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Molecular detection of human papillomavirus in Brazilian women with cervical intraepithelial neoplasia in a northeast Brazilian city

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ABSTRACT. We examined the prevalence of human papillomavirus (HPV) infection in Brazilian women with cervical intraepithelial neoplasia. Our goal was to identify the types of HPV and their association with risk factors. This prospective cross-sectional study included 97 samples collected from women aged 14-79 years at the public health units of gynecological care in São Luís, MA, Brazil. HPV detection was performed by nested polymerase chain reaction and sequence analysis. The study patients completed a structured questionnaire to provide information regarding their socio-demographic, clinical, and behavioral status. HPV prevalence was found to be 80.4%, with 17 virus types detected, including HPV 16, 18, 58, 6, and 11. Significant associations between HPV infection and age and frequency of doctor visits were identified. The study findings indicate the significance of age and low frequency of visits to the gynecologist as risk factors for genital HPV infection, suggesting that HPV infection-derived cervical cancer could

be prevented through orientation programs for women, which include sex education and information regarding screening tests. We also found an increased prevalence of high-risk HPV serotypes in cervical lesions, which reveals an association between cervical lesions and high-risk HPV.

Key words: Human papillomavirus; Polymerase chain reaction; Risk factors; Uterine cervical neoplasms

INTRODUCTION

Cervical cancer is the 3rd most commonly diagnosed cancer in women, 2nd only to breast and colorectal cancer, and the 4th leading cause of cancer-related deaths in women worldwide (Jemal et al., 2011). Cervical cancer accounts for 9% of new cancer cases among women, accounting for approximately half a million new cases and 200,000 deaths annually, with 85% of occurring in developing countries.

Epidemiological evidence suggests that human papillomavirus (HPV) is necessary for the development of cervical cancer (Andall-Brereton et al., 2011; Katki et al., 2013). Studies conducted over the past decade have clearly shown that HPV infection precedes the development of cervical cancer and have confirmed that sexual transmission is the predominant mode of HPV acquisition. The estimated global HPV prevalence among women is 11.7%, which shows some variation worldwide between 10 and 25%; the rates are higher for women in Africa, Eastern Europe, and Latin America (Ayres and Silva, 2010; Bruni et al., 2010). Overall, approximately 70% of cervical cancers are associated with either HPV type 16 or 18. Other tumorigenic serotypes include HPV 52, 31, and 58 (Bruni et al., 2010; Li et al., 2011).

HPV infection is a necessary condition for the development of cervical cancer, but the presence of HPV infection alone is not sufficient for malignant progression of cervical lesions. In addition to the HPV type, the evolution to malignancy is related to other risk factors (Fernandes et al., 2009).

A large number of women infected with oncogenic HPV types do not develop cervical cancer, indicating that other risk factors are associated with the progression of cervical lesions to malignancy (Fernandes et al., 2009). The development of precancerous lesions to invasive cancers also depends on susceptible host phenotypes, HPV genomic variability, multiplicity of HPV infections, co-infection with other agents (including *Chlamydia trachomatis* and HIV), and lifestyle (de Freitas et al., 2012).

Understanding the distribution of HPV tumorigenic serotypes may help to prevent cancer in other organs such as the anus, oropharynx, and esophagus. HPV infection in these organs may be related to sexual practices, allowing HPV to penetrate into organs other than the reproductive organs (Zandberg et al., 2013).

The diagnosis of cervical intraepithelial lesions and cancer is primarily based on cytological, histopathological, and clinical examinations.

MATERIAL AND METHODS

Study population

This prospective cross-sectional study included 97 women aged 14-79 years who were

patients at the public health units of gynecological care in São Luís, MA, Brazil, from February 2010 to January 2013. The women showed cytological evidence of atypical squamous cells or history of cervical cancer. Those who were pregnant or at less than 45 days postpartum were excluded from this study.

A standardized questionnaire was used to collect information on the demographic, social, family, and behavioral characteristics of the patients. These data included age, literacy, marital status, ethnicity, age of menarche, age at first intercourse, parity, lifetime number of sexual partners, methods of contraception, frequency of visits to the gynecologist before the diagnosis of intraepithelial lesions, and methods used to diagnose neoplasia.

The project was approved by the Research Ethics Committee, and informed consent was obtained from all subjects.

Specimen collection

For HPV DNA isolation, samples cervix were collected, placed in the hc_2 DNA Collection buffer (Qiagen, Hilden, Germany, USA), and frozen at -20°C until processing.

