

# Human papillomavirus and infections of the lower genital tract in women with abnormal cervical cytological examination

*Papilomavírus humano e infecções do trato genital inferior em mulheres com exame colpocitológico anormal*

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## ABSTRACT

**Aims:** To study the associations of positive human papillomavirus (HPV) DNA in the female genital tract with *Gardnerella vaginalis*, *Trichomonas vaginalis* and *Candida sp.* vaginitis, and with possible socio-demographic risk factors for HPV infections.

**Methods:** The study was conducted with 208 patients with abnormal cervical cytological examination assisted at the Gynecology Service of Central Clinic of Caxias do Sul University, Rio Grande do Sul, Brazil, analyzing the presence of HPV DNA by polymerase chain reaction and associating the results to diagnosis of vaginal infections obtained from the medical charts of the patients.

**Results:** HPV DNA was detected in 60.1% of all cases analyzed; 93% of them presented simple infections and 6.9% showed multiple infections. Seventeen viral types were identified, being HPV16 the most frequent (38.8%). At least one of the vaginitis investigated was observed in 57.2% of the patients. *Gardnerella vaginalis* was found in 45.7%, *Candida sp.* in 20.2%, and *Trichomonas vaginalis* in 3.8% of the patients examined. No statistical association between the presence of HPV DNA and other infection of the lower genital tract or socio-demographic risk factors was observed.

**Conclusions:** No association of HPV infection with other infections of the female genital tract or with socio-demographic features could be found. Multiple infections with up to five types of HPV were observed in a low percentage of women. It is difficult to clearly define a group at high risk for HPV infection. All women with abnormal cervical cytological examination are possible carriers of HPV and other infections of the lower genital tract.

**KEY WORDS:** PAPILOMAVIRUS INFECTIONS; HUMAN PAPILOMAVIRUS; VAGINITIS; *GARDNERELLA VAGINALIS*; *CANDIDA*; *TRICHOMONAS VAGINALIS*; UTERINE CERVICAL DYSPLASIA; CERVICAL SMEARS.

## RESUMO

**Objetivos:** Estudar as associações da positividade para o DNA de papilomavírus humano (HPV) no trato genital inferior feminino com as vaginites por *Gardnerella vaginalis*, *Candida sp.* e *Trichomonas vaginalis* e com a presença de possíveis fatores de risco para infecção por HPV.

**Métodos:** O estudo foi realizado com 208 pacientes com exame colpocitológico anormal atendidas no Serviço de Ginecologia do Ambulatório Central da Universidade de Caxias do Sul analisando a presença do DNA de HPV através de reação em cadeia da polimerase e associando os resultados aos diagnósticos de infecção vaginal obtidos nos prontuários das pacientes.

**Resultados:** Foi detectado DNA de HPV em 60,1% de todos os casos analisados, sendo que 93% apresentaram infecções simples e 6,9% apresentaram infecções múltiplas. Dezesete tipos virais foram identificados, sendo HPV16 o mais frequente (38,8%). Pelo menos uma das vaginites investigadas foi observada em 57,2% dos pacientes. *Gardnerella vaginalis* foi encontrada em 45,7%, *Candida sp.* em 20,2%, e *Trichomonas vaginalis* em 3,8% das pacientes examinadas. Não foi observada nenhuma associação estatística entre a presença do DNA de HPV e outras infecções do trato genital inferior ou fatores sociodemográficos de risco.

**Conclusões:** Não houve associação da infecção pelo HPV com outras infecções do trato genital feminino nem com características sociodemográficas. Múltiplas infecções com até cinco tipos de HPV foram observadas em uma pequena parcela das mulheres. É difícil definir claramente um grupo de risco para a infecção por HPV. Todas as mulheres com exame colpocitológico anormal são possíveis portadoras do HPV e de outras infecções do trato genital inferior.

**DESCRIPTORIOS:** PAPILOMAVIRUS HUMANO; INFECÇÕES POR PAPILOMAVIRUS; VAGINITE; *GARDNERELLA VAGINALIS*; *CANDIDA*; *TRICHOMONAS VAGINALIS*; DISPLASIA DO COLO DO ÚTERO; EXAME COLPOCITOLÓGICO.

