

Research Paper

High incidence of oncogenic HPV genotypes found in women from Southern Brazil

Ludmila G Entiauspe^{1,2}, Mariângela Silveira³, Emily M Nunes¹, Suélen P Basgalupp¹,
Dulce Stauffert⁴, Odir A. Dellagostin^{1,2}, Tiago Collares^{1,2}, Fabiana K. Seixas^{1,2*}

¹Grupo de Pesquisa em Oncologia Molecular e Celular, Centro de Desenvolvimento Tecnológico,
Universidade Federal de Pelotas, RS, Brasil.

²Programa de Pós-Graduação em Biotecnologia, CDTEC, Universidade Federal de Pelotas,
Pelotas, RS, Brasil.

³Programa de Pós-Graduação em Epidemiologia, Universidade Federal de Pelotas, Pelotas, RS, Brasil.

⁴Laboratório da Faculdade de Medicina, Universidade Federal de Pelotas, Pelotas, RS, Brasil.

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Abstract

Oncogenic HPV genotypes are strongly associated with premalignant and malignant cervical lesion. The purpose was to determine human papillomavirus (HPV) prevalence and genotypes, and to estimate cervical cancer risk factor associations. Cervical samples were obtained from 251 women seeking gynecological care at the Pelotas School of Medicine Clinic. This is a cross-sectional study. HPV-DNA was amplified by nested-PCR using MY09/11 and GP5/6 primers, and the sequencing was used for genotyping. Sociodemographic and behavioral risk factors were obtained by closed questionnaire, and its relationship to HPV infection prevalence were analyzed. Statistical analyses were performed using SPSS 16.0 software, and differences were considered significant at $p < 0.05$. As results, the prevalence of HPV infection was 29.9%. The most frequent genotype was HPV-16 (41.3%), followed by HPV-18 (17.3%), and HPV-33 (9.3%). Others nine HPV genotypes were also found. On this population, prevalence of oncogenic HPV genotypes was high, but does not seem to confer relationship with the risk factors investigated. Future investigations in larger populations are necessary, for the proposition of more appropriated monitoring strategies and treatment according to the Brazilian health service reality, as well as patients.

Key words: HPV, SDT, molecular epidemiology, cervical cancer.

Introduction

The human papillomavirus (HPV) is one of the most common etiologic agent of sexually transmitted diseases, with high prevalence rates. Actually, more than 100 HPV types have been described, of which 40 can infect the mucosa of the anogenital tract in both, men and women. According to their oncogenic potential, those viruses are classified as high- or low-risk genotypes (Bernard *et al.*, 2010; De Villiers *et al.*, 2011). The high-risk oncogenic genotypes are those that are strongly associated with premalignant and malignant cervical lesions, mainly HPV types 16 and 18, being also responsible for 70% of invasive cervical cancer (CC) cases in Brazil (Muñoz *et al.*, 2006).

In Brazil, cervical cancer is the second most common type of cancer in the female population, and the fourth major cause of death in women. For 2012, 17,540 new cases were expected, 17 cases per 100,000 women (MS, 2011). Highly accurate techniques and epidemiologic studies demonstrate the relationship between the development of cervical cancer and HPV. HPV-DNA has been detected in 99.7% of cervical cancer cases worldwide (Fernandes *et al.*, 2004).

Although HPV infection is considered an integral part of cervical cancer development, an association between HPV infection, oncogenic genotypes persistence, and other risk factors, determine the development of cervical intraepithelial neoplasia (CIN), and cervical cancer (Wang

et al., 2010). The risk factors include youth, a high number of sexual partners throughout life, early sexual activity, tobacco smoking, low socioeconomic status, and genetic factors (Marlow *et al.*, 2007; Kapeus *et al.*, 2009; Louie *et al.*, 2009), as well the 16 and/or 18 HPVs genotypes infection, responsible for 70% of invasive cervical cancer (CC) cases in Brazil (Muñoz *et al.*, 2006). In addition, infections caused by human immunodeficiency virus (HIV), bacterial vaginosis (BV), *Trichomonas vaginalis* (TV), and by *Chlamydia trachomatis* (CT) were involved as co-factors for cervical cancer development, adjuvants of the neoplastic process (King *et al.*, 2011, Nam *et al.*, 2009).

Based on these data, our study aimed to detect the prevalence of HPV infection, to identify HPV genotypes, and to analyze cervical cancer risk factors in a female population representative of Pelotas, Rio Grande do Sul, Brazil.

