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Molecular detection of oncogenic subtypes of human papillomavirus (HPV) in a group of women in the Amazon region of Brazil

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ABSTRACT. The natural history of cervical cancer is strongly related to the presence of human papillomavirus (HPV) infection, with its relationship with cervical cancer being a matter of concern. It is estimated that 70% of all cervical cancers worldwide are caused by HPV 16 and 18. Accordingly, the present study aimed to contribute to the identification of HPV subtypes circulating in a group of women of Manaus-Brazil. Cervical samples were collected from 49 women, following the eligibility criteria of the study, and DNA was then extracted from the samples, which were analyzed for the presence of the virus in the genetic material through the polymerase chain reaction (PCR) using generic primers (GP05/06). Finally, identification of the viral subtypes was performed using specific primers for the detection of the main subtypes already examined (16 and 18). Positive HPV DNA was detected in 100% of the samples included in the study. Human papillomavirus 16 was the most prevalent subtype in the majority of lesions, accounting for 29 (59.2%) of the positive cases, and HPV 18 was detected in four (8.2%) women. In these 4 cases there was co-infection, with the presence of both HPV 18 and HPV 16. Therefore, 40.8% (20 cases) in which HPV DNA was detected presented infection with other subtypes of HPV not included in the study. This data has clinical implications related to cervical cancer prevention, as the current prophylactic HPV vaccines are only effective against high-risk HPV 16 and 18 subtypes.

Keywords: cervical cancer; human papillomavirus; Amazon.

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Introduction

Infection by human papillomavirus (HPV) is the leading cause of cervical cancer and cervical intraepithelial neoplasia (CIN) worldwide. Nearly 500,000 cases of cervical cancer occur each year and 200,000 women die of the disease. Approximately 291 million women worldwide are HPV DNA carriers. Also, 80% of sexually active women will be infected with one or more HPV subtypes at some point in their lives, and the infection is often asymptomatic (Koutsky, 1997; Stanley, 2010).

Cervical cancer is the third most common cause of cancer deaths in Brazil (Almeida et al., 2017). Therefore, the use of HPV testing in cervical cancer screening attracts considerable attention. In 2018, 16,370 new cases of cervical cancer were estimated for each year of the 2018-2019 biennium. Regarding regional incidence, most cases of cervical cancer occur in the Northern region of Brazil, with 25.62 cases per 100,000 women (Almeida et al., 2017). Knowledge of HPV infection is essential for the control of cervical cancer. Albring, Brentano, and Vargas (2006) reported geographic variations in the HPV subtypes detected in cervical cancer tissue samples.

Cervical cancer predominantly affects squamous epithelial cells, which undergo changes that induce uncontrolled proliferation and progressive growth of the epithelium. With the progression of the disease, other tissues and organs of the body can be invaded in an average period of 10 to 20 years, and patient prognosis is poor (*Instituto Nacional de Câncer José Alencar Gomes da Silva [INCA]*, 2015).

Human papillomavirus (HPV) belongs to the genus *Papillomavirus* (family *Papillomaviridae*). More than 100 HPV genotypes have been described and approximately 40 subtypes infect the genital regions. Most skin or mucosal lesions caused by HPV have limited growth and regress spontaneously. However, some subtypes of HPV can lead to the formation of warts. Persistent cervical infection may pose a high risk for the

development of precancerous lesions that may progress to cancer within a few years (Workowski & Bolan, 2015).

More than 100 HPV subtypes have been identified and 20 of them have a predilection for squamous epithelium of the lower genital tract (cervix, vulva, perineal body, perianal and anal regions). Of these, 6, 11, 26, 40, 42, 53-55, 57, 59, 66 and 68 are classified as non-risk subtypes, i.e. they do not pose a risk for the development of cervical cancer. They are mostly associated with benign lesions such as condyloma and with Intraepithelial lesions such as Low-grade squamous intraepithelial lesions - LSIL. However, the medium- and high-risk subtypes are 16, 18, 31, 33, 35, 39, 45, 51, 52, 56 and 59, which are strongly related to High-grade squamous intraepithelial lesions - HSIL and cancer (Derchain, Longatto Filho, & Syrjanen, 2005).

