Invest Clin 54(4): 392 - 405, 2013

Amplification of human papillomavirus early genes for detection of nine genotypes in Venezuelan women.

Elvia Michelli[1,2], Luis Téllez[1], José-Andrés Mendoza[1], María-Eugenia Noguera[3], Melisse Milano[4], Reauben Vera[1] and Diana Callejas[5].

[1]Laboratorio de Microbiología y Salud Pública del Estado Mérida, Universidad de Los Andes, Mérida.
[2]Escuela de Ciencias, Universidad de Oriente, Cumaná.
[3]Dpto. de Obstetricia y Ginecología, IAHULA, [4]Dpto. de Anatomía Patológica, Facultad de Medicina, Universidad de Los Andes, Mérida.
[5]Instituto de Investigaciones Clínicas "Dr. Américo Negrette", Laboratorio Regional de Referencia Virológica, Universidad del Zulia. Maracaibo, Venezuela.

Keywords: HPV, genotypes, cervical cancer.

Abstract. Genotyping of human papillomavirus (HPV) by molecular methods may enhance assessment information for screening and following of cervical infection. In this study, cervical samples were obtained from 250 women, along with colposcopic and cytological evaluations. A Nested-PCR-Multiplex assay was used for HPV detection and genotyping for HPV E6/E7 early regions. Infection with HPV was detected in 26.0% of the samples, with 98.46% positive for at least one genotype. High-risk HPVs were identified in 98.44%. HPV18 infection was detected in 76.92% of samples and HPV16 in 36.92%, whether as individual or as multiple infections. These infections were seen more frequently in women under 35 years of age (64.7%). The Pap-smear examination showed that 16.92% (11/65) of the samples had cervical changes suggesting HPV infection, whereas the colposcopic evaluation was suggestive of HPV infection in 47.69% (31/65) of DNA-HPV positive samples. There was a high frequency of high-risk HPV genotypes, particularly HPV18, alone or in multiple-type infections. Colposcopy findings showed to have a high predictive value for the diagnosis of HPV infection. The results reflect that over 50% of HPV-positive patients had a normal colposcopy and/or cytology, highlighting the importance of including HPV testing along with genotype identification in routine gynecological evaluations.

Corresponding authors: Elvia Michelli. Universidad de Los Andes, Laboratorio de Microbiología y Salud Pública del Estado Mérida, Av. Las Américas, Mérida, Venezuela. E-mail: elviamichelli@yahoo.com

Amplificación de genes tempranos del virus del papiloma humano para la detección de nueve genotipos en mujeres venezolanas.

Invest Clin 2013; 54(4): 392 - 405

Palabras clave: VP H; genotipos; cáncer cervical.

Resumen. La genotipificación de virus del papiloma humano (VPH) por métodos moleculares puede proveer información valiosa para el monitoreo y seguimiento de la infección cervical. Se estudiaron muestras cervicales obtenidas a partir de 250 mujeres, en quienes se realizó, simultáneamente, evaluación citológica y colposcópica. Un ensayo de PCR, en formato Nested-múltiple para las amplificación de la región temparana E6/E7, fue realizado para la detección y genotipificación viral. La infección por VPH se detectó en 26,0% de las muestras, de las cuales, un 98,46% fue positivo para al menos uno de los genotipos probados; los VPH de alto riesgo se identificaron en un 98,44%. Los genotipos más frecuentes fueron VPH18 con un 76,92%, y VPH16 con un 36,92%, ya sea como infecciones individuales o múltiples. En cuanto a la edad, estas infecciones fueron más frecuentes en mujeres menores de 35 años, con un 64,7%. Los resultados citológicos mostraron que 16,92% (11/65) de las muestras cervicales tenían cambios sugestivos de infección por VPH; mientras que la evaluación colposcópica fue sugestiva de la infección por VPH en 47,69% (31/65) de las muestras ADN-VPH positivas. Se determinó una elevada frecuencia de los genotipos de VPH de alto riesgo, particularmente VPH18, solo o en infecciones múltiples. Los hallazgos colposcópicos mostraron un elevado valor predictivo para el diagnóstico de la infección por VPH. Los resultados reflejan que más del 50% de las pacientes VPH positivas tenían colposcopía y/o citología normal, evidenciando la importancia de incluir las pruebas de detección e identificación de VPH en la evaluación ginecológica de rutina.

Recibido: 10-12-2012 Aceptado: 17-10-2013

INTRODUCTION

Cervical carcinoma (CC) represents the second worldwide most frequent cancer in women, being the leading cause of cancer deaths among women in Latin America and the Caribbean (1). In Venezuela, CC is the second cause of mortality in women whose ages vary from 15 to 75 years old (2). In Mérida state, western Venezuela, morbidity and mortality frequency of CC has shown to be as high as in the rest of the country (3, 4). It is very well established today that infection with specific high-risk HPV genotypes are necessary, but not sufficient, to virtually cause all CC, and to be most likely responsible for other anogenital neoplasm and oral squamous cell carcinomas; thus, high-risk HPV genotypes emerge as one of the most important infectious carcinogens in humans (5).

Clinical trials based on the natural history of human papillomavirus (HPV) infection have demonstrated that most infec-

tions are usually asymptomatic and spontaneously cleared by the immune system, or produce transient minor lesions (6, 7). Nevertheless, a little fraction of untreated viral infections may persist and progress to different stages of what it's known as cervical intraepithelial neoplasia (CIN), a cancer precursor. Without intervention, a considerable fraction of women with CIN 3 can develop cancer, in about one to two decades after the initial infection. The DNA from high-risk HPV genotypes can be detected in virtually all cases of invasive cancer (8, 9). Therefore, women infected with high-risk HPV have a higher risk for the development of CC than those infected with low-risk HPV or women not infected (6, 10).