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Recebido em 16/05/2012; aceito em 30/12/2012

## INTRODUCTION

Cervical carcinoma is the second most common neoplasia in women worldwide and *Human papillomavirus* (HPV) is the most known sexually transmitted viral infection.<sup>1</sup> The association of HPV with cervical dysplasia has been reported<sup>2</sup> but only a small number of HPV-infected women progress to cervical neoplasias, suggesting that other co-factors may interact together with HPV, such as sexually transmitted diseases, smoking, hormone rates, sexual behavior and immune responses.<sup>3,4</sup>

Clinical reports indicate that there are associations between an imbalance of the vaginal flora and cervical neoplasia atypias, including bacteriosis, trichomoniasis and candidiasis.<sup>5</sup> The bacterial vaginosis caused by *Gardnerella vaginalis* produces major changes in the vaginal ecosystem, mainly because of over-population that replaces the lactobacilli normally present in vaginal secretions.<sup>6</sup> These microbiological changes are also produced by candidiasis, which modifies the normal production of hydrogen peroxide and lactic acid, reducing vaginal pH, increasing available substrate and suppressing restrictive effect of the lactobacillary flora.<sup>7</sup> Candidiasis may show symptoms such as itching and irritation, with inflammatory characteristics. Like candidiasis, trichomoniasis also develops inflammatory responses, generating interactions of the parasite responsible for this sexually transmitted disease with the squamous epithelium, increasing vascularization, edema, erosion of surface layers and in some cases even necrosis.<sup>8</sup>

The purpose of this study was to study the associations of positive HPV DNA in the lower female genital tract with *Gardnerella vaginalis*, *Trichomonas vaginalis* and *Candida sp.* vaginitis, and with possible risk factors for HPV infections in patients of the Gynecology Service of Central Clinic of Caxias do Sul University, Rio Grande do Sul, Brazil.

## METHODS

A total of 208 women were attended between November 2000 and November 2002 at the Lower Genital Tract Pathology Outpatient Clinic of the University of Caxias do Sul, Rio Grande do Sul state, Brazil. These patients were previously referred by primary health care by having changes in cytological diagnosis, either compatible with HPV infection, or presenting different grades (I, II, III) of cervical intraepithelial neoplasia (CIN).

Informed consent was obtained from patients and the study was approved by the institutional Ethics

Committee. Patients were questioned upon sexual and reproductive behavior, use of contraceptives and smoking habits.

## Collection of uterine cervical smears

Two samples of uterine cervical smears were collected from each patient with a cytobrush. One of them was placed on a slide for cytological examination and the other was dipped in 1 ml TE (Tris HCl 10 mM, EDTA 1 mM) for molecular analyses.

## Detection of HPV DNA

Viral DNA was obtained by extracting total DNA using the Gentra Systems commercial kit Puregene™ from Buccal Cells (Minneapolis, MN, USA). The samples were tested for the presence of HPV DNA by polymerase chain reaction (PCR), amplifying a segment of 450bp from a conserved L1 region of the virus delimited by primers MY9 and MY11 according to Hildesheim et al., 1994.<sup>9</sup> The samples were previously submitted to amplification 268bp segment of the human B-globin gene with primers PCO4 and GH20 to evaluate the quality of the DNA sample of the host cells, as described in Saiki et al., 1988.<sup>10</sup> A negative and a positive control were included to all amplifications, with exception of genomic DNA, with HPV16 DNA extracted from the cells of SiHa lineage (kindly given to us by Dr. LL Villa, Instituto Ludwig para Pesquisa sobre o Câncer, São Paulo, Brazil) (Paesi et al., 2009<sup>11</sup>).