HPV DNA extraction

For DNA extraction, we used QIAamp DNA Mini and Blood Mini kits (Qiagen) according to manufacturer instructions. A sample was homogenized in a 2-mL microtube with 400 µL buffer AL containing proteinase K and incubated at 56°C for 10 min. Next, 400 µL absolute ethanol was added to the sample, mixed, transferred to a spin column, and subjected to centrifugation at 5500 *g* for 1 min. Subsequently, 500 µL buffer AW1 was added to the column, which was centrifuged at 5500 *g* for 1 min, and 500 µL buffer AW2 was added and the sample was centrifuged at 15000 *g* for 3 min. Finally, 200 µL buffer AE was added, the column was centrifuged, and the collected sample was stored at -20°C.

Extracted DNA was quantified using a NanoVue unit (GE Healthcare Life Sciences, Little Chalfont, UK) and evaluated by amplification of the human β-globin gene.

Detection of HPV

HPV detection by PCR was carried out using a nested PCR approach with the primer pairs MY09/MY11 and GP5+/GP6+ (Invitrogen, Carlsbad, CA, USA) as described previously (Kleter et al., 1999).

The first round of amplification was carried out in a 25-µL reaction volume by using 5 µL DNA, 8.7 µL water, 2.5 µL 10X PCR buffer (10 mM Tris-HCl, pH 8.5, 50 mM KCl), 1.5 mM $MgCl_2$, 10 mM each dNTP, 30 µM each primer (MY09 and MY11), and 0.5 µL *Platinum Taq* DNA polymerase (Invitrogen). Amplification was performed according to the following protocol: 35 cycles at 94°C for 30 s, 51.5°C for 30 s, 72°C for 30 s, followed by a final step at 72°C for 7 min.

The second amplification was also carried out in a 25-µL reaction volume by using 5 µL amplified DNA, 9.7 µL water, 2.5 µL 10X PCR buffer, 1.5 mM $MgCl_2$, 10 mM of each dNTP, 30 µM of each primer (GP5+ and GP6+), and 0.5 µL *Platinum Taq* DNA polymerase. Amplification was performed as follows: 94°C for 5 min, followed by 45 cycles at 94°C for 45 s, 40°C for 60 s, and 72°C for 60 s, and a final step at 72°C for 10 min.

Amplification products were evaluated by electrophoresis on a 1.5% agarose gel in 1X TBE buffer for 30 min at 5 V/cm in a horizontal unit (Life Technologies, Carlsbad, CA,

USA). Bands were stained with 0.1% Gel Red (Invitrogen) and visualized using an ultraviolet transilluminator (BioRad Laboratories, Hercules, CA, USA). Samples positive for HPV DNA were further subjected to sequence analysis.

Sequencing was performed at the Laboratory of Molecular Carcinogenesis, National Cancer Institute José Alencar Gomes da Silva (INCA), using the ET Dye Terminator Cycle Sequencing kit and automated sequencer MegaBACE 1000 (GE Healthcare) according to manufacturer instructions. Each reaction contained 2 μL purified PCR product, 40 ng exon-specific oligonucleotides (sense or antisense), and 2 μL kit reagent.

When an indeterminate result was obtained by sequencing, the sample DNA was subjected to allele-specific PCR for HPV 16. PCR amplification was carried out as described above using the primers E6-R 5'-ACCTCACGTCGCAGTAACGTTG-3' and E6-F 5'-GSGCGACCAGAAAGTTACCAG-3' (Rocha et al., 2012). The cycling protocol consisted of an initial denaturation step at 94°C for 5 min, 40 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 7 min.

Data analysis

Data was analyzed using the Stata version 11 software for Windows. Data were initially subjected to descriptive analysis using the chi-square test. Multiple logistic regression was used to evaluate associations between HPV infection status and socio-demographic factors. The distribution of HPV genotypes in the study population is presented as frequencies and proportions. $P < 0.05$ was considered to be statistically significant.

RESULTS

The study included 97 women with cervical lesions; among them, 80 were diagnosed with low-grade lesions (82.4%) and 17 with high-grade lesions (17.6%), whereas 78 women were found to be positive for HPV DNA (80.4%). Seventeen HPV types were identified in 63 women; HPV type was not determined for 15 patients (Table 1). The most prevalent viral types were HPV 16 (31.1%), 18 (7.7%), 58 (6.4%), 6 (5.1%), and 11 (5.1%). High-risk viral types were detected in 69.2% of HPV-infected women (Table 1).

Table 1. HPV type distribution in 78 samples from HPV-positive women in São Luís, Maranhão, Brazil (2013).

Risk HPV	HPV type	N	%
High risk	16	29	31.1
	18	6	7.7
	58	5	6.4
	35	2	2.6
	45	2	2.6
	33	1	1.3
	56	1	1.3
	67	1	1.3
	68	1	1.3
	69	1	1.3
Probable high-risk	53	1	1.3
	66	1	1.3
Low-risk	6	4	5.1
	11	4	5.1
	81	2	2.6
	44	1	1.3
	62	1	1.3
	Undetermined	15	19.2
	Total	78	100

Risk factors associated with cervical lesions were age and frequency of visits to the gynecologist (Tables 2 and 3).