CC screening based on the Papanicolaou (Pap) smear test has been credited with a significant reduction in the incidence of this disease (10). Nevertheless, studies performed up to date, have concluded that cervical cytology is moderately accurate and that the average sensitivity to detect CC or precancerous lesions was considerably lower than generally believed; in addition, its sensitivity has shown to be highly variable among different countries (11).

Epidemiological and clinical studies had detected high-risk HPV between 95.0-99.0% of CC, which 70.0% of them are due to HPV16 and HPV18, representing major risk factors related with the progression of cervical lesion to CIN and/or CC (8, 12). Considering the central role played by infection with high-risk HPV genotypes in the etiology of CC, DNA testing for identification of these genotypes has been introduced for early diagnosis (13). Because HPV testing in combination with cytological evaluation, has proven to have higher sensitivity than cytological test alone for detection of CIN 3 and CC, the International Agency for Research on Cancer considered an acceptable alternative to Pap smears/cervical cytology for CC screening (14).

HPV genotyping is also a powerful tool as a primary evaluation test for viral infection and determination of carcinogenic HPV genotypes persistence, which could be used for specific persistent high-risk-HPV infection (13,15). Moreover, HPV genotyping is needed to predict how prophylactic vaccination of young women could affect the secondary prevention of cervical cancer (16). The aim of this study was to detect and genotype HPV, from cervical samples of Venezuelan women, along with the analysis of colposcopy and cytology examination.

MATERIALS AND METHODS

Study population

Cervical samples were obtained from 250 sexually active women (15 to 69 years-old), who attended a gynecology outpatient clinic at the Department of Obstetrics & Gynecology (Gyn/OB)-Los Andes University Hospital Autonomic Institute (IAHULA) in Mérida State, western Venezuela, between August 2008 and August 2011.

For the development of the research, randomly selected women were included, attending gynecology when undergoing routine screening. Likewise, the study excluded all women who, by the time of the interview and sampling, had any genital bleeding because of normal menstruation, metrorrhagia or menorrhagia, and women who showed changes in the cervix caused by surgical procedures that prevented proper sampling. Individuals and parent/guardian consent were obtained from each participant following the Helsinki and WHO ethics principles in human research (17). This study was also conducted in compliance with the local institutional ethical board.

Practitioners were instructed to obtain the sample from the cervix transformation zoneusing a DNA collection device (Digene® Corporation, Gaithersburg, MD,

USA). Samples were stored at 4°C and transported to the Microbiology and Public Health Laboratory, University of Los Andes of Mérida, Venezuela (ULA) within the same day of sample collection. Simultaneously, Pap-smear sampling and colposcopy were performed.

Colposcopy

All study participants underwent a colposcopic examination of the cervix, vagina, and vulva by the Gyn/OB specialists. Practitioners examined the cervix by inserting an unlubricated bivalve vaginal speculum, with the help of a halogen focus lamp; immediately, lugol iodine solution was applied to the cervix under direct vision. Lesions in the transformation zone (TZ) were assessed by applying 5.0% acetic acid and iodine solution under ×8 to ×12 magnification. An international nomenclature (IFCPC) was used to classify the colposcopic patterns (18).

Cytology

Pap-smear analysis of cervical cells was carried out by medical pathologists (Department of Pathology, Medicine School, ULA), adopting the conventional Bethesda terminology/classification (19, 20). Negative and benign changes were kept as originally categorized. Atypical changes were classified as atypical squamous cells of undetermined significance (ASC-US) and atypical squamous cells, cannot exclude HSIL (ASC-H). Mild dysplasia was classified as low-grade intraepithelial lesions (LSIL) of squamous or glandular type; moderate to severe dysplasia were classified as high-grade intraepithelial lesions (HSIL) of the squamous or glandular type.

DNA-HPV Specimen processing

Cells kept in the DNA collection device were centrifuged and two aliquots of 700 μL each were used for DNA isolation in silicon-based column technology using the QIAamp DNA Mini Kit (QIAGEN®, Hilden, Germany). Purified DNA was eluted in 100 μL of buffer in accordance with the manufacturer recommendations. DNA-quantification was performed at 260nm wavelength (UV1101/1101T, Biotech, Cambridge, UK) and purified DNA was stored at –20°C until processing.

Amplification of early genome regions (E6/E7) by PCR

DNA extraction processes and all pre- and post-PCR procedures were carried out in separate rooms and cabinets under the most stringent conditions. Buffer and blank controls were always included for the extraction protocol to obtain sufficient number of negative controls in order to monitor contamination. 100 ng of DNA were used for each PCR assay in a final volume of 25 μL.

A Nested-PCR-Multiplex assay was used for amplification of conserved early region from HPV genome. The first PCR amplified a segment of a 630bp-length of E6/E7 gene. These regions were amplified using the consensus sequences GP-E6 3F/5B/6B (21), and 10 pmol of primers PC04/GH20, with an initial denaturation step of 15 min at 95°C, followed by 40 cycles (1 min of denaturation at 94°C, annealing at 55°C/1 min and an extension step at 72°C/1 min) with a final extension step of 72°C/10 min program. A commercially available positive control was used in each PCR assay (HPV-4011, Maxim Biotech, USA).

In the second PCR, which allows the genotyping of HPV in a multiplex format, 1 microliter of the first PCR product was used as template. Amplicon sizes varied from 151 to 457bp for HPV16-18-31-45 (Coctail I) and 33-6/11-58-52 and 56 (Coctail II) (21). Commercially available positive controls were used in each PCR assay: HPV-C001 (HPV16/18), HPV-4011

(HPV18), HPV-4009-33 (HPV33) HPV-4012-11 (HPV11) (Maxim Biotech, USA). These reactions were performed in a program of 35 cycles (94°C/30 second, 56° C/30 second and 72°C/45 second) with a final extension step of 72°C/4 min; adapted after Sotlar *et al*. (21).

