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Prevalence of Human Papillomavirus Types Among Mexican Women with Intraepithelial Lesions and Cervical Cancer: Detection with MY09/MY011 and GP5+/GP6+ Primer Systems

[1,2]Gloria Fernández-Tilapa, [2]Berenice Illades-Aguiar, [2]Dinorah Nashely Martínez-Carrillo
[1,2]Luz del Carmen Alarcón-Romero, [1,2]Amalia Vences-Velázquez, [3]Marco Antonio Terán-Porcayo
[1]Elba Reyes-Maldonado, [1]María Lilia Domínguez López and [1]Ethel García-Latorre
[1]Departamento de Inmunología, Escuela Nacional de Ciencias Biológicas
Instituto Politécnico Nacional, México, D.F.
[2]Facultad de Ciencias Químico Biológicas, Universidad Autónoma de Guerrero
Chilpancingo, Guerrero, México
[3]Instituto Estatal de Cancerología, "Dr. Arturo Beltrán Ortega", Acapulco, Guerrero, México

Abstract: Squamous cervical carcinoma (SCC) is the most common cancer found in Mexican women. Human papillomavirus (HPV) infection is a prerequisite for this disease. In Mexico little is known about the prevalence of HPV-types and knowledge of the circulating genotypes by region is limited. The aim of this study was to determine the prevalence and genotypes of HPV in biopsies from women with intraepithelial lesions and SCC. A total of 211 cervical biopsies were studied. Histopathological analysis was done and HPV DNA was detected by PCR using MY09/MY11 (MY-PCR) and GP5+/GP6+ (GP+PCR) primers. Viral type was determined by RFLP's or sequencing. Tissues were classified as low-grade squamous intraepithelial lesion (LSIL) in 123 cases, high-grade squamous intraepithelial lesion (HSL) in 40 and SCC in 48. Prevalence of HPV was 55.3% in LSIL, 65% in HSIL and 91.7% in SCC by MY-PCR. These percentages increased to 83, 77.5 and 100%, respectively, when HPV-negative samples were analyzed by GP+PCR. In 138 of 211 samples (65.4%), HPV was detected by MY-PCR and 43 (20.4%) were positive by GP+PCR. In 166 (91.7%) of 181 infections high risk HPV-types were found. Twelve genotypes were detected (16, 18, 31, 33, 45, 52, 58, 59, 61, 66, 70, 81). Prevalence of HPV 16 in LSIL, HSIL and SCC, were 70.6, 54.8 and 70.8%, respectively and it was the most common type in all cases (67.9%). A significantly higher number of positive samples were detected with MY-PCR and GP+PCR. The high prevalence of HPV infection with high-risk types, especially HPV16, among Mexican women with SIL and SCC, has important implications in the treatment and prophylaxis.

Key words: Human papillomavirus, squamous cervical carcinoma, PCR, México

INTRODUCTION

Infection with high oncogenic risk types of human papillomavirus (HPV), especially types 16 and 18, has been established as a necessary cause for the development of squamous cervical carcinoma (SCC) and its premalignant lesions (squamous intraepithelial lesions, SIL)[1-4].

Walboomers *et al*., in 1999 conducted a worldwide study reporting that 99.7% of tissues with cervical carcinoma contained HPV DNA although percentage varied from 93 to 99.7%, depending on the primers used for the PCR[1,5,6].

In Mexico, SCC is the main cause of death by cancer in women over 25 and in Guerrero State the women experience a higher mortality rates by this disease compared with women who live in other states[7,8]. However, few studies about the distribution and frequency of the HPV genotypes in SCC and their premalignant lesions exist and they have been limited to just a few of the 32 states in the country. The prevalence of genital HPV infection in the world varies from one region to another [9-12].