Human papillomavirus transmission occurs by direct contact with the infected region. The HPV subtypes that infect the genital tract are transmitted through sexual contact and infect various sites such as the vagina, cervix, penis and anus. Condom use decreases the chances of transmission of HPV, but does not completely prevent it, since HPV can be transmitted through sexual contact even when there is no penetration (Nadal & Nadal, 2008). Therefore, the authors believe that preventive measures, such as cervical cancer screening, HPV vaccination (routine for girls since 2013 in the state of Amazonas and extended to boys nationwide from 2017) and the use of condoms, can drastically reduce rates of cervical cancer and other HPV virus-associated cancers over the next two decades (Torres et al., 2018; Queiroz, Rocha, Filho, & Santos, 2015).

Human papillomavirus leads to several consequences in human hosts depending on the viral subtype. Low-risk HPV subtypes (6, 11, and others) are associated with benign skin and mucosal epithelial growths, whereas high-risk HPV subtypes (mainly 16, 18, and 45) are associated with neoplastic lesions, especially cervical cancer, where HPV DNA integration (integration of viral DNA into host cell DNA) can be detected, through molecular methods, in up to 95% of the lesions (Nadal & Nadal, 2008). Genital warts in the mucous membrane or skin of the penis, anus, vulva, cervix etc. can be diagnosed by urological, gynecological or dermatological exams. However, cervical cancer precursor lesions due to HPV infection can only be diagnosed through the cytopathological examination known as a Papanicolaou (Pap) test.

Studies have revealed that the adoption of the Pap smear, with good sensitivity and specificity, has reduced the incidence and mortality from cervical cancer in several countries (Saslow et al., 2012). In Brazil, Pap smear screening is particularly recommended for women aged 25-64 years who are or have been sexually active. The first two screening tests should be carried out annually, if the results of both test are normal, further tests can be performed every three years (INCA, 2015).

Considering that cervical cancer is the type of cancer with the highest incidence in the northern region of Brazil and few studies have been conducted so far on this topic, the present study aimed to contribute to the identification of the viral subtypes of HPV circulating in the population of this Brazilian region, as well as to compare data from cytological exams (Pap test and colposcopy) with the results from molecular biology analyses.

Material and methods

This was a cross-sectional study conducted in the following places: the Codajás Outpatient clinic (PAM) and the João dos Santos Braga Outpatient clinic (PJSB) in Manaus/Brazil. The analyses were carried out in the Laboratories of Molecular Biology of the *Universidade do Estado do Amazonas* (UEA) and the *Universidade Federal de Ciências da Saúde de Porto Alegre* (UFCSPA).

The inclusion criteria used in this study were: women, 18-60 years of age, diagnosed with abnormal cervical cytology, with referral for colposcopy, who agreed to participate in the study. The exclusion criteria used were: women outside the age group, and those that did not live in the region of Manaus.

After approval of the study by the research ethics committee, the cytological results (clinical diagnoses from Pap smears and colposcopy exams) were obtained from all the patients that were referred for colposcopy because their Pap tests showed changes in their cervical cells and agreed to participate in the project. The biological material of the patients was collected with cervical brushes or swabs and later discarded as biological waste. Participant confidentiality was ensured at all the stages of the study.

The clinical material collected was placed in labeled 1.5 mL microtubes containing TE buffer solution (10 mM Tris-HCl pH 8.0 and 1 mM EDTA). The samples were kept in an ice-water bath and then sent to the Laboratory of Molecular Biology of the *Universidade do Estado do Amazonas* (UEA), and stored at -20°C prior

to DNA extraction and PCR procedures. A 500 μ L cell pellet aliquot of cervical sample was used for DNA extraction. Proteinase K digestion was carried out, as described by Doyle and Doyle (1987).

To evaluate the integrity of the extracted DNA and the absence of inhibitors, PCR was performed using the set of primers specific for human β -globin (GH20 5'-GAAGAGCCAAGGACAGGTAC-3' and PC04 5'-CAACTTCATCCACGTTTACC-3') of 268 base pairs. The following amplification protocol was used: 94°C for four minutes, followed by 35 cycles of denaturation at 94°C, for one minute; annealing at 55°C for one minute; and extension at 72°C for one minute, followed by an additional cycle at 72°C for five minutes. The success of DNA amplification was verified by 1.5% agarose gel electrophoresis, stained with 2 μ L GelRed [1 μ g μ L⁻¹] in 1X TEB buffer (Tris borate and EDTA) for 1 hour at 100 volts and visualized under ultraviolet light on a UVP™ transilluminator and photographed on a digital system.