All PCRs were performed on an ABI 2400 instrument (Applied Biosystems) and amplification products were visualized on 2.0% agarose gels containing 10 μL of ethidium bromide/100 mL agarose, under UV light (UV transilluminator, Vilber Lourmat, France).

Statistical analysis

Patient information was collected in a formulary designed for this objective. Data base and statistical analyses were performed using EPI Info 2008, version 3.5.1. A descriptive analysis of the variables was firstly carried out. The distribution of cervical results was determined by evaluating the proportion of normal and abnormal results. Chi-Squared test and P value were used to assess HPV DNA infection rate in each age group and to establish relationships between HPV DNA infection and colposcopic and/or cytological atypia. Any difference was considered statistically significant when $P<0.05$.

RESULTS

Cervical samples from 250 women, between 15 and 69 years of age (the median age of women was 33.74 years with standard deviation of 10.45 years), were tested for HPV infection. Viral detection was performed by PCR assay for E6/E7 regions. HPV genotypes were identified by a Nested-PCR-Multiplex assay for amplification of early viral genome region; in Fig. 1 is showed PCR amplification products of HPV E6/E7 region, then visualized on 2.0% agarose gels. Taken together, 26.0% (65/250) of samples was positive for HPV; from which, 98.46% (64/65) were positive for at least one of the genotypes assayed. The distribution of the HPV genotypes in the 65 HPV positive samples is summarized in Fig. 2: HPV18 (50/64) along with HPV16 (24/64) were the most frequent genotypes found in this study; 1 sample was negative when evaluating genotypes. It should be noted that the high-risk HPV genotypes were identified in 98.44% (63/64) positive samples.

Twenty seven patients (41.53%) were infected with one single HPV genotype, while 56.92% (37/65) showed multiple infection with two or more HPV genotypes, being the combination of HPV18/6/11 (14/65) the most frequent. Fig. 3 shows the distribution of HPV infection by groups of age; the prevalence of HPV infections was higher (60.0%), in women under 35 years old than in older women participating in the present study; statistical analysis did not show any significant association between the age of patients and HPV detection ($p=0.2635$; square-Chi Test).

Table I shows the varying degrees of dysplasia and the histological findings during colposcopy examination versus the HPV detection and genotyping. Colposcopy evaluation was suggestive of HPV infection in 47.69% (31/65) positive samples by HPV testing, while the remaining 52.31% HPV positive samples showed normal colposcopic findings. HPV18 was the most frequent genotype identified in women with positive colposcopy, both as individual (9/27) and multiple infection (12/37). There was not any significant association between Schiller test and the most frequent HPV genotypes: HPV18 ($p=0.8182$; square-Chi Test) and HPV16 ($p=0.4080$; square-Chi Test).

8.0% (20/250) of Pap smears showed cytological changes suggesting HPV infection, from which 95.0% (19/20) was de-

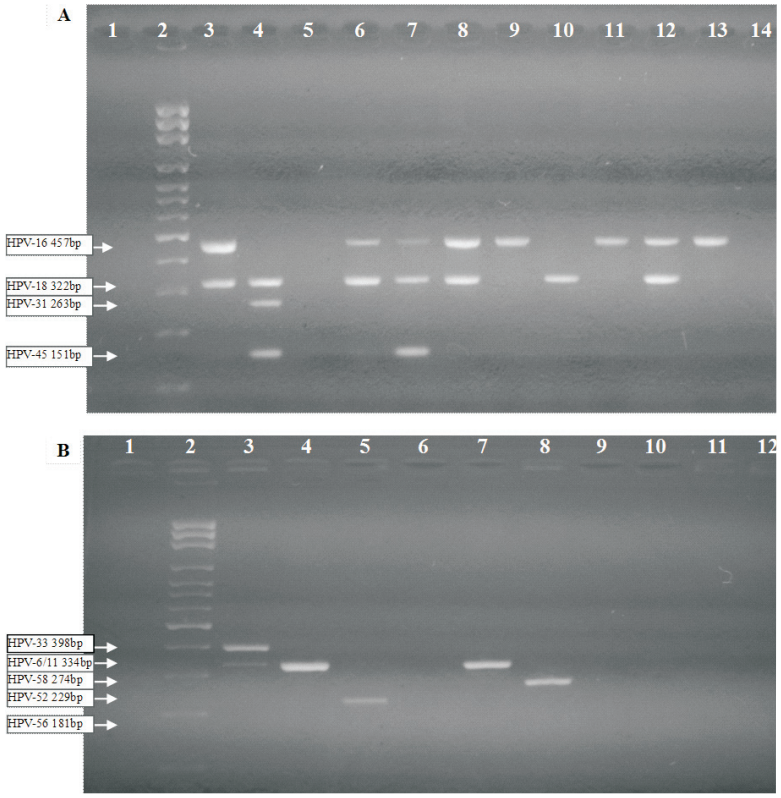

Fig. 1. Nested-PCR-multiplex assay: DNA from cervical samples was amplified by PCR [see methods]. [A]: cocktail 1 [HPV16, 18, 31, 45]. [B]: cocktail 2 [HPV 6/11, 33, 52, 56, 58]. [1] negative control. [2] molecular weight ladder. [3a] generic positive control [HPV-c001 mb], amplicon of 457bp; HPV16 positive control [HPV-4009-11-18 mb]; amplicon of 322bp; HPV18 internal positive control.[3b] positive control HPV-4012-33 mb, amplicon of 398bp, HPV33 positive control [HPV-4009-11 mb], amplicon of 334bp, HPV11 internal positive control. [4-14]: HPV positive and negative samples.

