Detection of high risk human papillomavirus by hybrid capture II® according cytological findings in women treated for squamous intraepithelial lesions of the cervix, period 2006/2010

Detección del virus del papiloma humano de alto riesgo por captura híbrida II® según hallazgos citológicos en mujeres tratadas por lesiones escamosas intraepiteliales de cuello uterino, período 2006/2010

Abstract

Objective: To determinate the frequency of high risk human papillomavirus (HR-HPV) by hybrid capture II ® (CH II®), according cytology results in women treated for squamous intraepithelial lesions of the cervix (SIL)**. Material and Methods:** A descriptive cross-sectional study of a series of cases that included 122 women treated, 79 (75%) for low grade SIL (LSIL) and 43 (35%) for high grade SIL (HSIL) attending at the HPV Laboratory at the Health Sciences Research Institute (IICS), National University of Asunción (UNA), for post-treatment control during period 2006/2010. **Results:** A total of 28% (34/122) of women treated for SIL were positive for HR-HPV, detecting viral infection in 20% of women with no SIL (NSIL) (22/108), in 83% of women with LSIL (10/12) and in 100% of women with HSIL (2/2). Of 34 women positive for HR-HPV, 10 women (29%) had high values (100 pg / mL or more) of relative viral load, detecting an increase of positive cases with severity of the lesion (28% NSIL, 30% LSIL, 50% HSIL). **Conclusion:** HR-HPV detection by CH II® and high relative viral load values especially in women with NSIL could help to identify treated women at risk of developing recurrence, thereby contributing to strengthening the cervical cancer prevention program.

Keywords: HPV. Post-treatment control. Hybrid Capture II ®. Paraguayan women. Relative viral load. Cytological diagnosis.

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Resumen

Objetivo: Determinar la frecuencia del virus de papiloma humano de alto riesgo oncogénico (HR-HPV) por captura híbrida II ® (CH II®) según hallazgos citológicos en mujeres tratadas por lesiones escamosas intraepiteliales (SIL) de cuello uterino. **Material y Método:** Estudio descriptivo de corte transverso de una serie de casos, en donde se incluyeron 122 mujeres tratadas, 79 (65%) por SIL de bajo grado (LSIL) y 43 (35%) por SIL de alto grado (HSIL) que concurrieron al Laboratorio de HPV del Instituto de Investigaciones en Ciencias de la Salud, Universidad Nacional de Asunción, para realizarse un control post-tratamiento, periodo 2006/2010. **Resultados:** Se observó un total del 28% (34/122) de mujeres tratadas por SIL positivas para HR-HPV, detectándose infección viral en un 20% de las mujeres con ausencia de SIL (NSIL) (22/108), 83% de las mujeres con LSIL (10/12) y 100% de las mujeres con HSIL (2/2). De las 34 mujeres positivas para HR-HPV, 10 mujeres (29%) presentaron valores altos (100 pg/mL o más) de carga viral relativa, detectándose un aumento de casos positivos con la severidad de la lesión (28% NSIL, 30% LSIL, 50% HSIL). **Conclusión:** La detección de HR-HPV por CH II®, así como los valores de carga viral relativa altos, en especial en mujeres con NSIL podrían ayudar a identificar mujeres tratadas con riesgo a desarrollar recidivas, contribuyendo así a fortalecer el programa de prevención de cáncer de cuello uterino.

Palabras claves: HPV. Control post tratamiento. Captura Híbrida II ®. Mujeres Paraguayas. Carga viral relativa. Diagnóstico citológico.

Introduction

Cervical cancer is the third most frequent type of cancer in women worldwide, including 529,828 new cases and 275,128 deaths in 2008, of which 31,712 occurred in Latin America and the Caribbean. A total of 85% of new cases take place in developing countries. The standard incidence rate by age in South America is 24.1 cases/100,000 women. In Paraguay, the incidence and mortality rates are 35.0 and 16.6/100,000 women, respectively. These rates are significantly higher than those recorded in neighboring countries such as Argentina, Brazil, Uruguay and Chile[1].

The human papillomavirus (HPV) is a factor involved in the development of cervical cancer. There are more than 100 types of HPV, of which approximately 40 infect the anogenital mucosa. These are categorized into high-risk viruses (HR-HPV) and low--risk viruses (LR-HPV), according to their oncogenic potential, and the HR-HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66) account for 95% of all cervical cancer cases[2-5].

The cytological diagnosis (Papanicolaou test) is a simple low-cost method used to detect the cytopathic effect of this virus, although not determining the viral type. As the HPV does not grow in traditional cell cultures, molecular methods have been recently used to identify the viral genotype, such as the Hybrid Capture II® test (HC-II®), which detects 13 types of HR-HPV and enables the viral load to be estimated[6,7].

