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# Herpes Simplex Virus and Human Papillomavirus Infection in Cervical Disease in Argentine Women

Luis Orlando Pérez, LIC., Gisela Barbisan, BEC., Martín Carlos Abba, Ph.D., Rubén Martín Laguens, M.D, Ph.D., Fernando Noel Dulout, PROF., and Carlos Daniel Golijow, Ph.D.

**Summary:** The aim of the present study was to determine that prevalence of herpes simplex virus (HSV) type 1 and 2 in cervical samples from Argentine women and to assess the role of HSV-2 in cervical cancer. A sample of 79 normal and 200 neoplastic cervical tissues (35 invasive cervical carcinomas, 75 high-grade squamous intraepithelial lesions, 79 low-grade squamous intraepithelial lesions and 11 abnormal squamous cells of undermined significance) was analyzed for herpes simplex and human papillomavirus DNA using the polymerase chain reaction method. Viral genotyping was performed by single strand conformation polymorphisms and restriction fragment length polymorphisms. The overall prevalence of HSV was 21.5% in controls and 29% in cases. Among women with normal cytology, herpes simplex prevalence in HPV positive (20.8%) women was approximately the same as in negative (21.8%) women. HPV- and age- adjusted ORs of high-grade squamous intraepithelial lesions and invasive cervical carcinomas for HSV-2 were 1.4 ($p = 0.6$) and 1.6 ($p = 0.5$), respectively. The obtained results indicated that herpes simplex virus may not be involved in cervical cancer development. Future investigations are needed to provided conclusive evidence on the role of this pathogen in cervical cancer. **Key Words:** Herpes simplex virus—Cervical cancers—Human papillomavirus infection.

Worldwide, carcinoma of the uterine cervix is the second most common cancer in women and the leading cause of cancer deaths among the female population in the developing world. Globally, it is estimated that approximately 500,000 new cases of cervical cancer are reported each year, and more than 200,000 women will die from this disease in 2005 (1). In Argentina, the annual incidence of cervical cancer was estimated at 23.5 per 100,000 women, and at 25.4 per 100,000 women in Buenos Aires province (year 2000) (1,2).

During the past 20 years, extraordinary advances in the understanding of cervical cancer have occurred. Although the infectious nature of this disease was long-suspected, several decades had to elapse before studies linked human papillomavirus (HPV) to cervical cancer. High-risk HPV types are now considered the main etiologic agents for cervical neoplasia, particularly the types HPV-16 and -18 (3). However, the presence of HPV infection alone is unlikely to be sufficient for the development of cervical cancer, and other sexually transmitted infections, such as herpes simplex virus (HSV) or *Chlamydia trachomatis*, may be involved (4,5).

The role of herpes simplex virus infection in cervical neoplasia is the subject of an active controversy. First, HSV-2 infection was considered the major cause of cervical cancer, but the epidemiologic data available in the 1980s, along with the lack of detection of HSV-2 DNA in cervical tissues, substantially weakened such hypothesis (6,7). To date, serologic studies have reported conflicting results about the relationship of HSV-2 and cervical cancer.

From the Departamento de Carcinogénesis (L.O.P., G.B., M.C.A., F.N.D., C.D.G.), Centro de Investigaciones en Genética Básica y Aplicada (CIGEBA), Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, La Plata, Argentina; Cátedra de Patología B (R.M.L.), Facultad de Ciencias Médicas, Universidad Nacional de La Plata, La Plata, Argentina.

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Address correspondence and reprint requests to Lic. Luis Orlando Pérez, Centro de Investigaciones en Genética Básica y Aplicada (CIGEBA), Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Calle 60 y 118 S/N, (B1900) La Plata, Buenos Aires, Argentina.

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Although some authors detected a significant association of HSV and cervical cancer using HPV-adjusted models, no evidence of such link was found by others (8–12).

On the other hand, laboratory studies have demonstrated that DNA sequences of HSV-2 are capable to transform epithelial cells in conjunction with HPV, leading to mutations and/or rearrangements of cellular genes, which may have critical growth regulation functions (12). However, the significance of HSV-2 in cervical cancer remained questioned, because HSV-2 DNA sequences were not consistently detected in cervical tissues. To date, a few studies have explored the relationship between HSV DNA and cervical biopsies using highly sensitive techniques, such as the polymerase chain reaction (PCR) assay, with variable results (13–18).

This study was designed to examine HSV and HPV infections, using PCR-based methods, in cervical samples obtained from women with normal cytology, atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesions (LGSIL), high-grade squamous intraepithelial lesions (HGSIL), and invasive squamous cervical carcinomas (ICC).