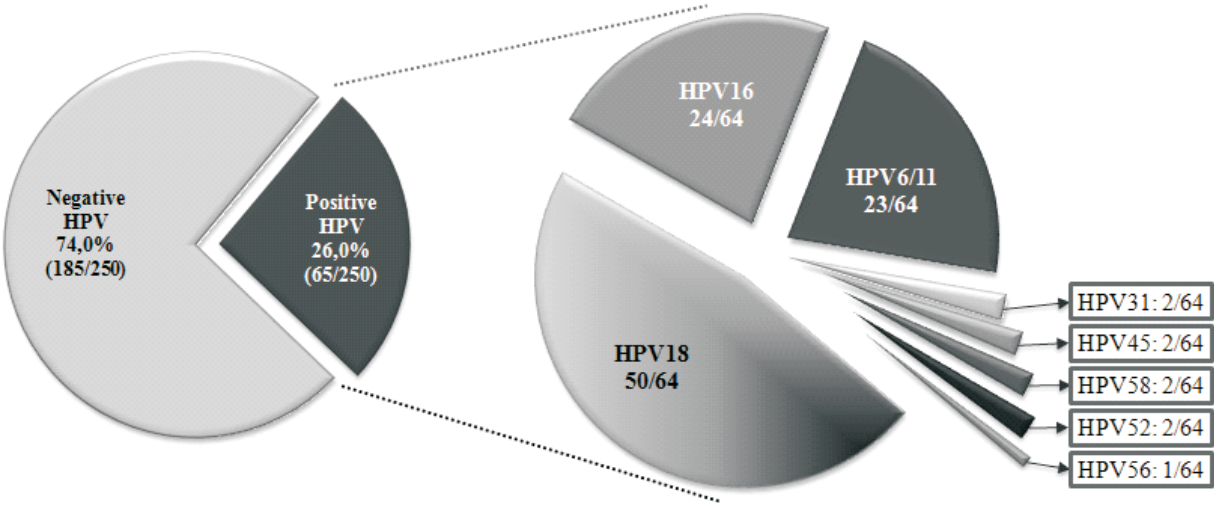

Fig. 2. Distribution of HPV genotypes identified from cervical samples of women attending the gyn/ob out patient public clinic [Los Andes University Hospital], Merida state, Venezuela.

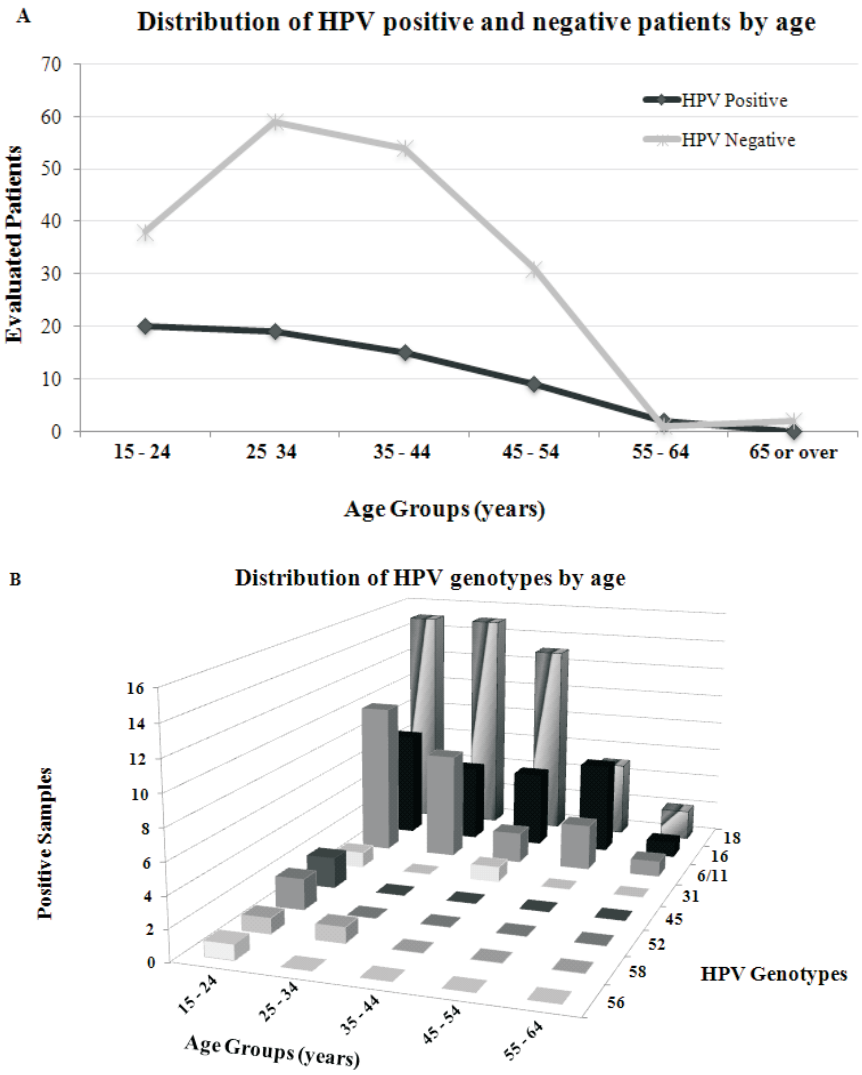

Fig. 3. Distribution of HPV infection by age: HPV infection detection [A] and HPV genotypes [B] by age groups of 10 years either one, from cervical samples of women attending the Gyn/Ob out patient public clinic [Los Andes University Hospital], Merida state, Venezuela.

scribed as LSIL. In samples from patients positive for HPV DNA, Pap-smear examination showed that 10 patients had LSIL (Table II), from which 7 were positive for HPV18. Statistical analysis did not show any significant association between the identification of HPV18 and the cytological findings associated to HPV infection ($p=0.3598$; square-Chi Test). Overall, colposcopy findings showed the best predicted value for diagnosis of HPV infection compared to Pap-smear examination.