## Viral typing

HPV-positive samples were typed using restriction fragment length polymorphism (RFLP) according to Bernard et al. 1994. Briefly, amplicons generated from primers MY9/MY11 were digested in 50 mM Tris-HCl and 10 mM MgCl<sub>2</sub> by the restriction enzymes *Bam* HI, *Dde* I, *Hae* III, *Hinf* I, *Pst* I, *Rsa* I and *Sau* 3AI (10 U/μL, GibcoBRL, Gaithersburg, MD, USA). A molecular standard (Φ X174 RF cleaved with *Hae* III) and a non-digested HPV sample were included in each gel. Gels were silver stained, and fragment patterns were compared with the prototypes.

## Co-infection genotyping

Cases of co-infection identified by RFLP were tested again by reverse dot strip analysis using similar parameters as Gravitt et al. 1998,<sup>12</sup> that identifies 27 different types of HPV in a single sample.

## Cytological and histopathological reports

Cytopathological analyses were performed by Papanicolaou method using a Nikon Labophot transmission microscope, based on the Bethesda system for cytological diagnosis.<sup>9</sup> Based on cytological and histopathologic analyses, samples were classified as mild dysplasia (CIN 1), moderate dysplasia (CIN 2), severe dysplasia (CIN 3), presence of HPV, vaginitis, leukocytes, squamous metaplasia and cervical cancer. Data on diagnosis of vaginitis were obtained from medical records of patients either compatible with HPV infection, or presenting different grades (I, II, III) of CIN in cytological diagnosis.

The reports contained the Papanicolaou smear screening of ectocervical scraping and endocervical curettage, harvested with Ayres spatula and endocervical brush, extended on a glass slide and fixed with polyethylene glycol, and finally stained by the Papanicolaou method. The samples were examined by an anatomic-pathologist and outcomes were classified according to the Brazilian Nomenclature for Cytopathology Reporting and Bethesda System 2011.<sup>13</sup> As recommended, the patients underwent colposcopic examination to check for changes or lesions in biopsies. The collected material was packed in 500 uL of 10% formalin, and later embedded in paraffin sections and sectioned in 4mm thick, hematoxylin-eosin stained and subjected to histological examination.

## Questionnaires and statistical analysis

Data used in the present study were obtained from the baseline questionnaires including questions on socio-demographic characteristics, tobacco and alcohol consumption, menstrual status, history of sexually transmitted diseases, sexual behavior and contraceptives administration. Interview data and sampling results were analyzed on SPSS program-version 16.0. Pearson chi-square test with a 0.05 level of significance was applied for statistical analysis.

## RESULTS

This study was performed with women 13 to 69 years old. The highest rate of HPV infection occurred in women aged up to 24 years (68,6%). Women older than 45 years showed the lowest rates of HPV infection (53.3%) (Table 1).

All patients reported active sex life, and most had basic education and income up to 792 US dollars.

**Table 1.** Age distribution of women with abnormal cytological examination, according to detection of viral DNA in the lower genital tract.

Age (years)	Human papillomavirus DNA detection		
	n (total: 208)	Negative (%)	Positive (%)
20-24	51	16 (31.4)	35 (68.6)
25-45	119	52 (43.7)	67 (56.3)
>45	38	17 (44.7)	21 (53.3)

Most patients were married, with three years average relationship. Menarche and beginning of sex life occurred between age of 12 and 18 years. The group studied had mostly up to two sex partners in their lifetime and a single partner during the previous year. They had at least one pregnancy and no abortion. Most of them reported moderate consumption of medications, drugs, alcohol and tobacco. Statistical analysis of the socio-demographic characteristics did not show a direct significant association with detection of HPV DNA (Table 2).

Pathological analysis of the samples showed that 11.7% presented cervical carcinoma, 51.5% exhibited moderate to severe cytological abnormalities (CIN 2, CIN 3), 26.7% had mild dysplasia (CIN 1) and 10.1% showed no neoplastic cells. Of the 208 samples analyzed, 125 were HPV-positive (60.1%). The remaining of the group did not have the 450 bp fragment characteristic of the virus (39.9%).

