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Human papillomavirus prevalence in women with normal cytology and with cervical cancer in Natal, Brazil

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Abstract. This study analyzed the prevalence of human papillomavirus (HPV) in cervical specimens obtained from women with normal cytology and with cervical cancer, in order to evaluate their correlation with health status and demographic characteristics, as well as sexual and reproductive activity in women treated at a cancer reference hospital in Natal, Northeast Brazil. A total of 158 women were divided into 2 groups according to their health status: group I comprised 110 women with normal cytology, and group II comprised 48 women with cervical cancer. Cervical smears were analyzed by cytological or histopathological examination for the detection of cytological alterations, and by PCR for HPV DNA detection using MY09/11 primers, followed by HPV genotyping by dot blot hybridization. Results showed overall HPV prevalence to be 24.5% in group I, with 19.1% of patients having single infection and 5.4% double infection. The HPV prevalence in group II was 85.4%, with 79.2% of patients having single and 6.2% double infection. We identified 10 different HPV genotypes, most with high oncogenic potential. HPV 16 was the most prevalent genotype in the two studied groups, followed by HPV 58 and HPV 18. High-risk HPV genital infection, chronological age, ethnicity, early onset of sexual and reproductive activities, multiple sexual partners and smoking increased the risk for cervical cancer.

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Introduction

Human papillomavirus (HPV) is among the most common causes of sexually transmitted infection in women worldwide, particularly in developing countries, where the most sexually active individuals are likely to be exposed to these viruses during their lifetime (1-3). Most HPV infections clear spontaneously, but some persist. Persistent infection by certain HPV genotypes, designated as high-risk HPV, may progress to malignant lesions. These types of viruses were identified as a necessary cause, although they are not sufficient, for the development of cervical cancer (3,4).

Genital HPV infection is a common event among sexually active women, particularly younger women, with the highest prevalence occurring up to 30 years of age, followed by a decline until age 45-50 and a second peak of incidence observed in the peri- or post-menopausal period (5-7). Most of the infections appear to be transitory and with little clinical significance (8). However, some of women with persistent high-risk HPV infection, especially with non-European HPV 16 variants, present a greater risk of progression to high-grade cervical lesions, particularly the infections with a high viral load (9,10). Tumor progression is a complex phenomenon that depends on multiple factors, including the type and variant of the virus, the combination of environmental factors, the host itself, genetic background and immune status, as well as sexual, behavioral and reproductive activities (10,11).

The present study evaluated the prevalence of HPV DNA in women with normal cytology and with cervical cancer treated in a cancer reference hospital in northeastern Brazil. The prevalence of the viral genotypes, as well as their association with socio-demographic characteristics, sexual and reproductive behavior and smoking habits of the participants were also analyzed.

Materials and methods

We analyzed cervical specimens of 112 women enrolled in the cervical cancer screening program of Luis Antonio Hospital,

a cancer reference hospital in Natal, Rio Grande do Norte State, and 48 women with suspected cervical cancer who were referred to the same hospital, between April 2001 and June 2002. Two specimens containing exfoliated cells of the uterine cervix were collected from the women attending the cancer-screening program using a cervical brush. One of these specimens was used for the Pap smear examination, based on the Bethesda system (12). The smears were analyzed by a trained pathologist from the Department of Pathology at the Federal University of Rio Grande do Norte. The other specimen was collected, placed in a tube containing a preserving solution [phosphate-buffered saline (PBS) combined with 0.25 U/ml of vancomicine + nistatine], and sent to a laboratory where it was processed for DNA extraction and analyzed for HPV detection and typing. Two samples of exfoliated cells with scant squamous cellularity were considered unsatisfactory for evaluation and were excluded from analysis.

A fragment of tissue was obtained by biopsy from women referred to the hospital with suspected cervical cancer. The fragment was analyzed through histopathological examination. Subsequently, another fragment of tissue was obtained from women with a diagnosis of cervical cancer at surgery following the conization procedure. The fragment was obtained and re-examined for result confirmation. In this study, 110 women were selected from those who had spontaneously used the cancer-screening program for a periodical Pap smear and who presented no cytological alterations, and 48 women were selected among those who had been referred to the service with suspected cervical cancer and who had confirmed diagnosis.