There are no treatments that can eliminate an HPV-related infection, they can only remove the lesions produced by this virus. The methods used for this treatment are as follows: Loop Electrosurgical Excision Procedure (LEEP), cryotherapy and cervical conization. Although HPV-related intraepithelial lesions can be treated, there is a chance of recurrence or even the development of cervical cancer. If the lesion extends to the endocervical surgical margin, the risk of failure is higher. Moreover, women who have lesion-free

surgical margins show a risk of treatment failure of 2-6%, regardless of the treatment used. The cumulative rate of invasion eight years after treatment is 5.8 cases/1,000 women, which is five times higher than that of the general population[8-14].

In addition to considering the compromised surgical margins as a recurrence factor, there are other factors that promote the persistence or recurrence of squamous intraepithelial lesions (SIL) after treatment, such as age, parity, cytological diagnosis and degree of lesion prior to treatment[15].

As a result, a continuous and thorough follow-up is important after the treatment. Recent studies suggest that the combined use of cytology and the HC-II® test increases effectiveness when selecting women at risk of developing residual or recurrent SIL after six months of treatment[16-19].

In Paraguay, previous studies have observed a high frequency of HR-HPV in women without cervical lesions and cofactors of risk (a high number of sexual partners and multiparity, among others) associated with the development of cervical cancer, comparable to or higher than those identified in other developing countries, which could partly explain the high incidence of such type of cancer in this country[20-23].

In Paraguay, there have been no studies on the frequency of HR-HPV in women treated for SIL. Thus, considering the high-risk sexual behavior of Paraguayan women, which could promote post-treatment viral infection and increase the risk of recurrence, the present study aimed to determine the frequency of HR-HPV using the HC-II® test, according to cytological results in women treated for cervical SIL, between 2006 and 2010.

Methods

Study population

A descriptive cross-sectional study of a series of cases was conducted with 122 women treated for cervical lesions who sought the Department of Public Health and Epidemiology of the *Instituto de Investigaciones en Ciencias de la Salud* (IICS –Health Sciences Research Institute) of the *Universidad Nacional de Asunción* (UNA – National University of Asunción), between 2006 and 2010, with a medical recommendation to have the HC-II® test performed for HR-HPV detection.

The treated women who went to the IICS-UNA originated from different public and private health centers of the Central Department of Paraguay. The present study included women who had a cytohistological diagnosis of their lesions prior to the treatment, were treated at least six months before, and had an updated cytological diagnosis performed after the treatment, when the HR-HPV was detected by the HC-II test. Women who sought the IICS, UNA, more than two years after the treatment had been performed were excluded from this study. The treatment applied depended on the medical criteria and was based on the Paraguayan Manual on Norms and Procedures for Cervical Cancer Control and Prevention, developed by the Ministry of Public Health[24].

Prior to treatment, of all 122 participating women, 79 had a confirmed histological diagnosis of low-grade SIL (LSIL) and 43 of high-grade SIL (HSIL). Of all women, 97 underwent LEEP and 25, cervical conization.

All women completed a questionnaire that included demographic, socioeconomic, gyneco-obstetric and sexual behavior characteristics. All information was processed while protecting patients' confidentiality. This research project was approved by the Research Ethics Committee of the IICS of the *Universidad Nacional de Asunción*, under number M07/10.

The minimum sample size of 144 women treated was calculated using the table in the 13E Appendix for a descriptive study with a dichotomous variable, considering 30% of expected frequency of HR-HPV after the treatment, an amplitude (ω) of 0.15 and a 95% confidence interval (95%CI)[25].

Detection of cervical lesions in women treated using cytological diagnosis

The cytological analysis was performed in the participating health centers. Samples were obtained by Pap smear and placed on polished microscope slides that were properly identified. These slides were fully immersed in a wide-rimmed container with 96% ethyl alcohol for 15 minutes. Slides were subsequently removed from the container and left to dry for ten minutes. Finally, fixing spray was applied to them. The cytological results were classified according to the 2001 Bethesda system[26].

Detection of HR-HPV in women treated using the HC-II® test

The study sample was collected in the IICS, UNA, using a cytobrush, which was introduced into a collection tube provided by its manufacturing company, Qiagen (Germany).