## MATERIAL AND METHODS

### Clinical Samples

A total of 279 cervical samples were collected from women attending 2 public hospitals conforming an anonymous specimens data bank in La Plata, Argentina. The specimens corresponded to women aged 15 to 67 years, comprising 79 normal cytologies, 11 ASCUS, 79 LGSIL, 75 HGSIL, and 35 squamous cervical cancers. Cervical exfoliated cells from the ecto-endocervix were collected using a cytobrush or spatula. Samples were eluted in 5 ml of phosphate-buffered saline (PBS), pelleted, and kept frozen at –80°C. Cervical-biopsy specimens were obtained for histological diagnosis for 30 LGSIL (38% of total), 38 HGSIL (50.7%), and 35 squamous ICC (100%) cases. Biopsy specimens were fixed in formalin and paraffin-embedded or kept freshly frozen.

### DNA Extraction

Paraffin-embedded samples were washed twice with xylol and finally with 100% ethanol, re-suspended in 350 µl of proteinase K digestion buffer (250 µg/ml), and incubated for 2 hours at 56°C. Cervical exfoliated cell pellets and frozen biopsies were suspended and washed twice with 1 ml of PBS, and incubated for 24 hours at 56°C in 400 µl of digestion (extraction) buffer (50 mM Tris-ClH pH 8,5; 1 mM EDTA; 1% Triton $\times$100 and 0,5% Tween 20) containing 250 µg/ml of proteinase K (Promega, Madison, Wisconsin, EEUU). After proteinase digestion, the samples were boiled for 10 minutes at 100°C for proteinase inactivation. DNA purification was conducted by the *Salting out* (direct protein precipitation methodology) procedure (19). Finally, the DNA was (re)suspended in distillate water, and its concentration was adjusted to 1 ng/µl. The samples were stored at –20°C until used. To determine DNA quality for PCR amplification, a fragment of the human thymidine kinase gene was amplified by PCR in all the samples (20).

### Human Papillomavirus DNA Detection and Genotyping

Papillomavirus DNA was detected in cervical tissues using nested PCR, with MY09/11 as external primers and GP5+/6+ as internal primers, according to the methods previously described (21,22). The most commonly used PCR methods for the detection of genital HPVs have used the MY09/11 and GP5+/6+ primer sets (MY/GP+). This strategy has been demonstrated highly sensitive and specific (22–24). HPV genotype was determined by the single strand conformation polymorphisms procedure in low ionic strength solutions (LIS-SSCP) (25). HPV types -6, -11, -16, -18, -31, -33, -34, and -51 were used as controls (kindly provided by Dr. de Villers and Dr. Ort). HPV types -6, -11, and -34 were considered low-risk HPV types, whereas HPV types -16, -18, -31, -33, and -51 were considered high-risk types.

### Herpes Simplex DNA Detection and Genotyping

Viral DNA polymerase gene primers were used to detect HSV-1 and -2 in cervical samples, as previously described by (26). PCR amplification was performed in a final volume of 25 µl, using 5 µl (1 ng/µl) of purified DNA, 2 mM $Cl_2Mg$, 200 µM of each deoxyribonucleoside triphosphate (dNTPs), 25 pmol of each primer, and 1.25 U of Taq DNA polymerase (Promega, Madison, Wisconsin, EEUU) in PCR buffer (20 mM Tris-HCl; pH 8.4 and 50 mM ClK). The reactions were cycled as follows: 1 cycle of 94°C for 2 minutes and 35 cycles of 1 minute at 94°C, 1 minute at 57°C, and 40 seconds at 72°C (finally, 1 cycle at 72°C for 5 minutes for elongation). Detection of the amplified fragments was made by electrophoresis onto a 2% agarose gel and ethidium bromide staining.

For HSV DNA-positive samples, genotyping was performed by the LIS-SSCP procedure, as previously described (27). SSCP patterns appearing in the gel were compared with the viral strains KOS (HSV-1 standard strain) and G (HSV-2 standard strain) DNA. HSV

genotyping was confirmed by restriction fragment length polymorphisms (RFLP) using the same PCR product, as previously described by (27).

### Statistical Analysis

Samples were divided into four age groups: younger than aged 23 years, 24 to 29 years, 30 to 39 years, and older than 41 years. The prevalence of HSV type 1 and 2 infection and HPV genotypes were compared in each grade of cervical disease by the chi-square test. The association of viral infections with cervical stage was performed using logistic regression analysis, controlling for the confounding effect of age.