All women were informed about the methods and objectives of the study and informed consent was obtained. A standardized questionnaire, administered to the participants by a trained interviewer, included questions about socio-demographic characteristics, sexual and reproductive behavior and smoking habits. Patient ethnicity was defined based on self-reports according to the criterion of the Instituto Brasileiro de Geografia e Estatística (IBGE) that classifies ethnicity into five categories: Caucasian, of African descent, mulatto, of Asian descent and native. In this study, the ethnicity groups including those of African descent, mulatto, of Asian descent and native categories were combined into a non-Caucasian category.

DNA extraction. The tubes containing the cervical specimens underwent vigorous agitation prior to removal of the brush and were centrifuged at 3000 x g for 10 min. The supernatant was removed and the resulting pellet was processed for DNA extraction, using rapid isolation of DNA from mammals, with proteinase K (12). Small fragments of tissues obtained by biopsy or collected at the surgical procedure were incubated in 200 μl of digestion buffer (0.01 M Tris-HCl, 0.02 M EDTA, 0.1 M NaCl, 0.5% SDS, pH 8.0) and 20 μl proteinase K 10 mg/ml at 42˚C overnight, 56˚C for 3 h and at 95˚C for 5 min. DNA extraction was performed using the phenol/chloroform method (13).

HPV DNA detection and typing. To analyze the quality of target DNA, DNA samples were quantified by 0.8% agarose gel electrophoresis, and aliquots with approximately 30 ng of DNA underwent polymerase chain reaction (PCR) using PCO3/PCO4 primers, as described by Saiki *et al* (14), specific for the amplification of 110 bp of the β-globin gene. All β-globin-positive samples were included in this study. Samples from 158 women, 110 with normal cytology (group I) and 48 with cervical cancer (group II), were analyzed by PCR for HPV DNA detection using the consensus primers MY09/M11, specific for the L1 ORF region of HPV as described by Manos *et al* (15). The products of PCR were electrophoresed on a 7% polyacrylamide gel, followed by silver staining, according to Sanguinetti *et al* (16). The amplicons were submitted for genotyping of the individual HPV types by dot blot hybridization using specific radioactive probes for the following types of HPV: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 66, 68, 70, 72 and 73, isolated or in cocktails, as described by Jacobs *et al* (17).

Statistical analysis. To estimate the risk of cervical cancer associated with various HPV types and other risk factors, odds ratios (OR) and 95% confidence intervals (CIs) were calculated, according to the adjusted univariate and multivariate regression model. Statistical significance within each group was evaluated by Pearson's χ^2 test. To perform statistical tests and to calculate the ORs and 95% CIs, we used the software SPSS, version 17.0. $P \leq 0.05$ was considered to be statistically significant.

The study protocol was approved by the Ethics Committee of the Universidade Federal do Rio Grande do Norte (CEP-UFRN).

Results

The distribution of the women enrolled in this study according to socio-demographic reproductive and behavioral characteristics showed that the age ranged from 15 to 65 years old, with a mean age of 29.4 (±9.609 years) for the women in group I and 47.3 (±9.109 years) in group II. Distribution by age group showed that the majority of the women in group I (55.5%) were up to 30 years old, of Caucasian ethnicity, married or in a stable relationship with their partner, non-smokers and had only one sexual lifetime partner. The majority of the women from group II (70.8%) were above 40 years old, of non-Caucasian ethnicity, married or in a stable relationship with their partner, smokers and had more than one sexual lifetime partner. Overall HPV prevalence was 24.5% for the women in group I, with a higher prevalence rate in those up to 30 years old. In group II, overall HPV prevalence was 85.4%, with rates varying from 76.9 to 100%. The statistical analysis through the χ^2 test revealed that the HPV prevalence rate was higher for the women in groups I and II among those who had more than one sexual lifetime partner (p=0.0000 and p=0.0004), and among the smokers only in group II (p=0.0008). No association was observed between age group, ethnicity, or marital status and HPV presence (Table I).

The statistical analysis by logistic regression showed that an HPV infection of high oncogenic potential increased the risk of development of cervical cancer independently of any other variable. We observed a tendency of augmentation of the number of cervical cancer cases proportional to the chronological age, with a higher risk of occurrence of the disease after age 40. Females of non-Caucasian ethnicity,

Table I. Socio-demographic characteristics and HPV prevalence according to health status.