Materials and Methods

Study population

This is a cross-sectional study. From May 2010 to May 2011, 251 women seeking gynecologic care at the gynecological ambulatory clinic at the Pelotas Federal University School of Medicine (UFPel) fulfilling eligibility criteria (not pregnant, sexually active, and not menstruating), and agreeing to participate in the study, were sequentially selected. The study used a closed questionnaire adapted from a previous study (Silveira and Santos, 2006), applied by a female trained interviewer. Routine gynecological exams (cervicitis indicators, visual inspection with acetic acid and Lugol's iodine), were realized, and included in the questionnaire, as well the patient's recorded information (last Pap test result).

The study was approved by the Ethics Committee of the Faculty of Medicine - Federal University of Pelotas in June 2009, and informed consent was obtained from all participants. All procedures were carried out in accordance with the guidelines of the Helsinki Declaration.

Sample collection and processing

Two cervical samples were collected from each patient. The first, with an Ayre's spatula was placed on slides for Bacterial vaginosis (BV) and *Trichomonas vaginalis* (TV) analysis by the Gram method. The second sample was collected with a cytobrush, and placed into 1.5 mL eppendorf tubes containing 300 μ L of Cell Lysis Solution (Puregene™ DNA Extraction Kit, *Genra Systems* Minneapolis, MN). The tubes were submitted to digestion using 1.5 μ L of Proteinase K (10 mg/mL, New England Biolabs, MA), and incubated overnight at room temperature. The genomic material (DNA) was extracted, according to manufacturer specifications. As control for extracted DNA quality, the human *TP53* gene PCR was performed, using the primers previously described (Lin *et al.*, 2008). PCR were performed in a final reaction volume of 12 μ L, and

was carried out with one cycle 94 °C for 3 min, followed by 40 cycles at 94 °C for 30 s, 57 °C for 30, 72 °C for 30 s and a final extension for 3 min at 72 °C (Thurow *et al.*, 2011).

HPV detection was carried out using nested-PCR (nPCR) technique, which is performed in two rounds: the first using MY09/11 primers (Manos *et al.*, 1989) and amplifying a 450 pb fragment, the second used GP5/6 internal primers (Snijders *et al.*, 1990), which amplify the 140 pb fragment. The MY90/11 PCR reaction was performed in a final volume of 25 μ L, and the conditions were as follows: 40 cycles of denaturation (1 min at 95 °C), annealing (1 min at 55 °C), and extension (1 min at 72 °C) (Gravitt *et al.*, 2000). In the second round, the conditions were 40 cycles of denaturation (30 s at 94 °C), annealing (30 s at 45 °C), and extension (30 s at 72 °C) (Husnjak *et al.*, 2000). Both PCR reactions were preheated for 9 min at 95 °C, and a final extension for 5 min at 72 °C. All PCR products were visualized on a 2.0% agarose gel with GelRed™ (Biotium Inc., CA).

HPV sequencing

HPV positive PCR products were purified previously using the Gel Band purification kit (GE Healthcare, USA) according the manufacturer instructions. The sequencing was performed in a MegaBACE 1000 DNA sequencer (GE Healthcare, USA), using Dynamic ET-terminator technology. Chromatograms were assembled and analyzed using the ContigExpress® module of the Vector NTI 10.0 suite (Invitrogen, USA). The assembled sequences were submitted to BLAST alignment (www.ncbi.nlm.gov/BLAST), against sequences available in GenBank.

Statistical analyses

Chi-square (χ^2) test was used to evaluate the association between HPV presence (and their HR/LR genotypes) with the variables of questionnaire. A multivariate analysis was made using logistic regression applied to a hierarchical model. The analysis was performed using SPSS 16.0 software (SPSS, Chicago, IL). Differences were considered significant at $p < 0.05$.

Results

Four hundred women were approached to participate, but only 251 were considered eligible for the study. The mean age was 33.3 years old (median 34 years old), ranging from 18 to 45 years old. Most of the samples did not present endocervical mucopurulent discharge (77.2%), friability (70.8%), or cervical motion tenderness (84.6%). The visual inspection with acetic acid (VIA) and visual inspection with Lugol's iodine (VILI) were negative for sub-clinical lesions in 85.9% and 71.1% of the participants, respectively. The Gram test detected BV in 9.2% of the women, and TV in 2.8%. Of the Pap smear results, 54.2% were normal, 38.6% inflammatory, 0.8% with ASCUS, 0.4%

showed presence HPV, 2.1% indicated NIC I, and 0.4% indicated NIC II. Colposcopies were abnormal in 5% of the participants (data not show). The behavioral and sociodemographic characteristics are showed on Table 1. HPV was detected in 29.9% (N = 75), HR-HPV in 25.3%, and LR-HPV in 2.9% of the samples (Table 2). The most frequent genotype was HPV-16 (41.3%), followed by HPV-18 (17.3%), and HPV-33 (9.3%). The genotypes HPV-6 (2.7%), -31 (2.7%), -43 (1.3%), -45 (1.3%), -54 (4.0%), -56 (4.0%), -58 (4.0%), -70 (1.3) and -81 (2.7%) were also found. Six samples (8.0%) could not classified by sequencing due to overlap of the sequence-peaks. These were categorized as HPV-X (Figure 1).