## RESULTS

All the samples studied were successfully amplified for the human thymidine kinase gene, demonstrating the high quality of the extractions and their viability for PCR analysis. Human papillomavirus prevalence increased with increasing severity of cervical lesions among 279 women from La Plata, Argentina. The prevalence range varied from 30% (24/79) among normal lesions to 100% (35/35) among squamous ICC. Similarly, high-risk HPV infections also increased according to lesion grade, accounting for 85% of the high-grade squamous intraepithelial lesions (HGSIL) and invasive cervical cancer (ICC) lesions. Among women who were HPV DNA-positive, 97.1% of ICC (n = 35), 86.5% of HGSIL (n = 74), 58.7% of LGSIL (n = 75), and 30% of ASCUS (n = 10) were infected with one or more oncogenic types (Table 1).

HSV DNA was successfully detected in women with normal cytology and those with neoplastic lesions. According to the obtained results, differences among HSV positivity by particular stages of cervical neoplasia were not significant. The overall prevalence of HSV was 21.5% among the normal tissues compared with 28.4% among the HGSIL and 32.3% among ICC. In all the stages of cervical disease, HSV-2 was the prevalent type, with 25.4% positivity, followed by 6.1% for HSV-1 and 2.5% for double infections (Table 2).

Among the 79 specimens corresponding to normal cytology, HPV and HSV positivity showed no significant correlation. The prevalence of HSV in HPV-negative samples (21.8%) was approximately the same as that obtained in HPV-positive specimens (20.8%) (Table 3). The analysis found that HSV-positive women were not at higher risk for HPV infection than HSV- negative women were (odds ratio (OR), 0.95; 95% confidence interval (CI), 0.29–3.05). Women with HSV-2 DNA had a slightly higher prevalence of HPV (33.4%) than HSV-2–negative women (30%), although the difference was not significant (OR, 1.1; 95% CI, 0.31–4.3). In addition, it is noteworthy that HPV and HSV DNA positivity did not seem to be associated with a particular age's interval ($p > 0.05$), although a small trend of decreasing prevalence by age was detected for overall HPV.

Compared with women with normal tissues, HPV DNA was strongly associated with a diagnosis of abnormal cytology, and ORs increased with advanced stages of cervical disease (Table 4). Contrarily, HSV-2 DNA was not statistically associated with cervical cancer, although a borderline significance was calculated for women with LGSIL ($p = 0.056$). More accurate odd ratios were obtained in the model adjusted for age and HPV, with ORs of 1.7 ($p = 0.38$), 1.4 ($p = 0.59$), and 1.6 ($p = 0.5$) for women with LGSIL, HGSIL, and ICC, respectively. Further analyzes were restricted to HPV-positive women to examine the effect of herpes simplex virus infection among HPV carriers. The obtained results were similar to those reached in the HPV-adjusted model.

## DISCUSSION

Herpes simplex is one of the most common sexually transmitted agents worldwide. In the United States,

**TABLE 1.** *Type-specific prevalence of human papillomavirus infections among HPV DNA-positive women, by stage of cervical disease*

| | HPV-positive normal* (n = 24) | HPV-positive ASCUS* (n = 10) | HPV-positive LGSIL* (n = 75) | HPV-positive HGSIL* (n = 74) | HPV-positive ICC* (n = 35) |
|---|---|---|---|---|---|
| High-risk HPV | 8 (33.4) | 3 (30) | 45 (60) | 73 (98.6) | 38 (108.6) |
| Low-risk HPV | 18 (75) | 8 (80) | 43 (57.4) | 22 (29.7) | 3 (8.6) |
| HPV-16 | 7 (29.2) | 3 (30) | 29 (38.7) | 42 (56.7) | 24 (68.6) |
| HPV-18 | 1 (4.2) | — | 11 (14.7) | 15 (20.3) | 6 (17.1) |
| Double infections | 2 (8.3) | 1 (10) | 13 (17.3) | 21 (28.4) | 6 (17.1) |

ASCUS, atypical squamous cells of undetermined significance; LGSIL, low-grade squamous intraepithelial lesions; HGSIL, high-grade squamous intraepithelial lesions; ICC, invasive squamous cervical carcinomas.

Data are numbers with percentages in parentheses.

* Percentages exceed total because of double infections.