Demographic variable	Group I Normal cytology				Group II Cervical cancer			
	n=110	(%)	HPV (+)	p-value[a]	n=48	(%)	HPV (+)	p-value[a]
Age (years)								
≤30	61	55.5	19		1	2.1	1	
31-40	37	33.6	6	0.3511	13	27.1	10	0.4572
41-50	5	4.5	1		19	39.6	18	
>51	7	6.4	1		15	31.2	12	
Ethnicity								
Caucasian	87	79.1	19		19	39.6	17	
Non-Caucasian	23	20.9	8	0.1996	29	60.4	24	0.5192
Marital status								
Single	32	29.1	11		9	18.8	8	
Married	78	70.9	16	0.1249	39	81.2	33	0.7433
More than one sexual partner								
Yes	29	26.4	17		34	70.8	33	
No	81	73.6	10	0.0000[b]	14	29.2	8	0.0004[b]
Smoking								
Yes	12	10.9	4		33	68.8	32	
No	98	89.1	23	0.4536	15	31.2	9	0.0008[b]

HPV, human papillomavirus. [a]P-value of the χ^2 test between analyzed variables and HPV prevalence. [b]Statistically significant.

who had their first sexual intercourse at age 14-17, who had sexual relationships with more than one partner in their lifetime, and were smokers, presented a higher risk of developing cervical cancer. Those who had their first pregnancy at age 21 or above, presented a lower risk of cervical cancer (Table II).

Ten different HPV types were identified: seven were classified as high-risk or of high oncogenic potential (HPV 16, 18, 33, 45, 56, 58 and 59); one as probably of high-risk, HPV 68; and two with indeterminate risk, HPV 55 and 57. In 2 samples of the women with normal cytology and 1 with cervical cancer, the HPV genotypes could not be identified with the probes used. Overall HPV prevalence in the women with normal cytology was 24.5%: 19.1% with single infection and 5.4% with double infection. Considering single and double HPV infection, HPV 16 was the most prevalent genotype in the women with normal cytology, with a prevalence rate of 15.4%, followed by HPV 58 with 5.4%. The most common association observed in this group was that between HPV 16 + 58 and HPV 56 + 57, each was detected in 1.8% of the samples.

In the group with cervical cancer, the overall HPV prevalence was 85.4%, with 79.2% of the cases with single infection and 6.2% with the double form of infection. Considering single and double HPV infection, HPV 16 was the most prevalent genotype found in 58.3% of the patients, followed by HPV 58 with a prevalence rate of 12.5% and HPV 18 with 8.3%. HPV 33 and 45 were the fourth most frequent genotypes, with prevalence rates of 4.2% each. The statistical analysis by logistic regression showed that single infection with HPV 16, 18, and 58 as well as double infection by HPV 16 + 58, increased the risk of development of cervical cancer (Table III).

Discussion

The association between cervical cancer and genital infection with high-risk HPV genotypes is clearly demonstrated (7,18-20). The Centers for Disease Control and Prevention (CDC) estimates that at least half of all sexually active individuals are likely to acquire HPV infection at some point in their lives, whereas at least 80% of women may acquire this pathogen before the age of 50 (21). Genital persistent infection with high-risk HPV has been firmly established biologically and epidemiologically as playing a causal role in all types of cancer of the uterine cervix; however, other cofactors are necessary for progression of the low-grade cervical lesions to cancer (4,11). In this study, we evaluated the role of HPV in cervical cancer, as well as the effect of certain characteristics of the patients with probable risk factors.

The mean age of the patients with cervical cancer was 47.3 years old, similar to that reported in a multicentric study covering countries on four continents (47.8%), but above the mean age described for African countries (33.9 years old) (22) and below the means described in São Paulo (southeastern Brazil, 52 years old), Belém (northern Brazil, 51 years old), and Goiânia (Central Brazil, 49 years old) (1,23,24). The mean age found in this study was also below the mean age reported in a multicentric study covering 9 developing countries, including Brazil, whose mean age was 49 years (25). These results suggest

Table II. OR for the association between selected variables and health status of the patients.