The bivariate analyses didn't show statistic significance in relation to behavioral characteristics, although it was observed that HPV infection occurred mostly in women who were ex-smokers (35.5%), had consumed alcohol at least once a week (34.9%), had never been pregnant (45.7%), began sexual activities between 13-15 years of age (35.4%), had from 2 to 4 sexual partners in life (26.9%), had STD detected in the last 12 months (35.3%), and whose last Pap test was in less than 3 years (32.3%). Cervicitis indicators showed that 30.1% of the samples did not present endocervical mucopurulent discharge, or friability at (31.6%); cervical motion tenderness was observed in 31.6% of the women (Table 3). The VIA inspection was positive in 34.3% of the participants, and VILI inspection

Table 1 - Distribution of socioeconomic, demographic and behavioral characteristics.

Variable	(%)	Variable	(%)
Age (years)		Current smoker	19.9
18 - 24	17.5	Ex-smoker	24.7
25 - 30	21.9	Never smoker	55.4
31 - 35	17.9	Alcohol consume (last 4 weeks)	
36 - 39	19.9	At least once a week	17.1
40 - 45	22.7	Less than once a week	29.5
Skin color		Never	53.4
White	65.7	Pregnancy	
Black	12.4	Yes	81.7
Others	21.9	No	18.3
Reading ability		Use of condom (in life)	
Yes	98.0	Yes	90.0
No	2.0	No	10.0
Schooling (years)		Sexual activity began (years)	
0	2.0	≤ 12	2.8
1-4	12.9	13 - 15	33.1
5-8	37.5	16 - 18	49.8
9-11	14.9	≥ 19	14.3
≥ 12	32.7	Sexual partners (in life)	
Marital status		1	30.8
Married/consensual union	81.3	2 - 4	44.4
Single	11.2	5 - 8	15.2
Separated/divorced/widowed	7.6	≥ 9	9.6
Live alone		STD detection (last 12 months)	
Yes	2.8	Yes	6.8
No	97.2	No	93.2
Family Income (minimum wages)		Last Pap test	
0 - 1	25.5	Never	4.4
2	41.3	< 3 years	74.9
3	23.5	> 3 years	15.1
≥ 4	9.7	Don't remember	5.6
Cigarette smoking			

Table 2 - HPV detection and oncogenic potential.

Variables	Number	(%)
HPV	251	
Positive		29.9
Negative		70.1
HPV oncogenic potential	69*	
High risk		89.9
Low risk		10.1

*HPV positives by nPCR.

in 29.6%. The presence of BV and TV was observed for 31.8% and 28.6% of the patients respectively. The cytological results for HPV presence, and NIC II was confirmed by nPCR for all ($p = 0.362$). The colposcopy results were abnormal in 41.7% of the HPV positive cases ($p = 0.379$). In relation to high or low risk HPV infection, and variables, it was observed that all high risk HPV infections showed a direct association (100%) with NIC I and NIC II Pap test results ($p = 0.470$). No statistical difference was also found in the multivariate analyses (data not show).

Discussion

Brazil has a population of 69.14 million women who are at risk for cervical cancer development, and current estimates indicate that 14.1% of the women in the general population have harbored a cervical HPV infection at some time in their lives. A total 70.7% of invasive cervical cancers are attributed to HPV-16 or -18 (WHO, 2010). HPV infection prevalence in Brazil varies between 7% and 43%, according to a systematic review of the literature performed in four Brazilian regions (Ayres and Silva, 2010). In this study, the prevalence was within the expected range (29.9%). Two other studies were realized in the same state in Southern Brazil: on the first was found a higher HPV infection prevalence (60%) in HIV negative women, (with or without cervical lesions) than reported here (Entiauspe *et*

al., 2010). In the second study Rosa *et al.* (2008) found that HPV infection was lower (12.3%). In the Northern regions, Noronha *et al.* (2011) conducted a screening study in 1021 women in the city of Belém, and observed an HPV prevalence of 12.4%.

