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Prevalence of Human Papillomavirus in Women from the State of Michoacan, Mexico, Showed High Frequency of Unusual Virus Genotypes

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ABSTRACT

Background: Human papillomaviruses (HPVs), the leading cause of cervical cancer, are distributed worldwide, with high prevalence in developing countries. Objective: The objective of the study is to know the prevalence and genotypes of HPV in women from the state of Michoacán and the Women's Hospital in Morelia, Michoacán. **Materials and Methods:** Cervical smear samples (159,288) were subjected to HPV detection by hybrid capture 2. A subsample of 484 patients from the Women's Hospital was studied by Papanicolaou test and linear array HPV genotyping, and when positive, patients were also examined by colposcopy and histopathology. **Results:** The overall prevalence for HPV in Michoacán State was 7.74%; 7.11% in 2009, 6.46% in 2010, 9.58% in 2011, and 8.43% in 2012. The highest prevalence was found in the age groups < 25 and 25-34 years. The prevalence at the Women's Hospital was 8.51%. Cytological examination revealed normal cytology in 64.44% of samples, 26.66 % with low-grade and 8.88 % with high-grade squamous intraepithelial lesion (HSIL). However, by colposcopy, normal tissue appearance was found only in 26.66%; 51% were reclassified as low-grade squamous intraepithelial lesion, 17.77% as HSIL, and in 4.4% atrophy was observed. The most prevalent genotype in single infections was HPV59, followed by HPV51 and HPV45. Double infections occurred with the following genotypes: 52-53, 51-59, 61-67, 66-11, 16-62, 53-62, 59-CP6108, 45-66, and 45-51. Triple infections were identified as: 6-31-39, 51-59-62, 51-62-81, 54-55-59, 16-58-71, and 16-59-62. **Conclusions:** The prevalent genotype found among women from Michoacán, HPV59, was different to the rest of the country. The high prevalence of HPV59 could be due to cases imported to Michoacán by agricultural workers migrating to the USA or may be associated to ethnicity differences. Implications of this finding for immunization programs should be explored.

Key words: Human papillomavirus infection. Human papillomavirus prevalence. High-grade squamous intraepithelial lesion. Low-grade squamous intraepithelial lesion.

INTRODUCTION

Human papillomavirus (HPV) is distributed worldwide and is related to more than 99% of all cervical cancer cases[1]; it can also infect the vagina, vulva, urethra, penis, or the perianal area[2]. The association of HPV with cancer depends on viral load, genotype, and whether or not the virus episome is integrated. HPV has a double-stranded DNA genome of approximately 8000 bp and three genomic parts: long control

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region (LCR) and early and late regions. The genome segment contained from the LCR to the *E6* and *E7* genes encodes oncoproteins described as essential for HPV-induced carcinogenesis *in vivo*[3]. E6 and E7 interact with tumor suppressor proteins p53[4] and pRB[5], respectively, resulting in alteration of the DNA repair process and cell-cycle control.

More than 150 HPV genotypes have been identified and classified into two distinctly different groups, low- or high-risk, based on their association with premalignant and malignant lesions[6]. The high-risk virus genotypes are 16, 26, 30, 18, 31, 33, 34, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 69, 70, 73, and 82, while the types 6, 11, 32, 40, 42, 44, 54, 55, 61, 62, 64, 71, 72, 74, 81, 83, 84, 87, 89, and 91 are considered as low-risk[7].

In 2011, Jemal et al. reported an estimated prevalence of new cases of cervical cancer close to half a million worldwide, and the calculated percentage of deaths slightly higher than 50%[8]. Africa, South-Central Asia, and South America had the largest incidence rates, while the highest mortality rates were documented in Africa, followed by Melanesia and Central America. In 2007, de Sanjosé et al. reported a crude prevalence of 20.5% in Central America (Costa Rica, Honduras, and Mexico)[9]. Bruni et al., in 2010, analyzed eight different studies and reported a crude prevalence of 20.6% in Mexico[10]. The associated risk factors in Mexico were the lack of formal education, low socioeconomic level, unemployment, poverty, no proper health care, and the patient's age[11,12].