Some studies conducted in Mexico report finding HPV DNA in 8.8 to 94.2% of low-grade squamous intraepithelial lesions (LSIL), in 77 to 92% of high-

Corresponding Author: Dra. Ethel García-Latorre, ENCB, IPN. Carpio y Plan de Ayala, s/n, Colonia Santo Tomás, México, D.F.11340. México

grade lesions (HSIL) and in 86.6 to 97.7% of tissues with SCC. The genotype 16 was the most frequent among women with cervical cancer or HSIL from some geographical areas of Mexico, in others HPV 58 was the most common. The prevalence of HPV 51, 33, 18, 31 and 58 differs with the diagnosis in each area [9-14]. The objective of this work was to determine the prevalence of HPV types in a group of women from the State of Guerrero, México, diagnosed with SIL and SCC.

MATERIALS AND METHODS

A cross sectional study was conducted between April and December 2003. After signing informed consent, 145 biopsies were taken from women who were attended in the Instituto Estatal de Cancerología located in Acapulco, Guerrero, recently clinically diagnosed with SIL or SCC, and 66 fragments of cervical tissue, obtained by conization, from women with findings during colposcopy, which suggested infection by HPV or SIL, who had attended the Clínica de Displasia Alameda in Chilpancingo, Guerrero. One fragment of tissue was separated for virological analysis and another for histopathology confirmation.

All tissues were kept in PBS at -70 ºC**.** DNA was extracted by digestion of the tissue with 200 mg/mL of proteinase K, followed by phenol-chloroform separation and precipitation with ethanol. To determine the quality and adequacy of extracted DNA, polymerase chain reaction (PCR) that used β-globin primers PCO4/ GH20 was performed [15].

DNA of HPV was detected by PCR with MY09/MY11 primers (MY-PCR) [16]. MY-PCR was carried out in 50 µL, with 1. 5 U of DNA polymerase Ampli*Taq* Gold (Roche, Applied Biosystems), $MgCl_2$ 2 mM, 200 µM of each dNTP and 50 pmol of each primer.

Conditions of amplification were: one cycle at 95 ºC for 10 min; 40 cycles at 95 ºC for 1 min, 55 ºC for 1 min, 72 ºC for 1 min, and one extension cycle of 5 min at 72 ºC [17]. Viral type was determined by RFLP's [18].

HPV-negative samples, as determined by MY-PCR, were amplified by PCR using GP5+/GP6+ (GP+PCR) primers [17,19]. The reaction was done in 25 µL, with 1 U DNA polymerase Ampli*Taq*, $MgCl_2$ 3.4 mM, 250 µM of each dNTP and 15 pmol of each primer. The thermal cycling conditions were: 95 °C for 4 min; 30 cycles at 94 °C for 20 sec, 38 °C for 40 sec, 71 °C for 80 sec and a final extension cycle at 71 °C for 4 min [20]. The products of the GP+-PCR were purified with isopropanol at 75%, sent for sequencing with the Abi Prism BigDye Terminator v3.1 Cycle Sequencing Kit, (Applied Biosystems) and reactions were run in the ABI Prism 310 Genetic Analyzer (Applied Biosystems). The HPV type was determined by comparison with the sequences available in GenBank (BLAST server)[21].

One pg and 1 ng of pHPV6 recombinant plasmid, which contains DNA of HPV 6, were included as positive controls. A negative control containing all PCR reagents, except DNA template was included in each PCR run and a reaction tube with 1 µg of DNA from peripheral blood mononuclear cells was also added to monitor for contamination[22].

To estimate concordance between methods, 66 of 138(47.8%) HPV DNA positive by MY-PCR samples were randomly selected and tested by GP+PCR.

Statistical analysis: To evaluate concordance between methods a *Kappa* statistic was calculated. A value of *Kappa* > 0.80 indicates complete agreement between the two PCR methods and values close to 0 indicate independence.

RESULTS

Of the 211 tissues from women enrolled in this study, 123 (58.3%) were cases with LSIL, 40 (19%) with HSIL and 48 (22.7%) with SCC. In 138 of 211 samples (65.4%), HPV DNA was detected by MY-PCR and 43 (20.4%) were positive by GP+PCR. Of all cases, 181 (85.8 %) were HPV positive by one or both PCR methods and the prevalence of infection by diagnosis was of 100% for SCC, 77.5% for HSIL and 83% for LSIL. The percentages of false negative cases by MY-PCR were of 8.3, 12.5 and 27.7% in SCC, HSIL and LSIL respectively, Fig. 1.

