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HPV 16 and 18 viral loads are greater in patients with high-grade cervical epithelial lesions

M. Ramírez-Flores[1], I. Delgado-Enciso[2], A.R.G. Fernández-Salinas[3], L.L. Valdez-Velázquez[4], J. Guzmán-Esquivel[5], L.M. Baltazar-Rodriguez[2]

[1] University Center of Biomedical Research, Universidad de Colima, Colima
[2] School of Medicine, Universidad de Colima, Colima; [3] Secretaria de Salud Publica de Estado de Colima, Colima
[4] School of Chemical Sciences, Universidad de Colima, Colima; 5 Hospital General de Zona 1 IMSS, Colima (Mexico)

Summary

Background: Cervical cancer is the second most common cancer in women worldwide. High-risk infection with HPV type 16 or type 18 is the most important risk factor associated with the development of cervical cancer. *Aims:* To determine the viral load of HPV-16 and HPV-18 in samples from women with cervical epithelial lesion in the State of Colima, Mexico. *Materials and Methods:* A cross-sectional analytic study was conducted that included 45 samples positive for HPV-16 and 45 samples positive for HPV-18 from patients with cervical cancer or precursor lesion. Real time PCR was employed to determine the number of copies /10^3 cells. Viral load was determined in the two groups of patients and correlated with tumor grade. *Results:* THe authors found that the HPV-16 viral load was greater than that of HPV-18 through a Mann-Whitney U analysis, resulting in a $p = 0.000$; as the malignancy of the cervical lesion progressed, the viral load increased, and HPV-16 showed a moderate positive association with an $r = 0.509$ and a $p = 0.000$, whereas HPV-18 showed a weak positive correlation with an $r = 0.372$ and a $p = 0.012$. *Conclusions:* The viral load of HPV-16 was greater than that of HPV-18. The HPV-16 viral load had a moderate positive association in relation to cervical lesion severity, whereas the viral load of HPV-18 had a weak positive correlation with respect to the cervical lesion grade.

Key words: Human papillomavirus; Viral load; Cervical lesion.

Introduction

Invasive cervical cancer (ICC) is an important cause of cancer-related morbidity and mortality among women worldwide, with geographic variation [1]. In 2013 in Mexico, 3,113 new cases of malignant uterine neck tumor and 37,633 new cases of human papillomavirus (HPV) were registered [2]. In 2012 malignant tumors of the uterine cervix were the second cause of death by cancer in the State of Colima. In women, 17.3% of the deaths from malignant cancer corresponded to uterine neck disease and 16.5% to breast cancer [3]. Genital infection from HPV is the most frequent sexually transmitted viral disease worldwide [1] and the most important etiologic factor for the development of invasive cervical cancer [4, 5]. At present, 120 types of HPV have been characterized that are based on their DNA sequence [6]. Of these, 51 types affect the genital epithelial mucosa and are divided into three groups based on their epidemiologic association with cervical cancer: 14 are high-risk, six are possibly high-risk, and 31 are low-risk [7]. The DNA of high-risk HPV types has been found in 99.7% of cervical cancer tissue, and persistent infection, particularly with HPV-16 and HPV-18, is acknowledged as a cause of cervical cancer [8]. Even though HPV is a necessary cause of the disease, the high prevalence of transient HPV infection makes the detection or absence of the virus an inefficient means for identifying women at-risk for developing cervical cancer [9]. Viral load has been suggested as a non-transient infection marker and high HPV loads in smears with normal cytology have been associated with the development of dysplasia and carcinoma *in situ* [10, 11].

Materials and Methods

Study population

The study was conducted at the University Center of Biomedical Research in Colima, Mexico. It was a cross-sectional comparative study in which samples were obtained from patients with histopathologic diagnoses of cervical intraepithelial neoplasia (CIN) I, CIN II, CIN III, and cervical cancer. The patients were recruited from the Dysplasia Clinic of the Hospital Regional Universitario in the city of Colima and the cervical samples were obtained through exfoliate cytology (cytobrush).