DISCUSSION

This study was designed to investigate the presence of HPV infection and to determine the respective genotype from cervix with or without lesions in women attending an outpatient clinic in Mérida, western Venezuela. The overall percentage of HPV positivity was 26.0%, higher than that found in the general population, which has been estimated between 9.0 and 13.0% worldwide (8). Regarding other reports from

TABLE I
CORRELATION BETWEEN RESULTS OF THE COLPOSCOPIC DIAGNOSIS AND HPV GENOTYPES IDENTIFIED IN CERVICAL SAMPLES OF WOMEN ATTENDING THE GYN/OB OUTPATIENT PUBLIC CLINIC [LOS ANDES UNIVERSITY HOSPITAL], MERIDA STATE, VENEZUELA

HPV diagnosis by Molecular Biology∞ (n/%)	Colposcopic changes suggesting of HPV infection		
	Schiller Test Positive (n/%)	Schiller Test Negative (n/%)	Total (n/%)
	31 (47.69)	34 (52.31)	65 (100.0)
HPV genotypes identified in individual infections (n/%)			27/65 (41.53)
HPV18	9 (52.94)	8 (47.06)	17 (100.0)
HPV16	5 (62.5)	3 (37.5)	8 (100.0)
HPV52	1 (100.0)	-	1 (100.0)
HPV58	1 (100.0)	-	1 (100.0)
HPV genotypes identified in multiple infections (n/%)			37/65 (56.92)
HPV18/6/11	5 (35.71)	9 (64.29)	14 (100.0)
HPV16/18	2 (22.2)	7 (77.8)	9 (100.0)
HPV16/18/6/11	1 (25.0)	3 (75.0)	4 (100.0)
HPV16/6/11	1 (50.0)	1 (50.0)	2 (100.0)
HPV18/31	2 (100.0)	-	2 (100.0)
HPV18/52	-	1 (100.0)	1 (100.0)
HPV18/58	-	1 (100.0)	1 (100.0)
HPV56/6/11	1 (100.0)	-	1 (100.0)
HPV18/45/6/11	1 (100.0)	-	1 (100.0)
HPV18/16/45	1 (100.0)	-	1 (100.0)
HPV6/11	1 (100.0)	-	1 (100.0)
HPV No typed	-	1 (100.0)	1/65 (1.54)

∞HPV diagnosis by PCR-E6/E7 (GP E6/E7).

Venezuela, the findings of this study are consistent with that conducted by Reigosa *et al*. (22) in Carabobo state, in which 58 asymptomatic women for cervical pathology studied, 35.50% were HPV positive. However, our results differ from reports of Caracas (23-25) and Sucre (26), in women with diagnosis of impaired cervical cytology or histopathology, which showed higher percentages (between 43.0-98.70%) of HPV infection prevalence.

The high percentage of HPV positive samples found in our study may be due to the nature of genital HPV infections, which are mainly transmitted through sex, an event that promotes its high transmission in sexually active women and men. It must be considered that most of these infections are transient, and therefore could be eliminated by the immune system in a relatively short period of time without apparent clinical consequences (5).

TABLE II
CORRELATION BETWEEN THE RESULTS OF THE CYTOLOGICAL DIAGNOSIS AND HPV GENOTYPES IDENTIFIED IN CERVICAL SAMPLES OF WOMEN ATTENDING THE GYN/OB OUTPATIENT PUBLIC CLINIC [LOS ANDES UNIVERSITY HOSPITAL], MERIDA STATE, VENEZUELA

HPV diagnosis by Molecular Biology∞ (n/%)	Cytological changes not suggesting of HPV infection (n/%)	Cytological changes suggesting of HPV infection (n/%)		Unsatisfactory smears (n/%)	Total (n/%)
		ASC-H↿	LSIL¥		
	53 (81.54)	1 (1.54)	10 (15.39)	1 (1.54)	65 (100.0)
HPV genotypes identified in individual infections (n/%)					27/65 (41.53)
HPV18	14 (82.35)	-	2 (11.67)	1 (5.88)	17 (100.0)
HPV16	6 (75.0)	-	2 (25.0)	-	8 (100.0)
HPV58	1 (100.0)	-	-	-	1 (100.0)
HPV52	1 (100.0)	-	-	-	1 (100.0)
HPV genotypes identified in multiple infections (n/%)					37/65 (56.92)
HPV18/6/11	12 (85.71)	-	2 (22.22)	-	14 (100.0)
HPV16/18	9 (100.0)	-	-	-	9 (100.0)
HPV16/18/6/11	4 (100.0)	-	-	-	4 (100.0)
HPV16/6/11	2 (100.0)	-	-	-	2 (100.0)
HPV18/31	-	-	2 (100.0)	-	2 (100.0)
HPV18/52	1 (100.0)	-	-	-	1 (100.0)
tHPV18/58	-	-	1 (100.0)	-	1 (100.0)
HPV56/6/11	-	-	1 (100.0)	-	1 (100.0)
HPV18/45/6/11	1 (100.0)	-	-	-	1 (100.0)
HPV18/16/45	1 (100.0)	-	-	-	1 (100.0)
HPV6/11	-	1 (100.0)	-	-	1 (100.0)
HPV No typed Genotype	1 (100.0)	-	-	-	1/65 (1.54)

∞HPV diagnosis by PCR-E6/E7 (GP E6/E7). ↿ASC-H: Atypical Squamous Cells cannot exclude High-grade Intraepithelial Lesion (HSIL); ¥LSIL: Low-grade Intraepithelial Lesion.