Variable	Health status		Logistic regression model			
	Normal	Cancer	Univariate		Multiple	
	n=110	n=48	OR	(95% CI)	OR	(95% CI)
HPV presence						
Yes	27	41	18.00	(7.24-44.80)[a]	9.97	(1.61-61.92)[a]
No	83	7	1.00	(Refs.)	1.00	(Refs.)
Age (years)						
≤30	61	1	1.00	(Refs.)	1.00	(Refs.)
31-40	37	13	21.43	(2.69-170.62)[a]	4.78	(0.94-24.31)
41-50	5	19	231.80	(25.48-2108.63)[a]	35.66	(5.68-223.95)[a]
≥51	7	15	130.71	(14.92-1194.44)[a]	40.42	(5.69-287.44)[a]
Ethnicity						
Caucasian	87	19	1.00	(Refs.)	1.00	(Refs.)
Non-Caucasian	23	29	5.77	(2.76-12.09)[a]	5.06	(1.54-16.63)[a]
Marital status						
Married	78	39	1.78	(0.77-4.09)	0.77	(0.21-2.79)
Single	32	9	1.00	(Refs.)	1.00	(Refs.)
Age at first sexual intercourse						
14-17	40	39	6.83	(1.46-32.02)[a]	0.09	(0.01-0.82)[a]
18-21	56	7	0.88	(0.16-4.68)	0.02	(0.00-0.26)[a]
>21	14	2	1.00	(Refs.)	1.00	(Refs.)
Age at first pregnancy						
Never pregnant	15	0	1.00	(Refs.)	1.00	(Refs.)
14-17	31	34	1.10	(0.67-1.78)	0.16	(0.01-1.96)
18-21	42	10	0.24	(0.12-0.48)[a]	0.29	(0.02-3.68)
>21	22	4	0.18	(0.06-0.53)[a]	0.04	(0.00-0.35)[a]
More than one partner						
Yes	34	34	5.43	(2.58-11.40)[a]	0.79	(0.17-3.76)
No	76	14	1.00	(Refs.)	1.00	(Refs.)
Smoking						
Yes	18	33	11.24	(5.09-24.83)[a]	1.25	(0.35-4.53)
No	92	15	1.00	(Refs.)	1.00	(Refs.)

OR, odds ratio; 95% CI, confidence interval. [a]Statistically significant.

that HPV infections occur earlier in women from the Northeast region of the country. An indication of this finding is that, among the women in this study with normal cytology infected with HPV, 50% of them were below 25 years of age.

Among the women with normal cytology, the HPV prevalence was significantly higher only in those who had more than one sexual lifetime partner, while in the women with cervical cancer the difference in HPV rate prevalence was significant for those who had a sexual relationship with more than one partner during their lifetime and for the smokers. In this study, chronological age, ethnicity and marital status did not show an association with augmentation of HPV prevalence rates in groups I or II.

In this study, the presence of HPV, conforming to what was expected and widely described (4,9-11,23), presented a strong association with cervical cancer occurrence, independently of other risk factors. We observed a tendency of an increase in the number of cases of cervical cancer with chronological age, confirming results obtained in São Paulo (23) and in Hong Kong, China (7). The higher number of non-Caucasian women observed among the carriers of cervical cancer is coherent with the results reported by Schoell *et al* (26) regarding the women in Miami, USA. However, it is probable that this may be more related to the social and economic conditions of the patients than to their ethnic characteristics.

In the present study, initiating sexual and reproductive activity at an early age, a sexual relationship with more than one partner during their lifetime, and smoking, presented an increased risk of cervical cancer, and appeared to be significant risk factors for the disease. These results are coherent, at least in part, with those reported by other investigators (11,23-24).

Table III. Distribution of the most prevalent HPV types according to health status and associated OR obtained by the univariate logistic regression model.