All patients were included that had a positive diagnosis for HPV-16 and HPV-18 determined through end-point PCR and that had not received antiviral treatment at the time of their inclusion. A general medical history was carried out, registering the following variables to be analyzed: age, age at menarche, age of first sexual encounter, number of pregnancies and children, family history of cancer, previous sexually transmitted infections, use of birth control, and a history of smoking. Place of residence, civil status, and educational level were also included. Study participation was vol-

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Table 1. — *Primers and probes for the real time PCR.*

Name	Primer / Probe	Sequence (5' to 3')
F16E7	Primer	AGCTCAGAGGAGGAGGATGAA
R16E7	Primer	GGTTACAATATTGTAATGGGCTC
S1.1	Probe	FAM-CCAGCTGGACAAGCAGAACCGG-TAMRA
F18E1	Primer	CATTTTGTGAACAGGCAGAGC
R18E1	Primer	ACTTGTGCATCATTGTGGACC
S1.2	Probe	FAM-AGAGACAGCACAGGCATTGTTCCATG-TAMRA
HMBS F	Primer	GCCTGCAGTTTGAAATCAGTG
HMBS R	Primer	CGGGACGGGCTTTAGCTA
S3	Probe	FAM-TGGAAGCTAATGGGAAGCCCAGTACC-TAMRA

untary and the patients signed statements of informed consent. The institutional review committee of the School of Medicine of the Universidad de Colima and the Health Department of the State of Colima approved the study, which was conducted in accordance with the Declaration of Helsinki (1964, amended in 2014 in Japan).

Sample collection and DNA extraction

The cytobrushes were deposited in 1.5 ml micropipettes containing 500 µl of lysis buffer (Tris-Cl 100 mM pH 8.8, EDTA 5 mM, SDS 0.5%, NaCl 100 mM, and 30 µl Proteinase K 10 mg/ml) as the transport medium. DNA extraction was developed through centrifuging the samples at 10,000 rpm for 25 minutes; 50 µl of Proteinase K (10 mg/ml) was added and the samples were incubated at 37°C for 48 hours. A phenol:chloroform:isoamyl alcohol solution (25:24:1) was used for extracting the DNA, which was precipitated with ethanol at 70% and 3M sodium acetate. Finally, the samples were re-suspended in 100 µl of TE 1X buffer. Spectrophotometry determined the quantity and purity of the DNA.

Standard curve preparation

To standardize the results, dilutions of 10^8 to 10^2 copies were prepared for HPV-16 using SiHa cell DNA, and for HPV-18 using HeLa cell DNA. Homo sapiens hydroxymethylbilane synthase (HMBS) (GenBank No. M95623) was used for normalization. Standard curves helped to determine the viral load for each of the samples.

Viral type quantification

Real time PCR amplification was developed at a total volume of 10 µl, containing: 1X of Master Mix (FastStart Taq DNA polymerase, reaction buffer, $MgCl_2$, and dNTP mix), 0.5 µM of each primer, 0.1 µM of the probe, and two µl of genomic DNA. Amplification and detection were developed using thermocycler version 1.5 as follows: pre-incubation: one cycle at 95 °C, ten minutes; amplification and quantification: 45 cycles at 95 °C, ten seconds, 57°C, 20 seconds, 72°C, one second; and cooling: one cycle 40°C, 30 seconds. The primers and probes were those proposed by Moberg *et al.* (Table 1) [12].

Table 3. — *Viral load according to lesion grade.*

	CIN I*	CIN II*	CIN III	Cervical Ca
	(copies/10^3 Cells)			
HPV-16	$6.81E^{+02}$	$3.66E^{+04}$	$3.99E^{+04}$	$4.18E^{+05}$
HPV-18	$3.27E^{+01}$	$2.2E^{+01}$	$4.83E^{+04}$	$2.04E^{+04}$

*statistically significant viral load difference.

Real time PCR data analysis

The Lightcycler version 4.0 software was used for sequence detection and a file with raw data was produced. The software was developed for calculating the threshold cycle, thus making the number conversion of the HPV copies per cell.

Statistical analysis

Frequencies and the Kolmogorov-Smirnov test were used for verifying data normalcy. The Mann-Whitney U test was used for the qualitative variable of viral load and the Spearman correlation coefficient was used for the variables of viral load and neoplasia grade.

Results

Forty-five samples that were positive for HPV-16 and 45 samples positive for HPV-18 were included in the study. In accordance with the histologic diagnosis, 26 (57.7%) of the HPV-16 samples were CIN I, five were CIN II (11.1%), eight were CIN III (17.7%), and six were cancer (13.3%). For the HPV-18 group, 31 samples were CIN I (68.8%), five were CIN II (11.1%), three were CIN III (6.6%), and six were cancer (13.3%). The mean ages were 39 (SD±10) and 40 (SD±11) years for the patients with HPV-16 and HPV-18, respectively. Table 2 shows the main socio-demographic characteristics. The viral load data were not parametric according to the Kolmogorov-Smirnov test.