It is important to emphasize the elevated frequency of high-risk HPV genotypes identified in this study, mainly HPV18 and/or HPV16. This distribution is consistent with previously published data in Merida, Venezuela, in which the predominance of HPV18 infections over other genotypes was evidenced (3); similarly, two recent studies developed in samples from patients diagnosed with cervical cancer have reported higher frequency of HPV18 over other genotypes identified (27, 28). On the other hand, the results of this study differ from reports previously published around the world, in which HPV16 has been consistently more identified. This variation can be explained by the fact that HPV genotypes reported in different regions worldwide may vary both in type and relative incidence (15, 29-31).

Investigations have shown that HPV16 and HPV18 infections preferentially progress to CC more readily than those due to other high-risk genotypes (8-9). Thereby, HPV testing have demonstrated to be a strong predictor of risk in the development of cervical neoplasia, supporting the hypothesis by which HPV testing effectively stratifies patients according to cancer risk (32). Moreover, HPV16 and 18 have been the most frequent genotypes identified in cervical smears from women harboring CC (8).

Among HPV-positive samples identified in this study, multiple high-risk genotype-infections were more frequent than single infections, showing the relevance of HPV genotype testing in patients with a positive report for HPV detection. Multiple HPV infection increases the probability of harboring a highly oncogenic HPV genotype (7). These co-infections seem to have a higher occurrence than expected by chance, suggesting a higher likelyhood of synergy amongst the co-infecting HPV genotypes (33).

HPV16/18 was the second more frequent combination in the present investigation. Previous publications concerning the etiologic role to HPV genotypes in neoplasic lesions and CC, have showed an elevated risk of progression of incident HPV16/18 infections to CIN and then to CC (34, 35). Similarly, case-control studies have yielded more important relative risks of invasive CC of 100 to 500 among women with these genotypes (8).

Epidemiologic studies have shown that sexually active young women at early ages are at greater risk of acquiring HPV (36, 37). The present study showed a prevalence of HPV infections higher in women whose ages were less than 35 years than in older women. This is a very relevant finding considering that HPV16 and HPV18 positive invasive cancers are more prevalent in younger women than invasive cancers related to other carcinogenic non-HPV16/18 genotypes. Even if HPV infections are very common among young women, and although they usually clear within one or two years (38), infections associated to cervical changes have been reported in 80.0% of women under 25 years of age, 66.0% in the age group of 26 to 35 years, and 51.0% in the group of 36 to 45 years old (37). In this study, the HPV prevalence declined in an age-related fashion; similar findings were presented in an epidemiological study of global data on age-specific prevalence of HPV infection overall, by Smith *et al.* (38), who observed a substantial decrease in HPV prevalence in older women.

Colposcopy evaluation of the population studied here was suggestive for HPV infection in a higher range than Pap smears, showing a better predicted value for diagnosis of HPV infection. In a meta-analysis developed by Mitchell *et al.* (39), a mean sensitivity of 96.0% and a mean specificity of 48.0% for colposcopy to detect histological

abnormalities, including CIN 1-3 and cancer were found.

It has been recognized that among women who are HPV positive but cytological negative, about 60.0% become HPV negative within six months. However, even in the presence of negative cytology, older women who are HPV positive have a greater risk of developing CIN 3 within 10 years, compared with younger women (21.0 vs 13.6%, respectively) (10). In this study, HPV18 was the most frequent genotype identified in patients with LSIL, the majority of these women were younger than 35 years old. It has been suggested that women with LSIL, positive for HPV16 and HPV18, have a higher risk to progress to CC than those positives for other HPV genotypes; together, HPV16 and HPV18 account for 35.0% of HPV positive LSIL but nearly 70.0% of worldwide CC (30); so that the specificity of HPV detection tests can be enhanced by setting an age limit at 35 years in cases of LSIL (40).

The variation of HPV distribution in LSIL and cancer, suggests that the distinction of high-risk HPV genotyping has the potential to improve the management of LSIL in clinical practice (29, 41). Thus, performing HPV testing and cytology screening simultaneously should improve the detection of prevalent disease; additionally, if Pap testing is negative in women infected with HPV, then there is future low risk for cervical neoplasia, so the screening interval for women with negative tests could be lengthened, and the subgroup of patients with positive results could be targeted for more frequent surveillance (32, 42).

In conclusion, the results obtained in this study showed a high frequency of HPV infection with specific HPV oncogenic-risk genotypes in women from Mérida state. This should be a key point when considering the implementation of the vaccination system against HPV infection,. Colposcopy should be considered a priority in women's gynecological routine examination, together with HPV testing; as well as prevention plans extended to men, aiming the education of sexual and reproductive health items in order to modify sexual practice and to avoid major risk factors associated to HPV infection.

ACKNOWLEDGMENTS

This study was supported by grants FONACIT-G-2005000408; Proyecto Misión Ciencia LUZPROLAB N° 2007001088 and CDCHTA-ULA N° M-945-08-07-A and M-946-08-07-C.

The authors thank, Dr. Pierina Petrosino and Dr. Asmiria Sotolongo from the Pathology Laboratory, Dept. de Anatomía Patológica, ULA, Mérida. The authors also appreciate the professional services of the gynecologists who gave time for this project [Dr. Nazira Monsalve and Dr. Manuel Santos from IAHULA].

