Correlation of cyto-colpo-histology in human papillomaviruses (HPV) lesions and the use of PCR technique in determining HPV infection


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Resumen

Introducción: El cáncer de cuello uterino es una patología prevalente más aun en países sub-desarrollados y existe una relación causal entre la infección persistente con algún tipo oncogénico de HPV y el desarrollo de dicha neoplasia. Se cuenta con un estudio de screening mundialmente aceptado y que también previene el carcinoma cervical que es el Papanicolaou y colposcopia, y en los últimos años se han incorporado estudios de biología molecular para el estudio del ADN viral.

Material y Métodos: Se estudiaron 60 pacientes. Se tomó como método gold standard a la biopsia de cuello uterino y a 29 pacientes se les realizó PCR para identificar ADN-HPV. La edad promedio de las pacientes fue de 30.95 años con mayor cantidad de pacientes en el rango etario de 21-25 años.

Resultados: El 70% tuvo citología negativa y 48 pacientes tuvieron biopsia positiva, 16 con resultado positivo de PCR. Obtuvimos una alta especificidad el Papanicolaou con respecto a la biopsia (90%), no así en comparación con el test de PCR (S=56% E=50%) debido al número pequeño de pacientes que se sometieron a este estudio.

Discusión: Las mujeres menores de 30 años son las que más

Abstract

Introduction: Cervical cancer is a prevalent pathology, especially in underdeveloped countries, and there is a causal relationship between persistent infection with some type of oncogenic HPVs and the development of said neoplasia. There are two universally-accepted screening studies which also prevent cervical cancer, i.e. Pap smear and colposcopy, and, in the last few years some molecular biology studies have been adopted for the study of viral DNA.

Material and methods: 60 patients were studied. Cervical biopsy was the gold standard method used and 29 patients were tested using the PCR technique to identify HPV-DNA. Patients’ average age was 30.95, most of whom belonged to the 21-25 years old age span.

Results: 70% of patients had negative cytology test and 48 patients obtained positive biopsy results, 16 had positive results in the PCR test. The Pap smear specificity was higher as regards the biopsy (90%), unlike the PCR test (S=56% E=50%) due to the small number of patients that underwent this study.

Discussion: Women younger than 30 years old have more chances of infection, but no of developing the disease considering its natural history. Therefore, we recommend continuing with the
chances tienen de contraer la infección, no así de desarrollar la enfermedad debido a la historia natural de la misma, por lo que aconsejamos continuar con el screening regular de Papanicolaou/colposcopia e introducir, con lapsos de 3 años o más en mujeres mayores a 30 años, los estudios de biología molecular.

Conclusiones: Se espera poder contar con mayores estrategias que nos permitan usar los beneficios del test ADN-HPV sin sobretratar a mujeres que probablemente atraviesan por una infección transitoria.

Palabras Clave: Papiloma Humano, PCR

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Introduction

Nowadays, cervical cancer is a highly prevalent pathology, especially in underdeveloped countries.

Every sexually active woman runs the risk of contracting cervical cancer in her life, and thus it can be considered a sexually transmitted neoplasia, since this is the only way of contracting the virus.

It is known that there is a steady causal relationship between persistent infection with some type of oncogenic HPVs and cervical cancer\(^{(1,2)}\), (99% of cancer is HPV-related), this risk being higher that the smoking-lung cancer association \(^{(21)}\). Said infection progresses from preneoplastic lesions (cervical intraepithelial neoplasia) to invasive cancer.

In human beings, it is an epitheliotropic virus which transfects the mucous membranes of the genital organs and the keratinized epithelium of the vulva, perineum, penis and the area of the anus and rectum\(^{(1,5,18)}\).

More than 100 types of HPVs have been identified, and the genital types have been divided according to their oncogenic potential:

<table>
<thead>
<tr>
<th>Risk Level</th>
<th>Types of HPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIGH RISK</td>
<td>16, 18, 31, 45, 33, 35, 39, 51, 52, 56, 58 and 59</td>
</tr>
<tr>
<td>MEDIUM RISK</td>
<td>26, 53, 66, 68, 72, and 82</td>
</tr>
<tr>
<td>LOW RISK</td>
<td>6, 11, 40, 42, 43, 44, 54, 61, 70, 72 and 81, (They are relate to low-grade squamous intraepithelial lesions (L-SIL) and condyloma acuminata, they are not capable of integrating into the human genome)(^{(1,18)}).</td>
</tr>
</tbody>
</table>

The union of the stratified squamous epithelium of the vagina and ectocervix with the endocervical canal columnar epithelium is known as original squamocolumnar junction. The cylindrical epithelium is replaced by undifferentiated, immature metaplastic epithelium thanks to the vaginal flora and the acidity of the environment, originating mature stratified metaplastic squamous epithelium, almost indistinguishable from the original epithelium, known as the new squamocolumnar junction (observed in a colposcopy)\(^{(1,5,7,18)}\).