The comparison of the viral load between HPV-16 and HPV-18 in accordance with cervical epithelial lesion grade was statistically significant in the CIN I (p = 0.001) and

Table 2. — *The most frequent socio-demographic aspects of the population studied.*

Variable	HPV-16 (%)		HPV-18 (%)	
Place of residence	Colima 48.9%	Out-of-town 51.1%	Colima 48.9%	Out-of-town 51.1%
Level of education	Primary 55.6%	Secondary or higher 44.4%	Secondary 40%	Professional or higher 60%
Civil status	Married 73.3%	Single 26.7%	Married 53.3%	Single 46.7%
Smoking	Positive 28.9%	Negative 71.1%	Positive 11.1%	Negative 88.9%
Previous STI infection	Positive 13.6%	Negative 86.4%	Positive 4.4%	Negative 95.6 %
Contraceptive use	No 28.9%	Yes 71.1%	No 40%	Yes 60%

STI= Sexually transmitted infections.

Table 4. — *Comparison of HPV-16 and HPV-18 viral loads.*

	N	Minimum (copies/10^3 Cells)	Maximum (copies/10^3 Cells)	Median (copies/10^3 Cells)	Standard deviation	Mann-Whitney U *p*
HPV-18	45	$5.05E^{-01}$	$5.79E^{+05}$	$4.17E^{+01}$	$9.13E^{+04}$	0.000
HPV-16	45	$2.08E^{+00}$	$9.87E^{+05}$	$1.16E^{+04}$	$2.49E^{+05}$	

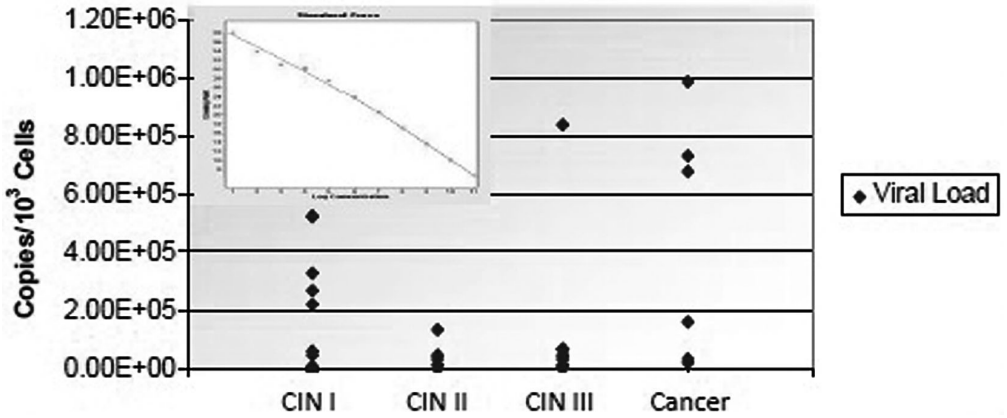

Figure 1. — HPV-16 viral load dispersion with their lesion grades.