In 2016, Lazcano-Ponce et al. reported a prevalence of high-risk HPV of 8.6% in 12 states of Mexico, with a 7% prevalence in Michoacán State.[13] In this study, we determined HPV prevalence in 159,288 women in Michoacán State and HPV genotype in 45 samples from the Women's Hospital in Morelia, Michoacán.

MATERIALS AND METHODS

Study population

The study population consisted of 159,288 women recruited in eight sanitary districts of Michoacán, Mexico (age 19-82 years) attending regular gynecological examination, between January 2009 and December 2012. A woman was considered eligible according to the following inclusion criteria: (i) resident of Michoacán State, (ii) currently without symptoms of any sexually transmitted infection (STI) or on treatment for any STI, and (iii) not vaccinated against HPV. After patients signed an informed consent, cervical samples were obtained following the protocol approved by the Ethics Committee of the State Hospital de la Mujer (Women's Hospital), Morelia, Michoacán. The women were stratified by age in the following groups: < 25, 25-34, 35-44, 45-54, 55-64, and ≥ 65 years.

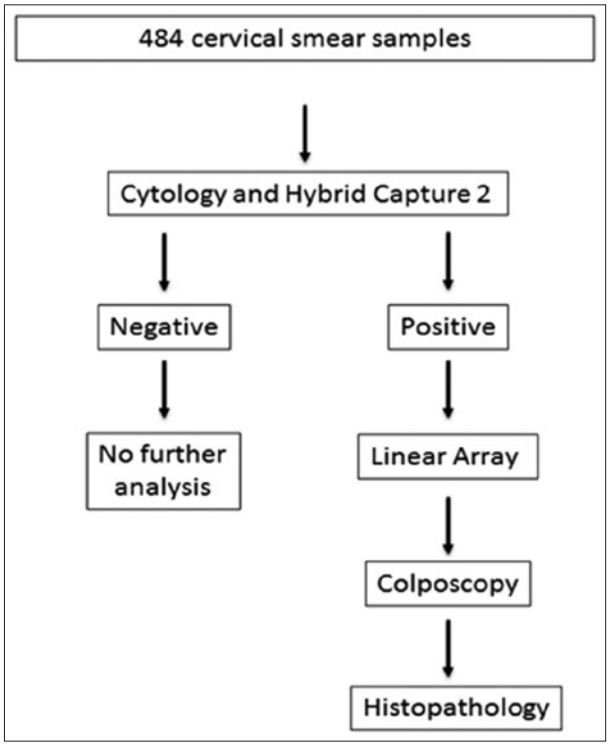
Cervical samples collection

Cervical smear samples were obtained and then cervical cytology and hybrid capture 2 analysis (HC2) were performed. Patients from the Women's Hospital additionally were submitted to colposcopy, and their samples were analyzed by histopathology and linear array analysis (Fig. 1).

HPV detection and genotyping

Detection of HPV was performed using the HC2 HPV Test (HC2 High Risk Probe, Digene, USA), which detects 13 cancer-associated HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 and includes

Figure 1. Flow diagram of the study.



a negative control (Carrier DNA, herring sperm) and calibrators A (1 pg/ml cloned HPV11 DNA) and B (1 pg/ml cloned HPV16 DNA). Plates were read in a DML200 Luminometer (Digene). Samples were considered positive if they were above the threshold of 1 pg HPV DNA/ml. Positive specimens were processed with the Linear Array HPV genotyping test following the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN) and read manually. The linear array detects 37 high- and low-risk HPV genotypes, including: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39, and CP6108, and the β-globin amplification as a positive control. The array was read manually according to the manufacturer, comparing the blue lines pattern against the reference strip.

Cervical Papanicolaou (PAP) analysis

Cervical smears were processed and cytological abnormalities were classified according to the Bethesda System diagnostic criteria.