HPV infection	Normal cytology (n=110)	Cervical cancer (n=48)		
	Patients, no. (%)	Patients, no. (%)	OR	95% CI
Type				
HPV 16	14 (12.7)	25 (52.1)	21.17	(7.70-58.22)[a]
HPV 18	1 (0.9)	3 (6.3)	35.57	(3.26-388.61)[a]
HPV 33	-	2 (4.2)	-	-
HPV 45	-	2 (4.2)	-	-
HPV 58	3 (2.7)	4 (8.3)	15.81	(2.94-85.17)[a]
HPV 59	1 (0.9)	-	-	-
HPV 68	-	1 (2.1)	-	-
Untyped HPV	2 (1.8)	1 (2.1)	5.93	(0.48-73.78)
Sub-total positive samples	21 (19.1)	38 (79.2)	21.45	(8.40-54.79)[a]
Double infection				
HPV 16 +18	1 (0.9)	1 (2.1)	11.86	(0.67-210.63)
HPV 16 + 58	2 (1.8)	2 (4.2)	11.86	(1.44-97.44)[a]
HPV 56 + 57	2 (1.8)	-	-	-
HPV 58 + 55	1 (0.9)	-	-	-
Sub-total positive double samples	6 (5.4)	3 (6.3)	5.93	(1.21-29.00)[a]
Total HPV-positive samples	27 (24.5)	41 (85.4)	18.00	(7.24-44.80)[a]
Total HPV-negative samples	83 (75.5)	7 (14.6)	1.0	(Refs.)

OR, odds ratio; 95% CI, confidence interval; HPV, human papillomavirus. [a]Statistically significant.

Findings of this study showed the overall HPV infection prevalence to be 24.5% in women with normal cytology, which is similar to that reported for Sub-Saharan Africa (24.7%) in a pooled analysis by Clifford *et al* (27). The prevalence rate found in this study was similar to that reported in the same region (19.5%) by Lorenzato *et al* (28) in Recife, and higher compared to the prevalence rate observed in other regions of southeastern (17.0%) (23) and southern Brazil (16.0%) (29), in which PCR-based assays using the consensus primer MY09/11 were also used for the detection of viral DNA.

We observed that of the women with normal cytology who were HPV PCR-positive, 92.6% were infected by at least one high-risk HPV genotype. Considering single infection and association with other genotypes, HPV 16 was the most prevalent genotype detected in 15.4% of the samples, followed by HPV 58 in 5.4%. The third most common genotypes were HPV 18, 56 and 57, each detected in 1.8% of the women. In a previous study performed in Recife on women with normal cytology (28), it was observed that 78.6% of HPV PCR-positive women were infected by genotypes of high-risk HPV, with HPV 16 being the most prevalent with 7.0%, followed by HPV 31 with 4.2% and HPV 58 with 1.4%. The HPV 16 prevalence found in the present study was almost equal to the estimate by Clifford *et al* (27) for South America (15.0%), but for HPV 58, 18 and 56, the prevalence rates were below those estimates for South America in the above-mentioned study.

The overall HPV prevalence in the women with cervical cancer was 85.4%, similar to that reported by Eluf-Neto *et al* (23) in the southeastern region (84.0%) and higher than that observed by Rabelo-Santos *et al* (1) in the Central region of Brazil (76.0%). Among the patients in this study who were HPV PCR-positive, 92.6% were infected by at least one high-risk HPV genotype. Considering single infection and association with other genotypes, HPV 16 was the most prevalent genotype, detected in 58.3% of the cases, followed by HPV 58 in 12.5% and HPV 18 in 8.3%, and then by HPV 33 and HPV 45, each detected in 4.2% of the samples. A previous study conducted in Recife, also in the northeastern region, by Lorenzato *et al* (28) found HPV 16, 31, 58, 33 and 18 with prevalence rates of 59.3, 11.9, 3.4, 5.1 and 5.1%, respectively, in women with cervical cancer. Furthermore, the HPV 16 prevalence reported by Rabelo-Santos *et al* (1) in women with cervical cancer in the North, South and Southeast regions of Brazil, were 43.5, 52.2 and 53.8%, respectively, below the value reported for the Northeast region. These results suggest that the women from Northeast Brazil may be more exposed to infection with high-risk HPV genotypes, particularly HPV 16, compared with those of other regions of the country. Based on these results, we believe that a vaccine capable of preventing HPV 16 and 18 may have a great impact on the burden of HPV-associated diseases in different geographic regions of Brazil, mainly the northeastern region.

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