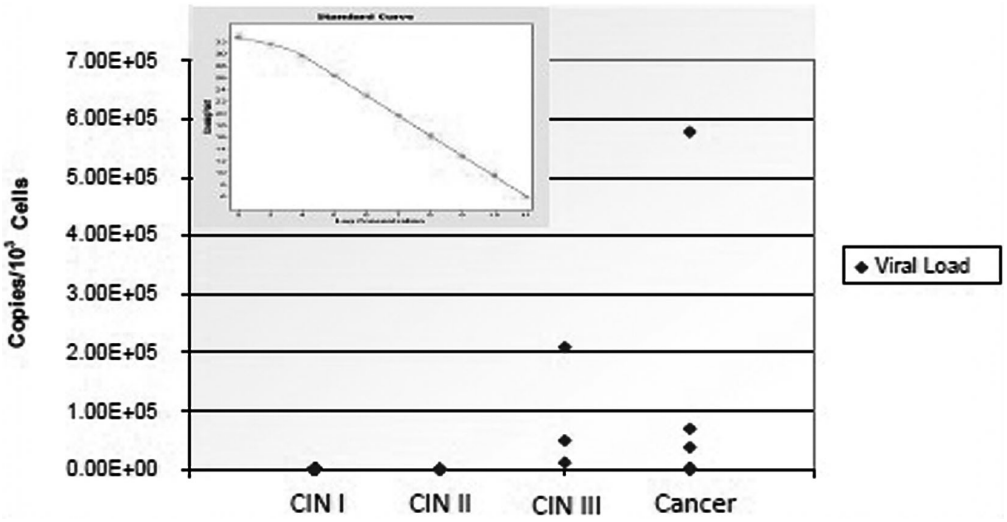

Figure 2. — HPV-18 viral load dispersion with their lesion grades.

CIN II ($p = 0.009$) groups, respectively (Table 3).

The HPV-16 and HPV-18 viral loads were compared using the Mann-Whitney U test, with a resulting $p = 0.000$, and therefore the difference between the viral loads of the two HPV types was considered statistically significant (Table 4).

The Spearman correlation coefficient was used to calculate the strength of the relation between the "lesion grade" and the "viral load" variables. For HPV-16, it produced a $r = 0.509$, with a $p = 0.000$, resulting in a moderate positive association (Figure 1), whereas for HPV-18 it produced a $r = 0.372$, with a $p = 0.012$, resulting in a weak positive correlation (Figure 2).

Discussion

This study on patients with different grades of cervical neoplasia showed that the normalized HPV-16 viral load was higher than that of HPV-18, with a statistically significant difference of $p = 0.000$. The present authors believe that this significant difference between the two HPV types is supported by the fact that HPV-18 may be associated with a more aggressive form of cervical cancer than other types of HPV [13].

Some researchers state that HPV-18 may be associated with a more rapid development of progression toward cervical cancer [14] and they have demonstrated that HPV-18 DNA was five-fold more efficient than HPV-16 DNA for the transformation of keratinocytes in vitro [15]. It has also been suggested that HPV-18 DNA is approximately ten- to 50-fold more efficient than HPV-16 DNA in the immortalization of epithelial cells, making it more aggressive [16]. Additionally, it has been observed that early viral protein expression, including the oncogenes, may be directed from a second promoter located within the long control region (LCR) of HPV-18 [17]. In summary, there is evidence suggesting that HPV-18 has a greater oncogenic capacity than HPV-16, perhaps due to the fact that the LCR, the main promoter of the E6 and E7 oncogenes of HPV-18, is more potent than the HPV-16 promoter [16]. If HPV-18 has a stronger promoter and thus a probable greater oncogenic potential; this might explain why an infection with a HPV-18 viral load lower than that of HPV-16 could be sufficient for altering the cervical epithelium and causing a CIN. One of the limitations of the present study lies in the comparison of the results with those already published. This is due to the fact that there is no current international consensus as to which units should be employed in the quantification of HPV viral loads. Among the different units found in the literature were quantity of virus per volume, viral load expressed in IU, viral load by viral count, an exponential format, a logarithmic format, viral load by weight, and copies/hge. This makes it impossible to discuss or compare the results in the literature, and the fact that different instruments and chemical processes are used for viral quantification and that distinct nomenclatures are used for describing the different grades of cervical lesion, only add to the difficulty. The present results in regards to the viral loads of HPV-16 and HPV-18 suggest that as the grade of malignancy increases, so does the viral load. These results are similar to those in the literature that report an increase in the odds ratio as the severity of disease increases [18]; it has also been concluded that there was both a high positive association between high-risk HPV infection and the development of CIN [19], as well as a significant increase in viral load when the grade of lesion malignancy increased [20].

Conclusions

HPV-16 viral load is greater than HPV-18 viral load in different grades of CIN. A lower viral load of HPV-18, compared with that of HPV-16, was sufficient for causing a cervical epithelial lesion of the same severity. The HPV-16 viral load had a moderate positive association with the grade of cervical lesion malignancy, whereas the HPV-18 viral load had a weak positive correlation with the grade of severity of the cervical lesion.

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Address reprint requests to:
J. GUZMÁN-ESQUIVEL, M.D.
Investigador A-D Instituto Mexicano
del Seguro Social, Del. Colima
Av. De los Maestros 149, Centro
Colima Col., CP 28000 (Mexico)
e-mail: pepeguzman_esquivel@outlook.com