chile), in which the prevalence of HPV was 14% in this age group[17,36,37]. It is well established that the most important risk factor for acquiring HPV infection is sexual activity[38]. In accordance to our study the number of lifetime sex partners has a direct effect on the risk of HPV acquisition. In the same line, albeit not analyzed in our data, another relevant variable affecting a woman's risk of HPV infection is the number of current and previous partners of her partner[39,40]. It has also been indicated that high parity is another risk factor associated with cervical cancer[41,42]. Although our data suggest that this may be true in women with 3-4 children, no definitive conclusion may be made.

These results will be useful for correct clinical diagnosis and prevention of cervical cancer due to early detection of HPV infection. Cervical cancer can be prevented and cured at both a low cost and risk as long as screening to facilitate early detection of precursor lesions, early diagnosis and appropriate treatment and follow up is available to all sectors of society.

In summary, our results demonstrate that PCR amplification allows detection of HPV infections more efficiently than PAP. Identification of the specific HPV genotype before evident cervical histological changes occur will allow the implementation of corrective or appropriate prophylactic measures and will have a direct impact on the natural history of the disease and subsequent development of cervical malignancy.

Conflict of interest statement

We declare that we have no conflict of interest.

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