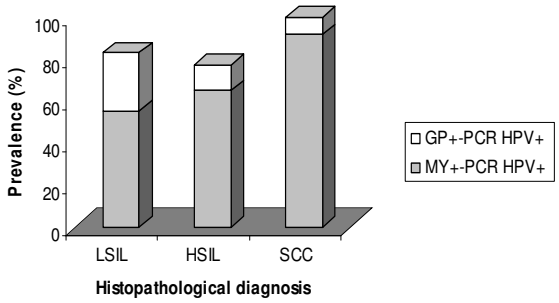

Fig. 1: Prevalence of human papillomavirus (HPV) infection among women with premalignant lesions and cervical cancer

In total, 139 samples (65.9%) were processed by MY-PCR and GP+PCR, including 66 (47.8%) of the 138 that were HPV-positive by MY-PCR, Table 1.

Table 1: HPV infection detected by MY-PCR and GP+-PCR distributed according to diagnosis

	HPV infection				
MY- PCR	Negative	Negative	Positive	Positive	
GP+ PCR	Negative	Positive	Positive	Not done	Total
	n (%)	*n* (%)	*n* (%)	*n* (%)	*n*
LSIL	21 (17)	34 (27.6)	52 (42.3)	16 (13.0)	123
HSIL	9 (22.5)	5 (12.5)	11 (27.5)	15 (37.5)	40
SCC	0 (0.0)	4 (8.3)	3 (6.3)	41 (85.4)	48
Total	30	43	66	72	211

Table 2: Prevalence of HPV types among Mexican women with LSIL, HSIL and SCC

HPV type	LSIL *n (%)*[a]	HSIL *n (%)*[a]	SCC *n (%)*[a]	Total *n (%)*[a]
Any	21 (17)	9 (22.5)	0	30 (14.2)
HPV Positive	102 (83)	31 (77.5)	48 (100)	181 (85.8)
High-risk types	98 (96.0)	23 (74.2)	45 (93.7)	166 (91.7)
Probably high-risk types	2 (1.96)	0	0	2 (1.1)
Low-risk types	1 (0.98)	4 (12.9)	0	5 (2.8)
Multiple infection	1 (0.98)	4 (12.9)	3 (6.3)	8 (4.4)
High-risk HPV				
16	72 (70.6)	17 (54.8)	34 (70.8)	123 (67.9)
18	7 (6.9)	1 (3.2)	6 (12.5)	14 (7.7)
31	5 (4.9)	3 (9.7)	3 (6.2)	11 (6.1)
33	1 (0.98)	1 (3.2)	0	2 (1.1)
45	4 (3.9)	0	0	4 (2.2.)
52	0	0	1(2.1)	1(0.6)
58	8 (7.8)	0	1 (2.1)	9 (4.9)
59	1 (0.98)	1 (3.2)	0	2 (1.1)
Probably high-risk HPV				
66	2 (1.96)	0	0	2 (1.1)
Low-risk HPV				
61	1 (0.98)	0	0	1 (0.6)
81 (CP8304)	0	3 (9.7)	0	3 (1.6)
70 (CP141 /LVX160)	0	1 (3.2)	0	1 (0.6)
Multiple infection	1 (0.98)	4 (12.9)	3 (6.3)	8 (4.4)
Total	102 (100)	31 (100)	48 (100)	181 (100)

[a] Percentage of each genotype is calculated with respect to the HPV-positive women in each diagnosis.

For MY-PCR^{positive} /GP+PCR ^{positive} agreement was of 100% (66) and for the MY-PCR^{negative} /GP+PCR^{positive} disagreement was of 58.9% (43). Overall concordance between methods, considering the 139 samples analyzed by MY-PCR and GP+PCR, the *kappa* value was of 0.4 for HPV DNA detection. In 30 cases, no viral DNA was detected by any method.