The area between the original squamocolumnar junction and the colposcopic junction is called Transformation Zone (area where most of the cervical neoplasias are originated).

The HPV genome integrates into the host’s chromosomes through E6 and E7 proteins, which immortalize the cervical epithelium keratinocytes,
which triggers genomic instability. E6 protein combines with the p53 gene, preventing apoptosis and increasing the telomerase activity. E7 protein combines with the retinoblastoma gene (tumor suppressor). More than three individual mutations are necessary for the malignant cell transformation.\(^{1,19}\)

HPV infection is necessary but not sufficient for inducing a carcinoma in an immunocompetent host, other influencing factors are: combined oral contraceptives, smoking, infections with other microorganisms, dietary factors.

Morphology of intraepithelial squamous lesions is characterized by the abnormal cell maturity, nuclear growth, atypia and mitosis. 60% of the cervical intraepithelial neoplasia (CIN) 1 recurs, 30% persists and 11% progresses to carcinoma in situ. In the case of CIN 2, 40% recurs, 40% persists, 20% progresses to carcinoma in situ and 5% progresses to invasive carcinoma, whereas for CIN 3\(^{1}\), there is 33% of recurrence, 56% of persistence and more than 12% of progression to invasive carcinoma.

Prospective trials in young women show high HPV acquisition rates and the average duration of these infections is less than a year.\(^{6}\)

In our country, cervical cancer is the second most frequently diagnosed type of cancer. It is estimated that 3000 new cases are diagnosed and approximately 1800 women die every year due to this disease. In 1980, the mortality rate in our country due to this pathology was 7.12/100000 women; in 2000, it was 7.59/100000 and in 2009, 7.50/100000 women.\(^{10}\)

According to the WHO, in their June 2010 Report, in Argentina the prevalence of HPV infection 16/18 with normal cytology is 6.8%, 37;6% with L-SIL and 67, % for H-SIL against 3.8%; 24.3% and 51.1% worldwide in the same categories.\(^{20}\)

Nowadays, we rely on screening methods accepted worldwide which, in turn, help to prevent cervical cancer –the pap smear and the colposcopy, which are used together in our country.

During the last few years, molecular biology techniques for studying HPV DNA have been implemented.

**Objectives**

This paper aims at:

1. Knowing the sensitivity and specificity of the screening methods available in our country for determining HPV infection.
2. Correlating cytology and colposcopic images suspicious for HPV with the histology results.
3. Assessing the PCR sensitivity and specificity for determining the presence of viral DNA in selected patients.
4. Determining the prevalence of infection according to the age span.

**Material and Methods**

Descriptive (series of cases), retrospective, non-randomized trial, in order to evaluate the presence of cyto-colpo-histologic lesions related to HPV infection, in women at the Lower genital tract Section of the Hospital Aeronáutico Córdoba.

60 female patients were studied who went to the Lower genital tract Section, Gynecology Department of the Hospital Aeronáutico Córdoba, between March 2010 and December 2011, for a routine cytology and colposcopy screening.

The study included all women between the ages of 18 and 55, with colposcopic lesion suspicious for HPV infection at the time of the exam, and who did not have a clinical history of said virus infection.
The Pap smear test was carried out using an Ayre spatula to obtain an ectocervix sample and a cytobrush for endocervical sampling (rotating the collection device one full turn –360°– in both cases). The samples are fixed in 96% alcohol, and then they are stained according to the Pap technique and observed through a microscope. After obtaining the cytology sample, the cervix is cleaned and stained with 5% acetic acid using a cotton swab and the observed through the colposcope. The same procedure is carried out with Lugol's solution.

Abnormal cytologic findings included the presence of 18:
- Koilocytes
- Ascus (Atypical squamous cells of undetermined significance)
- Mild, moderate, or severe dysplasia (CIN I-II-III)

Abnormal colposcopic findings were 14:
- Aceto-white epithelium
- Base
- Mosaic
- Any combination of the ones mentioned above.