Table 2. Multivariate analysis of social factors associated with cervical lesions in HPV-positive women from São Luís, Maranhão, Brazil (2013).

	OR	Z	IC		P value
Age (years)	2.57	2.47	1.21	5.43	0.013
Literacy	0.91	-0.42	0.62	1.35	0.671
Marital status	0.97	-0.12	0.67	1.4	0.903
Ethnicity	0.61	-1.08	0.25	1.49	0.281
Visit to the gynecologist	41.71	2.94	2.37	500.0	0.003
Age at menarche	2.21	1.36	0.7	6.99	0.173
Age first intercourse	1.16	0.22	0.29	4.58	0.825
Parity	1.6	1.38	0.81	3.15	0.167
Lifetime number of sexual partners	0.99	-0.04	0.63	1.54	0.966

OR = odds ratio; 95%CI = 95% confidence interval.

Table 3. Analysis of risk factors associated with cervical lesions in HPV-positive women from São Luís, Maranhão, Brazil (2013).

	Grade lesions				P value
	Low-grade		High-grade		
	N	%	N	%	
Age (years)					0.026
<30	44	55.0	4	23.5	
30 to 49	29	35.0	7	47.1	
>50	8	10.0	5	29.4	
Literacy					0.323
Illiterate	4	5.0	2	11.8	
Elementary Education (complete)	5	6.2	2	11.8	
Elementary Education (incomplete)	16	20.0	2	11.8	
High school (complete)	30	37.5	3	17.6	
High school (incomplete)	16	20.0	7	41.1	
University (complete)	7	8.8	1	5.9	
University (incomplete)	2	2.5	0	0.0	
Marital status					0.853
Single	22	27.5	6	35.3	
Married	38	47.5	7	41.2	
Consensual union	18	22.5	3	23.5	
Method diagnosed CIN					<0.001
Pap smear	66	82.6	0	0.0	
Directed biopsy (colposcopy)	7	8.7	17	100.0	
Diagnostic conizations	7	8.7	0	0.0	
Frequency of visit to the gynecologist before diagnosis of CIN					<0.001
Once every year	20	25	1	5.9	
Once every 3 years	0	0	6	35.3	
Never	60	75	10	58.8	
Use of barrier method					0.90
No	58	72.5	5	82.4	
Yes (Condom)	22	27.5	12	17.6	

DISCUSSION

In our study, 78 (84.4%) women with cervical lesions had HPV infection. This result is similar to the findings of Haghshenas et al. (2013) who examined 98 cervical samples and showed that 78 (79.59%) were positive for HPV DNA.

Among the 78 HPV-positive women identified in our study, 51(65.4%) were infected with high-risk HPV types. This frequency was similar to that reported by Brismar-Wendel et al. (2009) who conducted population-based screening and found high-risk HPV serotypes in 71% of low-grade intraepithelial lesions.

The most prevalent high-risk HPV types were HPV 16 and 18, confirming the previously reported type-specific HPV distribution in 432 patients with invasive cervical carcinoma (Kasamatsu et al., 2012). The study also showed that 73.1% of HPV-positive cases were HPV 16 and 18; other common types included HPV 45, 33, 31, 52, 35, and 39. Seroprevalence of these HPV phenotypes was increased in HIV-positive patients or in those receiving immunosuppressive therapy, which is known to increase the risk of co-infections and viral persistence (Naucler et al., 2011; Nicol et al., 2013). In addition to HPV types 16 and 18, serotypes 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 should be considered high-risk, and HPV 26, 53, and 66 can be classified as probable high-risk types (Muñoz et al., 2003).

The low-risk HPV types 6 and 11 are responsible for approximately 90% of genital wart (condylomata acuminata) cases, although the identification requires analysis of the innermost wart region because the surface area can contain multiple HPV types that are not necessarily involved in lesion pathogenesis (Hawkins et al., 2013). HPV 16 and 11 are sometimes observed in high-grade lesions and in anal cancer (Cornall et al., 2013). However, there are other types of low-risk HPV with a lower frequency, such as HPV 44, which were identified in our study in one patient. HPV 6 and 11 are also more prevalent in pregnant than in non-pregnant women, which is important for understanding the epidemiology of these HPV types in relation to the vertical transmission of HPV infection (Rombaldi et al., 2009; Naucler et al., 2011).

After HPV 16 and 18, HPV 45 is the 3rd most frequently detected serotype in cancer biopsies, and HPV 31 is most frequently detected together with HPV 16 in co-infections, regardless of the region of the female reproductive system (Baldez da Silva et al., 2012; Muñoz et al., 2003).