Table 2 shows the frequency of 12 genital HPV types detected in cervical specimens, 7 by both methods, (types 16,18,31,33,45,58,81), one was detected by only GP+PCR (HPV 59); 4 types (61,66,52 and 70 equivalent to CP141-7) and 8 cases of multiple infection were diagnosed by MY-PCR (data not shown). Five of the 66 DNA HPV-positives by MY-PCR and GP+PCR were typed by RFLP's and sequencing. Four showed HPV-16 and one HPV-45; agreement between tests for these samples was of 100%. Eight types (66.7%) were high-risk (types 16, 18, 31, 33, 45, 52, 58 and 59), one (8.3%) was of probably high-risk (HPV 66) and three (25%) were low-risk (61, 70 and 81). There were 181 infections, 166 (91.7%) of which were only high-risk HPV-types. Among HPV-positive women, 95.6% (173/181) had single infection and 4.4% had multiple infections. HPV DNA was detected in 43 samples only by GP+PCR, of

Table 3: Demographic characteristics of 211 women from the Guerrero State, México participating in the HPV DNA detection study

Characteristic	LSIL n=123	HSIL n=40	SCC n=48	Total n=211
	n(%)[a]	*n* (%)[a]	*n* (%)[a]	*n* (%)[a]
Age (years)				
≤ 20	5 (4.0)	2 (5.0)	0	7 (3.3)
20-29	36 (29.3)	12 (30.0)	2 (4.2)	50 (23.7)
30-39	48 (39.0)	12 (30.0)	7 (14.6)	67 (31.8)
40-49	26 (21.1)	10 (25.0)	10 (20.8)	46 (21.8)
≥ 50	8 (6.5)	4 (10.0)	29 (60.4)	41 (19.4)
Age at first intercourse (years)				
≤ 15	29 (23.6)	10 (25.0)	25 (52.0)	64 (30.3)
16-19	65 (52.8)	23 (57.5)	16 (33.3)	104 (49.3)
≥ 20	29 (23.6)	7 (17.5)	7 (14.6)	43 (20.4)
Lifetime number of sexual partners				
1	82 (66.7)	28 (70)	34 (70.8)	144 (68.2)
2	29 (23.6)	6 (15)	12 (25.0)	47 (22.3)
≥3	12 (9.8)	6 (15)	2 (4.2)	20 (9.5)
Number of live births				
0-1	46 (37.4)	16 (40)	6 (12.5)	68 (32.2)
2-3	40 (32.5)	10 (25)	6 (12.5)	56 (26.5)
≥4	37 (30.1)	14 (35)	36 (75.0)	87 (41.2)
Smokers				
No	112 (91.1)	34 (85)	45 (93.8)	191 (90.5)
Yes	11 (8.9)	6 (15)	3 (6.3)	20 (9.5)

[a] The percentage of each characteristic was calculated by diagnosis and in total women

which 42 (97.7%) had only high-risk types (data not shown). The most prevalent types were 16 and 18, with 67.9% (123) and 7.7% (14), respectively. HPV 16 was the most frequent in all groups.

The mean age was 38.6 years (range16-97 years) and more than 50% of them were less than 39 years old. The age at first intercourse was younger than 20 years for about 79% of the women in the study. For women with LSIL and HSIL the mean age at first intercourse was 18.1 years and 52% (25/48) of women with SCC had their first sexual intercourse at or before 15 years of age, mean 16.8. The majority of study participants informed one lifetime sexual partner (68.2%) and 80.6% (170) had given birth at least once. Only 9.5% (20) of the women were smokers at moment of the diagnosis, Table 3.

DISCUSSION

Knowledge of the frequency and distribution of the different genotypes of HPV in a population is an important first step toward the development of strategies for preventing cervical cancer and to make therapeutic decisions in patients with LSIL.

In our study we found a prevalence of 65.4% of HPV in all biopsies and were detected using the MY09/11 primers set but the percentage varied from 55.3 in LSIL, to 65 in HSIL and 91.7 in SCC. Nevertheless, in LSIL and SCC the percentage was higher than that recently reported by other authors, who used the same primers, but the prevalence of HPV found in HSIL was lower than that [9, 11].