Every patient with colposcopic lesion suggestive of HPV infection was called again for a biopsy and 29 patients were referred to be performed the HPV DNA test (in our case, PCR technique was performed). For the histological assessment, a biopsy of the affected region was carried out with punch forceps, it is fixed in 40% formaldehyde and, depending on the sample size, it was cut into two or more halves, and paraffin blocks were prepared, sections cut with microtome, hematoxylin-eosin (H&E) staining and observation through an optical microscope. Results were informed as follows: the presence of koilocytes as HPV-suggestive lesion; mild dysplasia as L-SIL; and moderate or severe epithelial dysplasia as H-SIL; the cases of carcinoma in situ, were informed as such.

If the HPV DNA test was requested, before the biopsy was performed, the corresponding sample for said test was taken and sent to the specialized laboratory to be examined.

The cervical biopsy was used as Gold Standard diagnostic test.

Epidemiologic data gathered were analyzed for all the variables using Microsoft Excel charts and they were statistically analyzed using OpenEpi (www.openepi.com) software.

**Results**

The average age of the patients studied was 30.95 years, with a standard deviation of 10.42 years.

The range was 37 years, with a minimum age of 18 and a maximum age of 55.

The histogram shows the distribution of patients according to their age, with a resulting left (negative) skew. 70% of patients (42) with colposcopic image suspicious for HPV infection obtained negative cytology for said infection and just 30% (18) was positive.
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Out of the 60 female patients studied, only 48 had a positive histology for HPV infection.

Charts:

**Chart N° 2**: Proportion of patients according to cytology

- cytology 30%
- cytology 70%

**Chart N° 3**: Distribution of patients according to histology result

Number of patients

<table>
<thead>
<tr>
<th>Biopsy result: negative/positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>bx negativa</td>
<td>29</td>
</tr>
<tr>
<td>bx positiva</td>
<td>31</td>
</tr>
</tbody>
</table>

29 patients were referred for HPV DNA evaluation, out of which 16 (55.17%) obtained a positive result and 13 (44.83%) did not present viral DNA.

The results obtained are included in the following tables:

**Table N° 1**: Pap smear sensitivity and specificity as compared to the Gold Standard

<table>
<thead>
<tr>
<th></th>
<th>SENSITIVITY</th>
<th>SPECIFICITY</th>
<th>PPV</th>
<th>NPV</th>
<th>DIAGNOSIS ACCURACY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>34.95%</td>
<td>90.91%</td>
<td>94.44%</td>
<td>23.81%</td>
<td>45%</td>
</tr>
<tr>
<td>CI 95%</td>
<td>22.92-48.69</td>
<td>62.26-98.38</td>
<td>74.24-99.01</td>
<td>13.48-36.53</td>
<td>33.09-57.51</td>
</tr>
</tbody>
</table>

**Chart N° 4**: Proportion of patients according to PCR result

29 patients were referred for HPV DNA test, out of which 16 (55.17%) obtained a positive result and 13 (44.83%) did not present viral DNA.

Once the patients were selected and with the definite results of the studies performed to each of them (cytology, colposcopy, biopsy, PCR technique) sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated, comparing the method used as gold standard with the cytology and the molecular biology study; and then the screening methods available in our research center with the HPV DNA test.

**Table N° 2**: HPV DNA (PCR) test sensitivity and specificity as compared to the Gold Standard

<table>
<thead>
<tr>
<th></th>
<th>SENSITIVITY</th>
<th>SPECIFICITY</th>
<th>PPV</th>
<th>NPV</th>
<th>DIAGNOSIS ACCURACY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>56.00%</td>
<td>50.00%</td>
<td>87.5%</td>
<td>15.38%</td>
<td>55.17%</td>
</tr>
<tr>
<td>CI 95%</td>
<td>37.07-73.33</td>
<td>15-85</td>
<td>63.98-96.5</td>
<td>4.326-42.24</td>
<td>37.55-71.59</td>
</tr>
</tbody>
</table>

**Table N° 3**: Pap smear + Colposcopy sensitivity and specificity as compared to the HPV DNA (PCR) test

<table>
<thead>
<tr>
<th></th>
<th>SENSITIVITY</th>
<th>SPECIFICITY</th>
<th>PPV</th>
<th>NPV</th>
<th>DIAGNOSIS ACCURACY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>66.67%</td>
<td>64.29%</td>
<td>66.67%</td>
<td>64.29%</td>
<td>65.52%</td>
</tr>
<tr>
<td>CI 95%</td>
<td>41.71-84.82</td>
<td>38.76-83.66</td>
<td>41.71-84.82</td>
<td>38.76-83.66</td>
<td>47.34-80.06</td>
</tr>
</tbody>
</table>

Out of the 16 patients with positive HPV DNA test, 50% was for low risk HPV, 37.5% for high risk HPV (prevailing subtype 58) and 12.5% was for intermediate risk.
All patients infected with high risk HPV were younger than 30 at the time of the study.