Concurrent infections

The presence of *Gardnerella vaginalis, Candida albicans,* and *Enterococcus* spp. was determined by culturing the samples in specific media for their identification. Specific primers were used to detect the pathogens by polymerase chain reaction: *Ureaplasma* spp. (5' GAG ATA ATG ATT ATA TGT CAG GAT CA 3' and 5' GAT CCA ACT TGG ATA GGA CGG 3'), *Chlamydia trachomatis* (5' TCC GGA GCG AGT TAC GAGA 3' and 5'AAT CAT TGC CGG GGA TTG GT 3'), and *Mycoplasma hominis* (5' CAA TGG CTA ATG CTG GAT ACG C 3' and 5' GGT ACC GTC AGT CTG CAA T 3'). Human β-globin was the positive control and the following primers were used: 5' GGT TGG CCA ATC TAC TCC CCG G 3' and 5' TGG TCT CCT TAG ACC TGT CTT G 3'.

Statistical analysis

Samples were stratified according to the patient's age, and results were analyzed using the software SAS. The crude prevalence was calculated and a contingency table comparison was done. χ^2 was calculated to associate the incidence between groups and a $p < 0.0001$ was considered significant. The crude prevalence from the Women's Hospital samples was calculated, and a statistical analysis was made as described above.

Diagnostic cytology and colposcopy validity tests

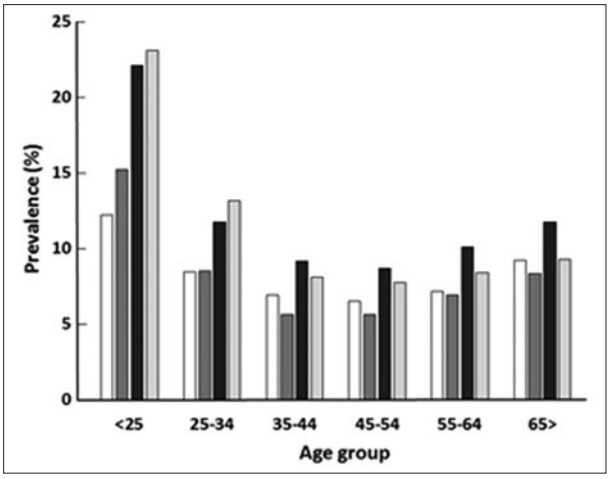
Results of cytology and colposcopy were compared with histopathology as the gold standard test for detection of lesions. For all tests, lesions were considered positive irrespective of their strength (high- or low-level). Diagnostic validity was performed with the OpenEpi v3.0, available online[14,15]. True positives, false positives, false negatives, and true negatives for cytology and colposcopy were defined against the histopathological assay.

RESULTS

Study population

During 4 years (2009-2012), 159,288 women were included from the eight sanitary districts of Michoacán State; cervical smear samples were obtained and DNA extraction was performed. The HPV overall prevalence was 7.74%; prevalence was 7.11% in 2009, decreased to 6.46% in 2010, rose to 9.58% in 2011, and dropped slightly to 8.43% in 2012. The patients in the age groups < 25 and 25-34 years showed the highest prevalence (Fig. 2). The χ^2 values were very high and $p < 0.0001$ indicating that age and locality are not independent, suggesting that in our group of study; there are external influences modifying the

Figure 2. Four-years age-specific human papillomavirus prevalence among 159,288 women in Michoacán State, México (white:2009; dark gray:2010; black:2011 and light gray:2012)..



frequencies such as access to medical services, educational level, the number of sexual partners, and the number of pregnancies and abortions.