REFERENCES

1. **Pan American Health Organization. (PAHO).** Towards comprehensive cervical cancer prevention and control: declaration from the regional meeting. Immunization Newsletter 2008, Washington DC.
2. **Ministerio de Poder Popular para la Salud (MPPS).** (Prensa MPPS). 2013. MPPS avanza en los métodos de detección temprana del Cáncer de cuello uterino. Available in: http://www.mpps.gov.ve.
3. **Muñoz M, Mendoza JA, Téllez L, Noguera ME, Moret O, López M, Toro M.** Detección de VPH-16 y 18 en muestras de cérvix de mujeres que acuden a centros asistenciales de la ciudad de Mérida, Venezuela. Rev Biomed 2003; 14: 61-68.
4. **Ministerio de Poder Popular para la Salud (MPPS).** Anuario de Mortalidad. 2007, República Bolivariana de Venezuela.
5. **zur Hausen H.** Papillomaviruses in the causation of human cancers –a brief his-

torical account. Virology 2009; 384: 260-265.
6. **Bosch FX, de Sanjose S.** The epidemiology of human Papillomavirus infection and cervical cancer. Dis Markers 2007; 23: 213-227.
7. **Fröberg M, Johansson B, Hjerpe A, Andersson S.** Human papillomavirus 'reflex' testing as a screening method in cases of minor cytological abnormalities. Br J Cancer 2008; 99: 563-568.
8. **Castellsagué X.** Natural history and epidemiology of HPV infection and cervical cancer. Gynecol Oncol 2008; 110: S4-S7.
9. **Gravitt PE.** The known unknowns of HPV natural history. J Clin Invest 2011; 121: 4593-4599.
10. **Kjaer S, Høgdall E, Frederiksen K, Munk C, van den Brule A, Svare E, Meijer C, Lörincz AT, Iftner T.** The absolute risk of cervical abnormalities in High-risk Human Papillomavirus–positive, cytologically normal women over a 10-year period. Cancer Res 2006; 66: 10630-10636.
11. **Cuzick J, Clavel C, Petry KU, Meijer CJ, Hoyer H, Ratnam S, Szarewski A, Birembaut P, Kulasingam S, Sasieni P, Iftner T.** Overview of the European and North American studies on HPV testing in primary cervical cancer screening. Int J Cancer 2006; 119(5):1095-1101.
12. **World Health Organization/Institut Català d' Oncologia (WHO/ICO).** Information Centre on HPV and Cervical Cancer. Human Papillomavirus and cervical cancer. 2007, Summary Report Venezuela Updated year 2007.
13. **Castle PE.** The potential utility of HPV Genotyping in screening and clinical management. J Natl Compr Canc Netw 2008; 6: 83-95.
14. **International Agency for Research on Cancer.** Cervix cancer screening. 2005, IARC handbooks of cancer prevention, vol 10, IARC Press, Lyon, France.
15. **Clifford GM, Rana RK, Franceschi S, Smith JS, Gough G, Pimenta JM.** Human Papillomavirus genotype distribution in low-grade cervical lesions: comparison by geographic region and with cervical cancer. Cancer Epidemiol Biomarkers Prev 2005; 14: 1157-1164.
16. **Wheeler CM, Hunt WC, Joste NE, Key CR, Quint WGV, Castle PE.** Human Papillomavirus genotype distributions: implications for vaccination and cancer screening in the United States. J Natl Cancer Inst 2009; 101: 475-487.
17. **Idänpään-Heikkilä J.** Ethical principles for the guidance of physicians in medical research-the Declaration of Helsinki. Bull WHO 2001; 79: 279.
18. **Walker P, Dexeus S, De Palo G, Barrasso R, Campion M, Girardi F, Jakob C, Roy M, from the Nomenclature Committee of the IFCPC.** International Terminology of Colposcopy: An updated report from the IFCPC. Obstet Gynecol 2003; 101: 175-177.
19. **Wright TCJ, Massad LS, Dunton CJ, Spitzer M, Wilkinson EJ, Solomon D, for the 2006 ASCCP-sponsored consensus conference.** 2006 Consensus guidelines for the management of women with abnormal cervical screening tests. J Low Genit Tract Dis 2007; 11: 201-222.
20. **Wright TCJ, Massad LS, Dunton CJ, Spitzer M, Wilkinson, EJ, Solomon D for the 2006 ASCCP-sponsored consensus conference.** 2006 Consensus guidelines for the management of women with cervical intraepithelial neoplasia or adenocarcinoma in situ. Am J Obstet Gynecol 2007; 197: 340-345.
21. **Sotlar K, Diemer D, Dethleffs A, Hack Y, Stubner A, Vollmer N, Menton S, Menton M, Dietz K, Wallwiener D, Kandolf R, Böltmann, B.** Detection and typing of human papillomavirus by E6 Nested Multiplex PCR. J Clin Microbiol 2004; 42: 3176-3184.
22. **Reigosa A, Alvarez M, De Vasconcelo M, Cristina R, Salas W, Rebolledo V, Voldman A**. Diagnóstico del virus papiloma humano en cuello uterino de mujeres que acuden a su consulta anual citológica. Salus 2004; 8(1): 26-31.
23. **Suárez C, Mijares A, Castillo L, Briceño J.** Tipificación del VPH en cáncer de cuello uterino en la población venezolana. Rev Ven Oncol 2006; 18(4): 221-225.