By GP+PCR we detected 20.4% (43) more HPV-positive cases that appeared negative by MY-PCR, 97.6% (42) of them were high risk HPV types. No GP+PCR ^{negative}/ MY-PCR^{positive} samples were detected, which may be due to the analytic sensibility of the GP+ primers which can detect one femtogram of HPV DNA [19]. The *kappa* value *(0.4)* denotes independence between methods.

With the use of the GP+PCR system the overall HPV prevalence increased to 83% in LSIL, greater than that found in other reports[11,13,14,23], but smaller than the one reported by Carrillo and coworkers and by Illades-Aguiar [9,12] in cervical samples. These differences may be due to the method used for the detection of viral DNA and to variations in the samples used.

Twenty-one cases (17%) of LSIL and 9 (22.5%) of HSIL were HPV-negative by both methods. This may be explained by the absence of infected cells in the tissue used for the PCR. In this study, the sensibility of MY-PCR was lower in LSIL, probably because a smaller number of HPV DNA copies exist. Under the premise that HPV is a necessary cause of cervical cancer, we should expect that all true pre-neoplastic lesions contain HPV DNA. Thus, the HPV-negative SIL could represent errors of diagnosis and low sensitivity of the HPV assay or both. Our results suggest that the use of MY-PCR and GP+PCR increases sensibility to detect the HPV DNA.

HPV 16 was clearly the predominant type in all groups (67.9% of the HPV-positive specimens). The proportion LSIL of HPV-positive attributable to HPV 16 was similar to those informed by Chaouki in cervical cancer cases from Morocco and was higher than that reported by Gonzáles-Losa and Carrillo in Mexican women from other geographical areas with the same diagnosis [9,11,24]. The prevalence of HPV 16 in HSIL (76.6%) and in SCC (70.8%) exceeds that reported by Herrero and Muñoz in HPV-positive cases from other countries and was higher than that reported by Carrillo and González-Losa in Mexican women from other geographic areas[5,6,9,11,25].

In our study HPV 18 was the second most frequent HPV type in SCC and third most common type in SIL's in our population. These findings agree with those of González-Losa and Carrillo [9,11]. HPV 58 was the second most common type in LSIL's and this place was taken by HPV 31 in HSIL's . Cancer-associated HPV types were present in more than 90% of the HPV-positives LSIL's. The LSIL's harboring cancer-associated HPV types are probably the most likely to persist and progress [25].

These results suggest that in the Guerrero State, Mexico, HPV 16 plays a very important role in the etiology of cervical lesions. We detected 4.4% of cases positive for more than one viral type, most frequently in HSIL (12.9%), lower than previously reported by Muñoz and González-Losa[6,11]. Three cases of SCC (6.2%) were found to have high risk type infection in co-infection with HPV 6 and 11. The percentage of low-risk types in SCC is greater than that reported by Muñoz [6] but smaller than reported by Herrero [25].

Is important to note that 23 to 25% of women with SIL and 52% with SCC had their first intercourse between 12 and 15 years of age and this finding is in contrast with the findings of Herrero, who found that the median age at first sexual intercourse was 18 years [25]. In our population cervical lesions appear at very early ages (SIL at < 20 and SCC at< 30 years); and 100% of these were HPV-positive to high-risk genotypes. Thus knowledge of the frequency and distribution of the types of HPV in the State of Guerrero is important to make decisions about therapy.

The overall prevalence of HPV DNA in clinical materials is underestimated if only a single detection method is used. Our results agree with the results of Qu who found that there was a differential amplification sensitivity of HPV types between the MY-PCR and GP+PCR primer systems [17]. In our hands the combined use of the MY09/MY11 and GP5+/GP6+ primers systems increased the sensibility to detect of HPV DNA in cervical tissues with premalignant lesions and SCC and the MY-PCR and GP+PCR methods complemented each other.

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