**TABLE 2.** *Type-specific prevalence of herpes simplex virus infections among women with normal tissues and cervical disease in Argentina*

| | Normal (n = 79) | Cases*† (n = 197) | ASCUS (n = 10) | LGSIL (n = 79) | HGSIL* (n = 74) | ICC* (n = 34) |
|---|---|---|---|---|---|---|
| HSV DNA negative | 62 (78.5) | 140 (71.1) | 8 (80) | 56 (70.9) | 53 (71.6) | 23 (67.6) |
| HSV-1 | 5 (6.3) | 12 (6.1) | — | 1 (1.3) | 7 (9.5) | 4 (11.7) |
| HSV-2 | 12 (15.2) | 50 (25.4) | 2 (20) | 22 (27.8) | 17 (23) | 9 (26.5) |
| Double infections | — | 5 (2.5) | — | — | 3 (4) | 2 (5.9) |

ASCUS, atypical squamous cells of undetermined significance; LGSIL, low-grade squamous intraepithelial lesions; HGSIL, high-grade squamous intraepithelial lesions; ICC, invasive squamous cervical carcinomas.

Data are numbers with percentages in parentheses.

* Percentages exceed total because of double infections.

† Three missing values.

asymptomatic carriers accounted for 21% of the general population aged 12 years and older, with an average of 26% in women and 18% in men (third National Health and Nutrition Examination Survey—NHANES III) (28). The prevalence of HSV was higher in general populations from the developing countries, in which the sub-Saharan Africa exhibits the higher prevalences, ranging from 30 to 80% in women and from 10 to 50% in men. Unfortunately, most of the studies from South and Central America are restricted to HSV-2 in women, with an estimated seroprevalence of 20 to 40%, whereas little information is available for HSV-1 infection (29).

To our knowledge, this is the first report on HSV DNA among the general population of Argentina. The prevalence of HSV-2 (15.2%) was relatively low compared with that reported for similar aged studies in non–high-risk populations from Latin America. In this sense, higher prevalences were found in the general population of Cali, Colombia (31.4%) (30), in women attending an antenatal class in Haiti (54%) (31), in a population-based study in Mexico City (29.8%) (32), and in women participating as control patients in cervical cancer studies from Sao Paulo, Brazil (42%) (33) and Costa Rica (39%) (34). Unlike the mentioned studies, in the present work HSV-2 positivity did not seem to increase with older ages. The prevalences were 36.8% among women younger than aged 23 years, 15.8% among women aged 24 to 29 years, 26.3% among women aged 30 to 39 years, and 9.1% among women older than aged 40 years. This situation could be explained by the fact that seroprevalence illustrates the cumulative exposure to an agent, whereas DNA analysis identifies only the active infection.

**TABLE 3.** *Correlation between herpes simplex virus and human papillomavirus DNA positivity among women with normal cytology*

| | Human papillomavirus DNA | | Odd ratio (95% confidence interval) |
|---|---|---|---|
| | Negative (n = 55) | Positive (n = 24) | |
| HSV DNA | | | |
| Negative (n = 62) | 43 | 19 | 1 |
| Positive (n = 17) | 12 | 5 | 0.95 (0.29–3.05)* |
| HSV-2 DNA | | | |
| Negative (n = 67) | 47 | 20 | 1 |
| Positive (n = 12) | 8 | 4 | 1.1 (0.31–4.3)* |

* $p > 0.05$.

A limited number of studies have used highly sensitive polymerase chain reaction-based methods to examine the association between cervical cancer and HSV infections. In this study, the data indicated that HSV infection was not statistically associated with HPV positivity among women with normal cytology, and patients with HSV types 1 or 2 were not at increased risk of cervical cancer. Recent researches have reported conflicting results concerning HSV-2 and cervical cancer development (8–12). These discrepancies may have derived from the different populations that were tested, or the diverse study designs that were used. In addition, it is possible that the methodology used has influenced the results. Serologic methods do not discriminate between current and past infections, or between genital and extragenital infections. Moreover, potential cross-reactivity between HSV-1 and HSV-2 may lead to HSV-2 miss-classification. A recent article comprising a large longitudinal study and a further meta-analysis of studies conducted in the Nordic countries did not find an association between HSV-2 antibodies and invasive cervical cancer (10). However, because that analysis was adjusted for HPV seropositivity, residual confounding from HPV exposure may be expected.