The samples were processed with the HC-II test, which detects 13 types of HR-HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. Following the manufacturer's protocol, the cervical samples were treated with sodium hydroxide, aiming to denature the DNA. The simple-stranded DNA was hybridized in a solution with a cocktail of RNA probes from the 13 types of HR-HPV. Each reaction mix including RNA-DNA hybrids was transferred to microplates sensitized with anti-hybrid antibodies, enabling their immobilization. The RNA-DNA hybrids bound to antibodies were put into contact with a second antibody conjugated with alkaline phosphatase. The non-binding material was removed by successively washing it, and a chemiluminescent reactive (Lumi-Phos 530) was subsequently added as substrate for the alkaline phosphatase. The luminescence produced by this reaction was measured with a luminometer. The unit of measurement of light was expressed as relative light units (RLU). Positive and negative controls originated from Qiagen were used in triplicate.

The relative viral load was obtained by comparing the sample RLU with those of the positive control (RLU/PC). Samples with RLU/PC ≥ 1.0 pg/mL were considered as positive. The relative viral load was divided into four categories according to their values in pg/mL: 1 ≤ value < 10 pg/mL (low relative viral load); 10 ≤ value < 100 pg/mL (average relative viral load); 100 ≤ value < 1,000 pg/mL (high relative viral load); and value ≥ 1,000 pg/mL (very high relative viral load)[27].

It should be emphasized that professionals who participated in the sample processing and reporting of results from the HC-II® test were blind to the cytological results. Consequently, the results of both methods were analyzed independently.

Statistical analysis

Data analysis was performed using descriptive statistics procedures and the Epi Info software, version 3.2 (CDC, Atlanta, USA). Chi-square analysis was performed to identify the association among type of treatment, length of time since the treatment and frequency of HR-HPV infection, using the Epi Info software, version 3.2. A p-value ≤0.05 was considered to be significant in all data analyses performed.

Results

The mean age of the 122 participating women was 34 ± 10 years (95%CI 17-67). Information about socioeconomic characteristics, obstetric history and sexual behavior could be obtained from 86% of the study population (105/122 women) (Table 1).

In all, 28% (34/122) of participants treated for SIL were positive for HR-HPV, and viral infection was detected in 20% of women without SIL (NSIL) (22/108), 83% of those with LSIL (10/12) and 100% of those with HSIL (2/2) (Table 2).

It should be emphasized that there were no significant differences among frequencies of HR-HPV according to type of treatment, and 27/97 (28%) women treated with LEEP and 7/25 (28%) treated with cervical conization had the viral infection (p = 0.7).

With regard to the length of time since the treatment, there were no significant

Table 1 - Socioeconomic characteristics, obstetric history and sexual behavior among women treated for cervical squamous intraepithelial lesions.
Tabla 1 - *Características socioeconómicas, antecedentes obstétricos y conducta sexual de las mujeres tratadas por lesiones escamosas intraepiteliales de cuello uterino.*

Variables*	n (%)
Level of education	
Complete primary school	32 (30)
Secondary school and higher	73 (70)
Smoking habit	
Yes	7 (7)
No	98 (93)
Use of hormonal contraceptive	
Yes	35(33)
No	70(67)
Pregnancy	
Presence	80 (76)
Absence	25 (24)
Number of pregnancies	
>2	34 (32)
≤2	46 (44)
Age of first pregnancy (in years)	
≤19	28 (27)
>19	52 (50)
Age of first sexual intercourse (in years)	
<19	57(54)
≥19	48 (46)
Number of sexual partners	
>2	40 (38)
≤2	65 (62)

* Information about socioeconomic characteristics, obstetric history and sexual behavior could be obtained from 86% of the study population (105/122 women).
**Fue posible obtener información acerca de las características socioeconómicas, antecedentes obstétricos y conducta sexual del 86% de la población en estudio (105/122 mujeres).*

Table 2 - HR-HPV results for CH II® in women treated for cervical squamous intraepithelial lesions according cytologic diagnosis.
Tabla 2 - *Resultado de HR-HPV por CH II® en mujeres tratadas por lesiones escamosas intraepiteliales según el diagnóstico citológico.*

Cytological diagnosis	n	HR-HPV infection	
		Positive n (%)	Negative n (%)
NSIL	108	22 (20)	86 (80)
LSIL	12	10 (83)	2 (17)
HSIL	2	2 (100)	0 (0)
Total	122	34(28)	88(72)

HR-HPV: high risk human papilloma virus; NSIL: no squamous intraepithelial lesion, LSIL: low grade squamous intraepithelial lesion; HSIL: high grade squamous intraepithelial lesion.
HR-HPV: virus del papiloma humano de alto riesgo; NSIL: ausencia de lesión intraepitelial escamosa; LSIL: lesión intraepitelial escamosa de bajo grado; HSIL: lesión intraepitelial escamosa de alto grado.