Based on the HPV-positive patients, viral typing was performed on 72 samples. Sixteen different HPV genotypes were identified, four of them of low risk (HPV 6, 11, 61 and 62), 10 types classified as high risk (HPV 16, 18, 31, 33, 39, 45, 52, 56, 58 and 82) and two probably as high-risk (HPV 53 and 66) according to “Phylogenetic Epidemiologic Classification and HPV Types”, proposed by Muñoz et al.<sup>1</sup> Four samples could not be identified by the current method. Only five cases showed multiple virus infection and were identified by methods described by Gravitt et al.<sup>12</sup>

The cytopathological frequency found for bacterial vaginosis caused by *Gardnerella vaginalis* was the highest, and an intermediate frequency was found for *Candida sp.* vaginitis. More than half of all cases analyzed (119/208) had at least one of the three vaginal flora alterations investigated. No statistically significant results were found when associations between HPV-infected patients and positive tests for *Gardnerella vaginalis*, *Candida sp* and *Trichomonas vaginalis* were investigated (Table 3).

**Table 2.** Frequency of possible risk factors for human papillomavirus infections in women with abnormal cytological examination, according detection of viral DNA in the lower genital tract.

Risk factors	Human papillomavirus DNA detection		
	n (total: 208)	Negative (%)	Positive (%)
<b>Education</b>			
Up to primary	152	64 (42.1)	88 (57.9)
Secondary/Higher education	22	9 (40.9)	13 (59.1)
<b>Income</b>			
More than 792 dollars	160	67 (41.9)	93 (58.1)
Less than 792 dollars	16	6 (37.5)	10 (62.5)
<b>Marital status</b>			
Single	28	15 (53.6)	13 (46.4)
Married	113	43 (38.1)	70 (61.9)
Other	33	16 (4.4)	20 (55.6)
<b>Age at 1<sup>st</sup> sexual intercourse</b>			
Up to 18 years	154	58 (37.7)	96 (62.3)
More than 18 years	43	21 (48.8)	22 (51.2)
<b>N<sup>o</sup> of partners in lifetime</b>			
≤2	83	38 (45.8)	45 (54.2)
3-5	62	24 (38.7)	38 (61.3)
>5	26	10 (38.5)	16 (61.5)
<b>Pregnancies</b>			
No	34	15 (44.1)	19 (55.9)
Yes	168	66 (39.3)	102 (60.7)
<b>Smoking</b>			
No	114	52 (45.6)	62 (54.4)
Yes	89	29 (32.6)	60 (67.4)

**Table 3.** Association of positivity for human papillomavirus DNA with *Gardenerella vaginallis*, *Candida* sp. and *Trichomonas vaginallis* in women with abnormal uterine cervical cytological examination.

Vaginitis	Negative HPV DNA 83 (39.9%)		Positive HPV DNA 125 (60.1%)		Total n 208	p*
	n	%	n	%		
	<i>Gardnerella</i>					
No	47	41.6	66	58.4	<b>113</b>	0,294
Yes	36	37.9	59	62.1	<b>95</b>	
<i>Candida</i>						
No	63	38.0	103	62.0	<b>166</b>	0,190
Yes	20	47.6	22	52.4	<b>42</b>	
<i>Trichomonas</i>						
No	77	38.5	123	61.5	<b>200</b>	0,503
Yes	6	75.0	2	25.0	<b>8</b>	
<i>Gardnerella</i> or <i>Candida</i> or <i>Trichomonas</i>						
No	33	37.1	56	62.9	<b>89</b>	0,342
Yes	50	42.0	69	58.0	<b>119</b>	

HPV: human papillomavirus.  
\* Pearson test.

## DISCUSSION

The analysis of HPV infection showed that not every women with suspected infection by cytological tests carries HPV DNA. Similar results were described in a study with Mexican women, where patients at a colposcopy clinic showed HPV prevalence of 56.2%.<sup>14</sup> Prevalence values higher than those presented in

this work were shown in Peru, where women with a previous diagnosis of cervical lesion had a 72.9% prevalence of HPV.<sup>15</sup> Higher HPV prevalence were observed when samples were related to invasive cervical cancer, as shown in studies conducted in nine different countries, with 90% prevalence of HPV by PCR in 1918 confirmed cases of cervical cancer.<sup>1</sup> Samples collected from patients with suspected viral

infection who had negative results for HPV DNA indicates that PCR is a reliable diagnostic method compared to clinical examinations, as cytological and histological tests.