**Discussion**

The tests used to determine the presence of viral DNA are more sensitive and not only do they identify women with cervical disease, but also those at risk of developing CIN (Cervical intraepithelial neoplasia) in the next 10 years. When combined with cytology, in patients having both tests with negative results, there is a higher probability of no risk of cervical cancer.²,²⁹,¹¹

PCR (polymerase chain reaction) is a molecular biology technique thanks to which great numbers of copies of a specific DNA fragment are obtained. It is based on DNA polymerases natural property to replicate DNA strands, for which high-and-low-temperature cycles are used in order to separate the strands and then leave them to unite again, this time using a primer (complementary sequence in the DNA template). It is a highly sensitive technique since a small DNA amount is necessary in order to obtain a great number of copies. For HPV detection, GP5+/GP6+ primers are used, which amplify approximately 150 base pairs from the L1 region of 22 HPV types (6-11-13-16-18-30-33-35-39-40-43-45-51-52-54-55-56-58-59-66), and if result is positive, primers are used to detect E6 of HPV 6-11-16-18-31-33¹⁷. The result obtained was expressed according to the HPV number involved in the lesion, if viral DNA presence was positive, or according to the lack of detection of DNA in the analyzed sample.

Genomic material detection tests are recommended for patients with abnormal findings in the cytology and/or colposcopy. They are also recommended for cervical cancer primary screening in women older than 30 years of age, since their use in adolescents is controversial considering that this population shows new and transitory infections, but no malignant pathology due to the long lag period between infection and development of carcinoma, and they can be considered high-risk patients when the infection is destined to resolve itself.¹¹,²¹

Women older than 30 years of age who underwent cytology/colposcopy screening and HPV DNA test obtained a CIN 2-3 risk decrease of approximately 40% and a similar carcinoma decrease.¹¹

In our center, Pap smear and colposcopy are used as screening methods to detect cervical carcinoma, studies which combined have 91% sensitivity and 96% specificity; but in most gynecology centers only cytology is used as screening method, which has a sensitivity of 48%, much lower than HPV DNA tests (95%).¹⁴

PCR technique for HPV is very sensitive, since the presence of just 10 copies of viral genome turns the test positive, as compared to the 5000 copies necessary for a positive hybrid capture.¹⁷

In our study, the cervical biopsy was considered as the gold standard method used in the population examined in order to determine the presence or not of HPV lesion, with 34.69% sensitivity and 90.91% specificity of the Pap
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Correlation of smear and colposcopy as regards the biopsy, with a diagnostic accuracy of 45% and a PPV of almost 95%. This information matches world literature.

As regards HPV DNA test sensitivity and specificity against the biopsy, results were 56% and 50%, with a diagnostic accuracy of 55% and a PPV of 87.5%. Similar sensitivity and specificity results were obtained for Pap smear and colposcopy as regards PCR technique; therefore, sample size of this preliminary study is not considered pertinent, since not all patients were performed viral DNA studies.

Conclusions

HPV infection is a disease afflicting our society which increases the risk of cervical neoplasia with the consequential high mortality rate, especially in underdeveloped countries.

Women under the age of 30 have more chances of infection worldwide, but said infection is usually transitory, therefore only frequent screening methods are recommended (pap smear and colposcopy followed by biopsy eventually), since, as was stated above, the lag period between the infection and the manifestation of the disease takes many years.

It is indeed recommended in women over the age of 30 to add the genomic study to the regular screening, at least every 3 years, since there are more chances of a viral DNA positive result to correlate with the persistent infection in said age span, with the previously mentioned consequences.

The number of patients subjected to viral DNA studies is still to be increased in order to obtain a better correlation of our studies with the reviewed literature and re evaluate already examined patients, especially those whose molecular biology study was positive, either younger or older than 30 years old. The aim is to follow the behavior of the disease according to its natural history.

The challenge is to develop clinical strategies which enable the use of HPV-DNA test benefits, without alarming or overtreating a large number of women, while efficiently using the screening methods on which we count, since as was demonstrated earlier, they are very useful in patients of all ages, their cost-benefit ratio is acceptable and are available to the entire population.

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