A major obstacle in determining an association between HSV-2 and cervical cancer has been the apparent absence of HSV-2 DNA in cervical cancer biopsies. Whereas previous analyzes were based on hybridization techniques, only a few studies evaluated the role of HSV-2 in cervical cancer tissues by using highly specific PCR assays (13–18). In this sense, Vecchione *et al.* (16) failed to detect HSV-2 sequences in 41 cervical lesions, including 25 low-grade squamous cervical intraepithelial

**TABLE 4.** *Risk estimation of human papillomavirus and herpes simplex virus infections for cervical disease among women in La Plata, Argentina*

| | HPV DNA OR* (95% CI) p value | Herpes simplex virus DNA | | Herpes simplex virus 2 DNA | |
|---|---|---|---|---|---|
| | | OR (95% CI) p value | OR† (95% CI) p value | OR (95% CI) p value | OR† (95% CI) p value |
| Normal | 1 | 1 | 1 | 1 | 1 |
| ASCUS | 30 (3.4–260) <0.01 | 0.9 (0.17–5) 0.9 | 1.5 (0.2–11) 0.660 | 1.4 (0.2–7.4) 0.7 | 0.8 (0.06–8.7) 0.8 |
| LGSIL | 50 (15.7–156) <0.01 | 1.5 (0.73–3) 0.3 | 1.6 (0.57–4.5) 0.367 | 2.1 (0.9–4.7) 0.056 | 1.7 (0.5–6.01) 0.38 |
| HGSIL | 215 (27–1730) <0.01 | 1.4 (0.68–3) 0.35 | 1.28 (0.4–3.8) 0.655 | 1.66 (0.7–3.7) 0.22 | 1.4 (0.4–4.7) 0.59 |
| ICC | † | 1.7 (0.7–4) 0.26 | 1.6 (0.4–6.4) 0.48 | 2.01 (0.7–5.3) 0.1 | 1.6 (0.3–7.2) 0.5 |

ASCUS, atypical squamous cells of undetermined significance; LGSIL, low-grade squamous intraepithelial lesions; HGSIL, high-grade squamous intraepithelial lesions; ICC, invasive squamous cervical carcinomas; OR, odds ratio; CI, confidence interval.

* OR adjusted for age.

† OR adjusted for age and HPV.

neoplasias, and 16 high-grade squamous intraepithelial neoplasias. Other studies have focused their attention on HSV-2 transforming sequences, specifically those within the *Bgl*II N locus of the viral genome. By using this approach, HSV-2 DNA was detected in 14% of squamous cervical carcinomas and 27% of cervical adenocarcinomas from Indonesian and Swedish patients (13), and in 25% of cervical dysplasia, 20% of carcinoma *in situ*, and 23% of squamous cervical carcinomas from 46 patients from Thailand (14). On the other hand, in another study conducted among 589 women (200 with cervical cancer, 65 with high-grade squamous intraepithelial lesions, 80 with low-grade squamous intraepithelial lesions, and 244 controls), none of the cervical samples scored positive for the HSV-2 *Xho* (*Bgl*II N) DNA sequence (18).

Using primers that allowed the amplification for both HSV-1 and HSV-2 DNA sequences, Baldauf *et al.* (17) examined 41 cases and 33 controls, finding a prevalence of 12% in controls (4/33), 19% in CIN patients (4/21), and 25% in carcinomas (5/20). Although it was a relatively small sample size, these results indicated that women with both HPV and herpesvirus infections were at increased risk for cervical cancer development, but the infection with one virus or the other was not significant. Synergistic interactions between HPV and HSV were found by Hildesheim et al. (35) in a large case-control study conducted among Latin American patients. In agreement with these results, early *in vitro* experiments provided evidence about an effect modifier role. It was demonstrated that HPV-immortalized epithelial cells transfected with HSV-2 DNA became tumorigenic in nude mice, and several mechanisms of interaction were suggested (12). However, loss of HSV DNA in the transformed cell phenotype lead researchers to suggest a *"hit and run"* mechanism for cancer induction, suggesting that the expression of the HSV genes would be necessary for the initiation of the transformation process, but not for its progression (36). Future *in vitro* studies are required to reveal the molecular mechanisms underlying the transformation abilities of HSV-2 DNA.

The present study provides additional information concerning HSV DNA in normal and neoplastic cervical tissues. Women from La Plata with cervical cancer were not associated to herpes simplex DNA, after controlling for age and HPV DNA. However, there exists the possibility that other confounding sources, such as sexual behavior including woman's age at first intercourse or number of sexual partners, may have partially biased the obtained results. Indeed, this study has a relatively small sample size, although it has the advantage that the whole spectrum of cervical stages was examined.

In conclusion, the present study reports a lack of association between HSV DNA and cervical cancer. However, the potential relationship between these viruses and cervical disease awaits further evaluation in larger studies to clarify the true role of HSV in cervical carcinogenesis.

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