The age group with the highest HPV infection in this study (up to 24 years old) is in agreement with described higher infection peak around the age of 25 years old.<sup>16</sup> Infections occur mainly in young and sexually active women, normally younger than 25 years old<sup>17</sup> and support the findings that infections are established in early ages. According to data collected by questionnaires, we found that most of the women investigated had few lifetime sexual partners and had their first sexual intercourse earlier compared to results obtained in other regions of Brazil and worldwide.<sup>18</sup>

Multiple infections of HPV with up to five types were observed only in a low percentage of women (6.9%), which is much lower compared to studies of multiple HPVs also described with African (24%), German (28.1%), and Brazilian (14.7%) women.<sup>3,19,20</sup> Genotyping by reverse hybridization line probe showed a rate of 78.9% of cases with up to ten different types of HPVs described with immunosuppressed patients in Brazil.<sup>21</sup> Possibly the profile of the immune system has greater influence on the stabilization of HPV than co-infections. This is quite clear when one evaluates immunosuppressed HPV-positive women, usually presenting greater HPV infection than in healthy women.<sup>22</sup>

The evaluation of multiple infections is clinically relevant since many viral types presented in a patient sample show a greater predisposition to uterine cervix lesions.<sup>23</sup> Seventeen different viruses reported here were in agreement with the literature.<sup>3,24</sup> Confirming previous investigations,<sup>11,25</sup> high frequency of HPV 58 was found to be the second most prevalent virus after HPV 16. HPV frequencies observed in female patients in Latin America and Europe reveals that HPV 58 in female population is not as prevalent as our findings.<sup>1</sup>

Controversial data are observed, however, for vaginal co-infections and sexually-transmitted diseases in relation to risk factors for developing cervical cancer.<sup>3,16,26</sup> High frequency of co-habitation of other microorganisms with HPV in the vaginal tract led to investigations on the role of these organisms in relation to HPV infections. In our study, no significant relationship between *Gardnerella vaginalis*, *Candida sp* and *Trichomonas vaginalis* and the presence of *Human papillomavirus* DNA was found in the smear of cervical cells. The frequencies of 45.7%, 20.2% and 3.8% show no association with the rates of HPV infections (60.1%). Changes in pH and small physiological variations are probably not enough to influence persistent HPV

infection in the female genital tract. Considering the multitude of different etiological agents involved such as viruses, bacteria, protozoans, and yeast, patient's immune system is probably activated very differently in each case of infection. In addition, *Gardnerella* and *Candida* are etiological agents that live together symbiotically in the vaginal flora and are well known to the immune system. This is not what happens with HPV and *Trichomonas*, which are completely new to the host. This difference triggers specific and individual physiological responses. At the same time, the environment where these microorganisms are established must be taken in account. Since HPV is an epitheliotrophic virus, it needs to find its way through the tissue, using microlesions to install in the epithelial compartment.<sup>24</sup> These results indicate the need to investigate HPV infection also as a temporal matter related to the appearance of vaginitis and establishing a correlation between time of infection and development of disease.

For a long period, clinical research sought to establish a profile of a patient at high risk of HPV and vaginal infections, as for instance, use of contraceptives, smoking, or sexual promiscuousness. Since these microorganisms can be sexually transmitted, patients with a low risk of infection may have a high-risk partner and thus increase the possibility of becoming infected, which is shown in an isolated study with high rates of HPV DNA positive partners with risk-taking behavior.<sup>27</sup>

Cervical lesions can develop in women with persistent infections associated to high risk types of HPV. Identifying co-factors that may participate in the etiology of HPV and contribute for disease development, however, would bring important additional information on preventing cervical lesions. No association could be found in this study with HPV and socio-demographic features, as well as with other microorganisms living in the female genital tract. The results indicate that it is difficult to clearly define a group at risk for HPV infection, which means that all women with abnormal cytological examination are possible carriers of HPV and other vaginal infections.

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