The crude prevalence of HPV in samples from the Women's Hospital (484 women attending regular gynecological examination) was 9.29% (45/484) with the following age distribution: < 25 years, 1.03%; 25-34 years, 1.65%; 35-44 years, 3.3%; 45-54 years, 2.27%; 55-64 years, 0.61%; ≥ 65 years, 0.413%. Age was significantly associated with HPV infection. Cytological examination of the 45 positive samples revealed normal cytology in 64.44% (29/45), 26.66% (12/45) with low-grade squamous intraepithelial lesion (LSIL), and 8.88% (4/45) with high-grade squamous intraepithelial lesion (HSIL) (Table 1). To confirm these results, a colposcopy study was conducted. Only 26.66% of the samples had normal tissue appearance (12/45), while 51% were reclassified as LSIL (23/45), 17.77% as HSIL (8/45), and atrophy was observed in 4.4% (2/45). In women whose samples showed tissue alterations, a tissue biopsy was performed for histopathological examination. Within the LSIL samples, 69.56% (16/23) were classified as cervical intraepithelial neoplasia 1 (CIN 1). Among the HSIL samples, 25% (2/8) were CIN 2, 50% (4/8) were CIN 3, and one sample showed micro invasive cancer (Table 1).

To evaluate the validity of cytology and colposcopy to predict HPV infections that cause lesions, diagnostic validity tests were performed. Clearly, colposcopy showed higher values of sensitivity, negative predictive value, and diagnostic precision, and correlated better with the histopathological assay. In contrast, cytology had a high level (58.62%) of false negative results (Table 2), thus rendering a low concordance.

Table 1. HPV genotype, cytology and histopathology analysis.

Age	Genotype	Cytology	Colposcopy	Histopathology
19	6, 31, 39	HSIL	HSIL	HSIL (CIN 2)
20	52, 53	Normal	LSIL	Normal
21	45, 51	LSIL	LSIL	Normal
22	45, 51	HSIL	HSIL	Normal
22	59	LSIL	LSIL	LSIL (CIN 1)
28	59	Normal	Normal	Normal
29	59	Normal	LSIL	LSIL (CIN 1)
30	59	HSIL	LSIL	Normal
32	35	Normal	Normal	Normal
32	16, 62	LSIL	HSIL	HSIL (CIN 3)
32	66, 11	Normal	LSIL	LSIL (CIN 1)
34	51	Normal	LSIL	LSIL (CIN 1)
34	59	Normal	LSIL	LSIL (CIN 1)
35	51, 62, 81	Normal	Normal	Normal
36	51	Normal	LSIL	LSIL (CIN 1)
37	51, 59, 62	Normal	Normal	Normal
37	59, HPV CP6108	LSIL	LSIL	LSIL (CIN 1)
38	39	Normal	Normal	Normal
38	51, 59	LSIL	LSIL	LSIL (CIN 1)
38	61, 67	Normal	Normal	Normal
38	31	HSIL	HSIL	HSIL (CIN 3)
40	51	Normal	LSIL	LSIL (CIN 1)
40	53, 62	LSIL	LSIL	LSIL (CIN 1)
41	31	LSIL	LSIL	LSIL (CIN 1)
42	59	Normal	LSIL	LSIL (CIN 1)
44	45	Normal	Normal	Normal
44	59	Normal	LSIL	Normal
44	59	Normal	LSIL	LSIL (CIN 1)
44	54, 55, 59	Normal	Normal	Normal
45	51	Normal	Normal	Normal
45	45, 66	LSIL	LSIL	LSIL (CIN 1)
46	58	HSIL	HSIL	HSIL (CIN 3)
46	16, 58, 71	Normal	LSIL	LSIL (CIN 1)
46	59	Normal	LSIL	LSIL (CIN 1)
47	16, 59, 62	Normal	Normal	Normal
48	45, 66	LSIL	HSIL	Microinvasive carcinoma
49	51	Normal	Normal	Normal
52	51	LSIL	LSIL	LSIL (CIN 1)
53	67	Normal	HSIL	HSIL (CIN 3)
53	58	Normal	LSIL	LSIL (CIN 1)
55	52	Normal	LSIL	Normal
59	59	Normal	Normal	Normal
59	51	LSIL	HSIL	HSIL (CIN 2)
71	59	Normal	Normal	Normal
73	45	Normal	Normal	Normal