Table 3 - Frequency of women treated for cervical squamous intraepithelial lesions positive for HR-HPV by CH II® accoding relative viral load values.
Tabla 3 - *Frecuencia de mujeres tratadas por lesiones escamosas intraepiteliales positivas para HR-HPV por CH II® según valores de carga viral relativa.*

Cytological diagnosis	Relative viral load values (pg/mL)				Total n
	Low (1 <10) n (%)	Average (10 <100) n (%)	High (100 <1000) n (%)	Very high (≥1000) n (%)	
NSIL	10 (45)	6 (27)	4 (18)	2 (10)	22
LSIL	5 (50)	2 (20)	2 (20)	1 (10)	10
HSIL	0 (0)	1 (50)	1 (50)	0 (0)	2
Total	15 (44)	9 (26)	7 (21)	3 (9)	34

NSIL: no squamous intraepithelial lesion; LSIL: low grade squamous intraepithelial lesion; HSIL: high grade squamous intraepithelial lesion.
NSIL: ausencia de lesión intraepitelial escamosa; LSIL: lesión intraepitelial escamosa de bajo grado; HSIL: lesión intraepitelial escamosa de alto grado.

differences in frequencies of HR-HPV between women who had a test to detect viral infection until one year after their treatment (32% 25/77 women) and those who did so after one year (20%, 9/45 women), p = 0.1.

In terms of the relative viral load, of all women positive for HR-HPV, 29% (10/34) had high or very high values. According to the cytological diagnosis, 28% of women with NSIL, 30% with LSIL and 50% with HSIL had high or very high relative viral loads. It should be emphasized that none of the relative viral load values in women with HSIL was low (Table 3).

Discussion

There is a high incidence of pre-neoplastic lesions and cervical cancer in Paraguay, which are considered to be a public health problem. The present study showed preliminary results of HR-HPV detection with the HC-II® test, according to the cytological diagnosis performed in women treated for SIL.

In this study, the frequency of women treated for SIL and positive for HR-HPV (28%) was similar or higher than those found in Brazil (33% or 22/67 women and 22% or 16/74 women), Korea (18% or 44/243 women) and Belgium (29% or 21/72 women), where women positive for HR-HPV had a high frequency (from 18% to 44%) of recurrence of SIL[16,28-30]. This suggests that post-treatment HR-HPV detection can be a useful tool in the follow-up and guidance of treated women.

With regard to the cytological diagnosis, 20% of women with NSIL were positive for HR-HPV. This lack of agreement between the HC-II® test and the cytological diagnosis could have been due to the fact that the former enables the viral presence to be detected even when there is no lesion[6,22]. Previous studies suggest that women with NSIL and positive for HR-HPV have a higher risk of developing cervical lesions. Koutsky et al. observed that 30% of women with a normal cytological diagnosis and positive for HR-HPV developed intraepithelial lesions in the course of two years. It should be emphasized that 80% (86/122) of women with NSIL had negative results for HR-HPV and, considering the high negative predictive value of the HC-II test, have a reduced risk of developing high-grade lesions[14,16-18,31-35].

The results found in the present study suggest that the frequency of HR-HPV in treated women does not vary according to the type of treatment and length of time spent to perform the post-treatment control (until two years), which could be due to other factors playing a role in the persistence of post-treatment HR-HPV infection.

Previous studies observed that women with HSIL infected with HPV-16, 18, 33 and 45 and those with multiple types of HPV prior to LEEP or cervical conization have a significantly higher frequency of viral infection recurrences after the treatment[15,36,37].

With regard to the relative viral load, previous studies suggest that patients with a persistent and high relative viral load have an increased risk of developing HSIL in a short period of time, even in the absence of detectable cytological changes[38,39]. In terms of the cytological diagnosis and in agreement with the results observed in the present study, the greater the severity of SIL, the higher the frequency of women with a high or very high relative viral load. It should be emphasized that there were no low relative viral load values among women with HSIL, which indicates that women positive for HR-HPV with a low relative viral load have a lower probability of developing HSIL, as observed in other studies. Thus, the present results point out that relative viral load values could contribute to post-treatment guidance and control of women, identifying those at a greater risk of developing cervical lesions again[38,39].

The present study had certain limitations, due to the impossibility of completing the sample comprised of 144 women and the lack of data on women's relative viral load prior to the treatment. Nonetheless, the results observed suggest that the use of the HC-II® test for HR-HPV detection and determination of relative viral load as a complement to the cytological diagnosis could contribute to the identification of women treated for pre-neoplastic lesions at a greater risk of having recurrences. The data obtained could help to strengthen the Paraguayan Cervical Cancer Prevention, Detection and Treatment Program, developed by the Ministry of Public Health and aimed at improving the service provided by Primary Health Care units.

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