While high-grade HPV 58, 35, and 33 are the 6th, 7th, and 8th most common types, respectively, in invasive cervical cancer patients, other genotypes can also be classified as carcinogenic, such as HPV types 53 and 66, which are found in high-grade intraepithelial lesions (Meyer et al., 1998). Additionally, HPV 44 and 81 are regarded as low-grade serotypes because of their low frequency in patients with cervical neoplasia or cancer (Muñoz et al., 2003).

HPV 62 is considered to be a rare, highly divergent HPV type prone to mutagenesis. HPV 62 infection was detected in intraepithelial lesions similar to those produced by highly oncogenic HPV subtypes. This HPV serotype is relevant not only for women but also for their male sexual partners, as indicated by a case of penile cancer in a 23-year-old man associated with HPV 62 infection. Such cases are primarily detected in younger populations; however, additional studies are needed to confirm this association (Meyer et al., 1998).

The unusual HPV serotype 69, which was previously associated with low-grade lesions, was found in a clinically aggressive plantar wart in a human immunodeficiency virus-positive patient. Thus, rare serotypes such as HPV 69 can also cause extremely cancerous dysplastic lesions that require immediate treatment and histopathological analysis if necessary (Whitaker et al., 2009).

Multiple infections were verified in 19.2% of HPV-infected women (undetermined HPV). This finding was similar to the previously reported frequencies of 17.9% (Goldman et al., 2013) and 20.4% (Carozzi et al., 2012). Women infected with multiple HPV serotypes are more prone to persistent infections with high viral loads and are therefore considered to be

at high-risk for developing cervical cancer (Xi et al., 2009). More than half of HPV-positive men are infected with multiple types of HPV, which can be transmitted to their female partners (Rositch et al., 2012).

Patients with high-grade cervical lesions have tested positive for HPV DNA in 64 to 83% of cases (Corrêa et al., 2012). HPV genotyping has important applications in the screening, evaluation, and monitoring of low-grade cervical intraepithelial neoplasia (Peralta-Zaragoza et al., 2013).

Risk factors associated with cervical lesions in this study included the patient's age and frequency of visits to the gynecologist. Cervical lesions were more frequently observed in patients younger than 30 years, thus confirming the results obtained in other studies (Brismar-Wendel et al., 2009; Demers et al., 2012; Wiley et al., 2012). However, older subjects showed an increased prevalence of high-grade lesions compared to younger subjects who primarily had low-grade lesions ($P < 0.026$).

The results also show that the Pap test was the most common diagnostic tool for low-grade lesions ($P < 0.001$). Cities that have implemented preventive screening showed a reduction in the incidence of cervical cancer (Bleggi et al., 2003).

A retrospective study based on cytopathological results revealed a high frequency of cases referred for colposcopy and histology (Albuquerque et al., 2012). An inevitable consequence of following this course carries the burden of secondary services and increased frequency of dispensable procedures (de Andrade, 2012). Such wasting of public resources may explain the low frequency of Pap screening in Latin America, which is approximately 50% (Soneji and Fukui, 2013). Additionally, there may be a lack of knowledge regarding the importance of preventive HPV screening in the fight against cervical cancer, particularly among younger people with lower income and educational levels (de Lima et al., 2012).

However, it is necessary to increase the range of preventive screening in the general population, particularly among women who have never undergone this examination and are therefore more likely to develop cervical cancer (Nascimento et al., 2012). The most important constraint is the low coverage by Pap screening. Although the number of women tested has increased, the testing frequency remains insufficient to result in an impact because of major regional economic and social inequalities (de Andrade, 2012).

Educational measures have been shown to reduce the number of cervical cancer cases, as adequate information can induce changes in sexual behavior and/or attitudes to health care. This information can be provided by any health professional, and it has been observed that doctor visits are often followed by preventive examination (Sogukpinar et al., 2013). In this study, we found that women who had never visited the gynecologist have a greater risk of developing both low- and high-grade cervical lesions. Medical consultation ensures not only the monitoring and prevention of the onset and progression of neoplastic cervical lesions but also the use of preventive measures such as condom use and behavioral changes (Soneji and Fukui, 2013). This is important because women who engage in risky behavior have a greater chance of developing cervical lesions as HPV is primarily sexually transmitted. One study found that condom usage not only reduced the risk of acquiring HPV but also resulted in HPV clearance by 30% (Pierce Campbell et al., 2013).

Therefore, to prevent the spread of HPV infections, it is essential to use condoms during sexual intercourse, even with only 1 sexual partner, as well as to implement other preventive measures. These measures are particularly important for women who have never undergone testing for cervical lesions.

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