HSIL: high-grade squamous intraepithelial lesion;
LSIL: low-grade squamous intraepithelial lesion; CIN: cervical intraepithelial neoplasia 1; HPV: human papillomavirus

Genotyping

In the linear array HPV genotyping tests, we detected 62% single infections (28/45) and 38% (17/45) had more than one genotype. Stratification by age for single infections and coinfections, respectively, was as follows: < 25 years, 11.11 and 44.44%; 25-34 years, 19.35 and 6.45%; 35-44 years, 4.91 and 3.82%; and 45-54 years, 4.26 and 2.42%. In the intervals of 55-64 and[3] 65 years, only single infections were detected: 4.76 and 5.55%, respectively (Fig. 3).

In single infections, HPV59 was the most prevalent (39.28%), followed by HPV51 (25%); HPV31, HPV45, and HPV58 (7.14% each); and HPV35, HPV39, HPV52, and HPV67 (3.57% each). The most prevalent genotype in double infections was HPV45, detected in four samples, associated twice with 66 and with 51. The other coinfections identified were 52-53, 51-59, 61-67, 66-11, 16-62, 53-62, and 59-CP6108.

Table 2. Concordance of results of cytology and colposcopy for HPV detection.

Method	TP	FP	FN	TN	% Sensitivity (95% CI)	% Specificity (95% CI)	% PV+ (95% CI)	% PV– (95% CI)	Diagnostic precision	LRPT	LRNT	Cohen's kappa
Cytology	13	3	12	17	52 (33.5-69.97)	85 (63.96-94.76)	81.25 (56.99-93.41)	58.62 (40.74-74.49)	66.67 (52.07-78.64)	3.467 (1.569-7.658)	0.564 (0.469-0.678)	0.3541 (0.08472-0.6234)
Colposcopy	25	6	0	14	100 (86.68-100.0)	70 (48.1-85.45)	80.65 (63.72-90.8)	100 (78.47-100.0)	86.57 (73.82-93.74)	3.333 (2.404-4.621)	0.0	0.7212 (0.441-1.002)

Fn: false negatives; Fp: false positives; LRNT: likelihood ratio of negative test; LRPT: likelihood ratio of positive test; PV+: positive predictive value; PV–: negative predictive value; Tn: true negatives; Tp: true positives, HPV: human papillomaviruses; CI: confidence interval.

In triple infections, the detected genotypes were 6-31-39, 51-59-62, 51-62-81, 54-55-59, 16-58-71, and 16-59-62 (Fig. 4).

Coinfections were more frequent in the age groups < 25 and 25-34 years, representing 44.44% and 6.45%, respectively. Low-risk HPVs were always detected in association with a high-risk HPV. An interesting finding was that among triple infections; the histopathology revealed normal tissue in four cases: LSIL in one, and HSIL in another.

Concurrent infections

Even though we identified in some samples the following pathogens: *G. vaginalis, C. albicans, U. urealyticum, M. hominis, C. trachomatis, Enterococcus* spp., alone or

Figure 3. Age-specific prevalence of human papillomavirus mono- or coinfections in 484 women.