24. **De Guglielmo Z, Ávila M, Veitía D, Cavazza M.** Evaluación, mediante RCP, de la infección por el virus de papiloma humano en muestras de pacientes con diagnóstico clínico o citológico. Rev Obstet Ginecol Venez 2008; 68: 240-247.
25. **Correnti M, Medina F, Cavazza ME, Rennola A, Ávila M, Fernándes A.** Human papillomavirus (HPV) type distribution in cervical carcinoma, low-grade, and high-grade squamous intraepithelial lesions in Venezuelan women. Gynecol Oncol 2011; 121(3): 527-531.
26. **Salazar E.** Detección del virus de papiloma humano en pacientes con lesiones intraepiteliales escamosas de cuello uterino. Rev Obstet Ginecol Venez 2007; 67: 47-54.
27. **Park JS, Kim YT, Lee A, Lee Y, Kim KT, Cho CH, Choi HS, Jenkins D, Pirog EC, Molijn AC, Ramakrishnan G, Chen J.** Prevalence and type distribution of human papillomavirus in cervical adenocarcinoma in Korean women. Gynecol Oncol 2013; 130(1): 115-120.
28. **Haghshenas M, Golini-moghaddam T, Rafiei A, Emadeian O, Shykhpour A, Ashrafi, GH**. Prevalence and type distribution of high-risk human papillomavirus in patients with cervical cancer: a population-based study Infect Agents and Cancer 2013; 8:20. Available in: http://www.infectagentscancer.com/content/8/1/20
29. **Clifford GM, Gallus S, Herrero R, Muñoz N, Snijders PJ, Vaccarella S, Anh PT, Ferreccio C, Hieu NT, Matos E, Molano M, Rajkumar R, Ronco G, de Sanjose S, Shin HR, Sukvirach S, Thomas JO, Tunsakul S, Meijer CJ, Franceschi S, IARC HPV Prevalence Surveys Study Group.** Worldwide distribution of human papillomavirus types in cytologically normal women in the International Agency for Research on Cancer HPV prevalence surveys: a pooled analysis. Lancet 2005; 366: 991-998.
30. **Clifford GM, Smith JS, Plummer M, Muñoz N, Franceschi S.** Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. Br J Cancer 2003; 88: 63-73.
31. **Conesa-Zamora P, Ortiz-Reina S, Moya-Biosca J, Doménech-Peris A, Orantes-Casado FJ, Pérez-Guillermo M, Egea-Cortines M.** Genotype distribution of human papillomavirus (HPV) and co-infections in cervical cytologic specimens from two outpatient gynecological clinics in a region of southeast Spain. BMC Infect Dis 2009; 9: doi:10.1186/1471-2334-9-124.
32. **Katki HA, Wentzensen N.** How might HPV testing be integrated into cervical screening?. Lancet Oncol 2012; 13: 8-10.
33. **Mendez F, Muñoz N, Posso H, Molano M, Moreno V, van den Brule AJC, Ronderos M, Meijer C, Muñoz A, INCH Papillomavirus Study group.** Cervical coinfection with human papillomavirus (HPV) types and posible implications for the prevention of cervical cancer by HPV vaccines. J Infect Dis 2005; 192: 1158-1165.
34. **Winer RL, Kiviat NB, Hughes JP, Adam DE, Lee S-K, Kuypers JM, Koutsky LA.** Development and duration of human papillomavirus lesions, after initial infection. J Infect Dis 2005; 191: 731-738.
35. **Insinga RP, Dasbach EJ, Elbasha EH, Liaw KL, Barr E.** Progression and regression of incident cervical HPV 6, 11, 16 and 18 infections in young women. Infect Agent Cancer 2007; 2: 15.
36. **de Sanjose S, Diaz M, Castellsagué X, Clifford G, Bruni L, Muñoz N, Bosch FX.** Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: a meta-analysis. Lancet Infect Dis 2007; 7: 453-459.
37. **Johnson AM, Mercer CH, Beddows S, de Silva N, Desai S, Howell-Jones R, Carder C, Sonnenberg P, Fenton KA, Lowndes C, Soldan K.** Epidemiology of, and behavioural risk factors for, sexually transmitted human papillomavirus infection in men and women in Britain. Transm Infect 2012; doi:10.1136/sextrans-2011-050306.
38. **Smith JS, Melendy A, Rana RK, Pimenta JM.** Age-specific prevalence of infection with human papillomavirus in females: A global review. J Adolesc Health 2008; 43: S5-S25.

39. **Mitchell MF, Schottenfeld D, Tortolero-Luna G, Cantor SB, Richards-Kortum R.** Colposcopy for the diagnosis of squamous intraepithelial lesions: a meta-analysis. Obstet Gynecol 1998; 91: 626-631.
40. **Ronco G, Cuzick J, Segnan N, Brezzi S, Carozzi F, Folicaldi S, Dalla-Palma P, Del Mistro A, Gillio-Tos A, Giubilato P, Naldoni C, Polla E, Iossa A, Zorzi M, Confortini M, Giorgi-Rossi P, NTCC working group.** HPV triage for low-grade (L-SIL) cytology is appropriate for women over 35 in mass cervical cancer screening using liquid based cytology. Eur J Cancer 2007; 43: 476-480.
41. **Mayrand M-H, Duarte-Franco E, Rodrigues I, Walter SD, Hanley J, Ferenczy A, Ratnam S, Coutlée F, Franco EL, for the Canadian Cervical Cancer Screening Trial Study Group.** Human Papillomavirus DNA versus Papanicolaou screening tests for cervical cancer. N Engl J Med 2007; 357: 1579-1588.
42. **Cuzick J, Szarewski A, Cubie H, Hulman G, Kitchener H, Luesley D, McGoogan E, Menon U, Terry G, Edwards R, Brooks C, Desai M, Gie C, Ho L, Jacobs I, Pickles C, Sasieni P.** Management of women who test positive for high-risk types of human papillomavirus: the HART study. Lancet 2003; 362: 1871-1876.