In this study, the most prevalent genotype was HPV-16, followed by HPV-18. These findings are consistent with Oliveira-Silva *et al.* (2011), which in a study performed in Rio de Janeiro (with PCR for DNA-HPV detection in 297 HPV positive women), noted a prevalence of 28% for HPV-16, and 14.4% for HPV-18, differing by only a third from the most prevalent genotype, which was reported for HPV-45 (7.6%), and in our study was observed for HPV-33 (9.3%). However, Mendonça *et al.* (2010) carried out a study with 248 women in the city of Recife, and also observed that the HPV-33 genotype was the third most prevalent (13.9%), a similar percentage to the present study. The high prevalence of oncogenic genotypes found in this study indicates the need for frequent monitoring, and suggests nPCR technique for DNA-HPV detection as an alternative to early identification of women at high risk for cervical cancer development. Previous studies have already demonstrated that the use of primers pair MY09/11 and GP5/GP6 in a nPCR assay increases the sensitivity of HPV detection compared with PCR assay (Seaman *et al.*, 2010; Coser *et al.*, 2011).

Recent data (Vigitel, 2011) show that there has occurred a large decrease in the number of smokers in Brazilian population, going from 16.2% in year of 2006 to less than 15% in 2011. These data show that the state capital of Rio Grande do Sul, Porto Alegre, has the largest number of smokers (23%), higher than the one found in this study. The survey research also identifies that there was an increase in Pap tests realized in the last three years (89.6%) in women who had more than 12 years schooling. This study also observed a higher percentage of Pap tests realized during the same period (< 3 years), but didn't find a relation with higher schooling. Some studies correlate potential risk fac-

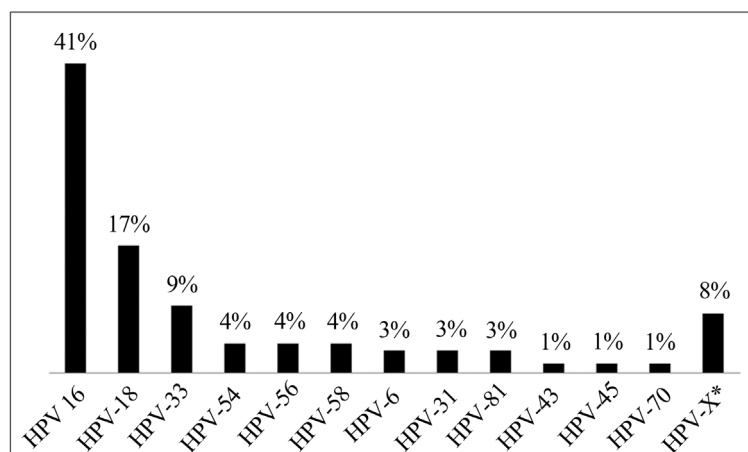


Figure 1 - HPV genotypes identified by sequencing.

Table 3 - Frequency distribution of HPV infection according Cervicitis indicators, visual inspection, bacterial infection and Pap test results.

Variables	HPV infection	
	Positive (%)	Negative (%)
Endocervical mucopurulent discharge		
Yes	28.1	71.9
No	30.1	69.9
	p = 0.773	
Friability		
Yes	26.0	74.0
No	31.6	98.4
	p = 0.379	
Cervical motion tenderness		
Yes	31.6	68.4
No	29.7	70.3
	p = 0.813	
Visual inspection with acetic acid (VIA)		
Positive	34.3	65.7
Negative	28.6	71.4
	p = 0.497	
Visual inspection with Lugol's iodine (VILI)		
Positive	29.6	70.4
Negative	29.1	70.9
	p = 0.946	
Vaginitis		
Positive	31.8	68.2
Negative	29.0	71.0
	p = 0.783	
<i>Trichomonas vaginalis</i>		
Positive	28.6	71.4
Negative	29.3	70.7
	p = 0.967	
Pap test results		
Normal	28.1	71.9
Inflammatory	34.1	65.9
ASCUS	0	100.0
HPV	100.0	0
NIC I	40.0	60.0
NIC II	100.0	0
Inconclusive	25.5	75.0
	p = 0.362	
Colposcopy		
Normal	29.7	70.3
Abnormal	41.7	58.3
	p = 0.379	

tors with HPV infection. In this study, it wasn't possible to observe this relationship. One of the explanations could be the increasing awareness of condom use and smoking health harms that may be contributed to these findings.

Since the study is clinical based, the results should be considered as preliminary, giving an indication of the high prevalence of HPV infection by oncogenic genotypes found in women from Pelotas, but not being capable of correlating the infection with behavioral and socio-demographic factors. Further population based studies from the same geographical area are needed. The current research helps to identify regional differences of HPV genotypes, given the large area and population differences exist in Brazil, and is a contribution to the medical care of the population of Pelotas and to research centers studying human papillomavirus.

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Conflict of Interest

None declared.

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