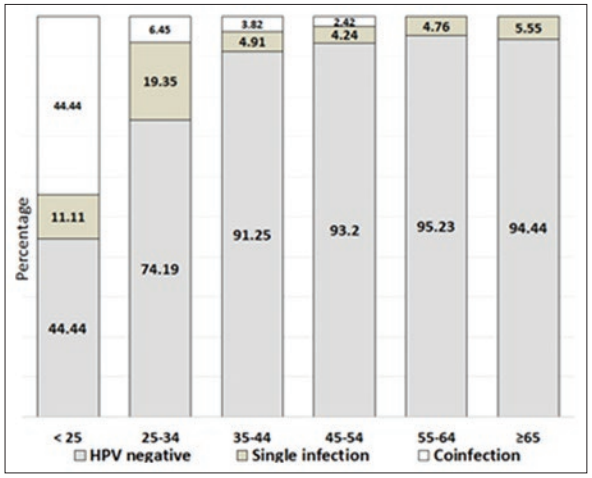
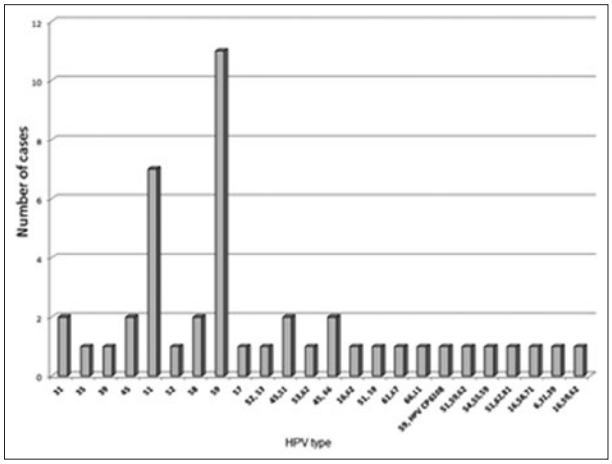


Figure 4. Human papillomavirus type prevalence among women in the Women's Hospital, Morelia, Michoacán, Mexico.



some in double infections (*G. vaginalis* + *C. albicans, G. vaginalis* + *U. urealyticum, G. vaginalis* + *C. albicans* + *U. urealyticum*), we found no correlation with severity of the viral infection.

DISCUSSION

The worldwide prevalence rate of HPV is higher in less-developed regions. Bruni et al. reported a crude prevalence of 20.6% in Central America (Mexico, Honduras, Guatemala, Costa Rica, Belize) in women with normal cytology. However, we found an overall prevalence of 7.74 in the state of Michoacán, Mexico.

Cervical cancer is the second most common cancer among Mexican women[16]. The traditional routine method to evaluate cervical tissue is PAP smear since 1941 and it has been very useful to detect cell abnormalities and reduce mortality rates. However, in our study, we found that 64.44% of the samples analyzed by PAP were classified as without lesions. After colposcopy study, 51% of samples were reclassified as LSIL. Granados-García et al.[17] found false negative PAPs in 42.6% of samples, while we found 26.66% of false negatives (Table 2). Altogether, these results suggest that colposcopy is a better assay than cytology to predict HPV infections causing lesions in the histopathology assay. Cytology was not as good as colposcopy because of its high level of false negative results as compared to histopathology, thus rendering a low concordance.

Despite these data, it is clear that the PAP coverage in national programs has reduced the mortality of cervical cancer.[18] Moreover, it was reported that PAP combined with HPV test increases the capability to identify cervical cancer[13,19,20].

In 2014, Salcedo et al. analyzed 2,956 samples and found an overall prevalence of HPV of 67.1% and 40 different HPV genotypes[21]; HPV16 was the most prevalent (39.4%), followed by HPV18 (7.5 5%), HPV31 (7.1%), HPV59 (4.9%), and HPV58 (3.2%). On the other hand, Aguilar-Lemarroy et al., in an analysis of 822 samples, found HPV16 to be the most prevalent type in all the studied groups, as in the study by Salcedo et al.[21], but also identified HPV18, HPV45, HPV52, HPV58, and HPV39 types[22].

All the works mentioned above clearly showed a higher prevalence of types 16, 18, 31, 33, 35, 45, 52, 58, and 59. In contrast, we found the highest prevalence for type 59 (Fig. 4), followed by 51, 45, 31, 58, 35, 39, 52, and 67. The HC2 assay does not detect HPV53, HPV61, or HPV67 although the manufacturer reports that there is cross-reaction with HPV53 and HPV61. In addition, Castle et al. found cross-reaction with HPV67[23]. These facts could explain why we identified positive samples with types not included in the HC2 test and that were later detected by linear arrays. The high prevalence observed for HPV59 could be due to cases imported to Michoacán State since a large number of men have migrated as agricultural workers to the USA. Thus, if poor sex education prevails and migrant men have unprotected intercourse abroad, they may be importing new genotypes on their return. We observed the highest prevalence of HPV infections in young women < 25 and 25-34 years old, which probably corresponds to the more sexually active women. It should be noted that HPV16 was only found in three coinfections and HPV18 was not found at all. Aguilar-Lemarroy et al. also found HPV16 more frequently, associated in coinfection with types 18, 39, and 70 in samples from cervical cancer[22]. Remarkably, none of the samples in our study contained any of the high-risk HPV types, including single, double, and triple infections.

In San Luis Potosí, 700 women who underwent colposcopy presented type 33 as the most prevalent followed by types 16, 18, and 51[24]. These results contrast with our findings since HPV51 was the second most common genotype.

Flores-Miramontes et al.[25] found that types 16 and 51 had the highest prevalence among women without cervical lesions; in contrast, we observed that HPV59 was more frequent in this type of sample. They also detected HPV16 as the most prevalent in women with CIN 1.

González-López et al.[26], in a retrospective analysis, found that CIN 1 was more frequent in women aged 35-44 years old, and CIN 3 in women aged 45-59. We found that CIN 1 was more frequent in women aged 25-34 and 35-44 years, and CIN 2 in women aged 45-64 years.

One unexpected result in our study was the observation by histopathology of normal tissue in women coinfected with three types, either high-high-low risk, or low-low-high combinations. This may be related to

the major histocompatibility complex (MHC)[27-29] and health of the patient, the homeostasis of the immune system, or the historical evolution of the infection, which we did not explore in this study.

On the other hand, there were 51% of LSIL, similar to the proportion previously reported. However, regarding the samples classified as HSIL, the proportion we found was lower than that previously published. This could be due to the fact that those studies were performed in women diagnosed first with intraepithelial lesions or squamous cell carcinoma and CIN or invasive carcinoma[30].

The HPV vaccines used initially were designed to protect against types 16-18 or 6-11-16-18. A study carried out in Mexican and some foreign women immunized with the quadrivalent vaccine showed effective protection against the genotypes included in the vaccine and also prevented against CIN grade 2/3, adenocarcinoma *in situ*, condyloma, and vaginal intraepithelial neoplasia[31]. However, the efficacy of this vaccine should be determined in long-term evaluation programs. Skinner et al. in 2016 studied the progression of HPV infection to detectable CIN in 2838 women and followed the persistent infections, finding that women infected with types 16, 31, 33, and 45 had a higher risk to develop CIN 2[32]. Salmeron et al. are performing a study in Mexico that will allow a more objective estimate of the efficacy of cervical cancer prevention programs and to establish the number of doses required for an effective protection[33].

In our study, the genotype found to be prevalent in Michoacán was different to the rest of the country. Moreover, none of the HPV genotypes that we identified more frequently are included in the current vaccines. Thus, it is critical to consider in the seroprevalence studies, the differences according to race, geographical location, and ethnicity[34]. In addition, it seems that the 9vHPV vaccine does not prevent infection or disease caused by genotypes different to those included in the vaccine[35].

In view of the variations in genotypes found in Mexico, there is a need to obtain a complete map of prevalence in our country and strengthen health education programs and counseling forums for women to provide more information about HPV, its transmission, and vaccination[36,37]. In addition to defining the efficacy of vaccines against HPV genotypes different to those included in the vaccine, the serum antibody response to vaccine HPV genotypes should be analyzed in future studies to determine antibody neutralization capabilities against genotypes not included in the vaccine preparations in immunized women. Furthermore, T-cell responses should be evaluated and genotyping of MHC molecules should be performed in patients since the current Mexican population is mostly of Mestizo ethnicity, mixed American Indian, and European descendent.

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