After the extraction and evaluation of the genomic DNA from the cervical samples, two PCR procedures were performed: one for HPV DNA detection and the other for identification of the HPV subtype. The HPV-positive samples were identified as viral subtypes 16 and 18. Generic primers GP05 (5'-TTTGTTACTGTGGTAGATACTAC-3') and GP06 (5'-GAAAAATAAACTGTAAATCATATTC-3'), described by de Roda Husman, Walboomers, van den Brule, Meijer, and Snijders (1995), were used in the HPV DNA detection by PCR. They amplify a conserved region of 170 base pairs (bp) of the HPV L1 gene.

A total volume of 20 μ L containing 1X PCR buffer, 200 μ M of each deoxynucleotide (dATP, dGTP, dCTP, dTTP), 1.6 mM of MgCl₂, 5 pmol μ L⁻¹ of each primer (GP05 and GP06), 0.5 U of Taq DNA polymerase and ~ 50 ng of DNA was used in the amplification reaction. The following amplification protocol was used: 95°C for one minute, followed by 40 cycles of 95°C for one minute; 57°C for one minute; and 72°C for one minute, followed by an additional cycle of 72°C for five minutes. Positive and negative controls were included in each run. The presence of HPV 16 and 18 in samples positive for consensus primers GP05/06 were determined in separate amplification reactions (PCR), using specific primers (Swan et al., 1999) HPV 16 (5'-TTGCAGATCATCAAGAACACGTAGA-3' / 5'-GTAGAGATCAGTTGTCTCTGGTTGC-3') and HPV 18 (5'-CAACCGAGCACGACAGGAACG-3' / 5'-TAGAAGGTCAACCGGAATTTTCAT-3'), which amplify a conserved region L1 ORF of 119 and 172 base pairs, respectively. Accordingly, 20 μ L of reagent mixtures were prepared with 0.5 U of Taq DNA polymerase, 200 μ M of each deoxynucleotide (dATP, dGTP, dCTP, dTTP), 1X PCR buffer, 1.6 mM MgCl₂, 5 pmol μ L⁻¹ of each primer and ~ 50 ng of DNA. The other reaction conditions were the same as in the PCR to identify the presence of HPV. In each reaction, positive and negative controls were included.

The PCR products were analyzed on 1.5% (w/v) agarose gel in solution, stained with 2 μ L red gel [1 μ g μ L⁻¹], in 1X TEB buffer (Tris-borate and EDTA) using 100 Volt current for approximately 40 minutes. Aliquots with a final volume of 6 μ L were composed of 3 μ L of the PCR product and 3 μ L of sample buffer (0.5% bromophenol blue and 20% glycerol). Invitrogen 100 bp DNA Ladder was used as a marker. The HPV subtypes were analyzed by comparing the bands generated in the gel with the positive controls of each reaction. The gels were visualized by fluorescence under ultra violet light in UVP™ transilluminator and photographed using a digital system.

Absolute frequencies were calculated for the HPV subtypes. Confidence intervals for the laboratory and clinical tests results were calculated at the 95% level (95% CI). Regarding age analysis, the mean and standard deviation (SD) were calculated and Student's t-test (parametric test) was applied, since the normality hypothesis was confirmed using the Shapiro-Wilk test (Sônia, 2004). The Epi Info version 7.2 for windows program was used for the data analysis, which was developed by the US Center for Disease Control and Prevention – CDC (www.cdc.gov/epiinfo) and distributed free of charge. The level of significance defined in the statistical tests was 5%.

Results and discussion

A total of 49 samples were obtained from women whose Pap tests and/or colposcopy were conducted in March-November 2016 and showed abnormal cervical cytology suggestive of atypia, high-grade lesions or low-grade lesions. In the Codajás Outpatient clinic health unit, 31 (63.3%) samples were collected and in the João dos Santos Braga Outpatient clinic health unit, 18 (36.7%) samples were collected. Patients were aged 18-60 years, with a mean age of 34.1 years (Figure 1). Samples from women who presented abnormal test results in their Pap and colposcopy exams performed at the healthcare service of the Codajás and João dos Santos Braga health units, were analyzed in the present study. The health units are considered model outpatient care systems as they provide assistance in several specialties.

Regarding age, the majority of the HPV infections were detected in women aged 26-60 years. These results corroborate the findings of several studies on the detection of these pathogens (Fernandes et al., 2010; Tamim, Finan, Sharida, Rashid, & Almawi, 2002).

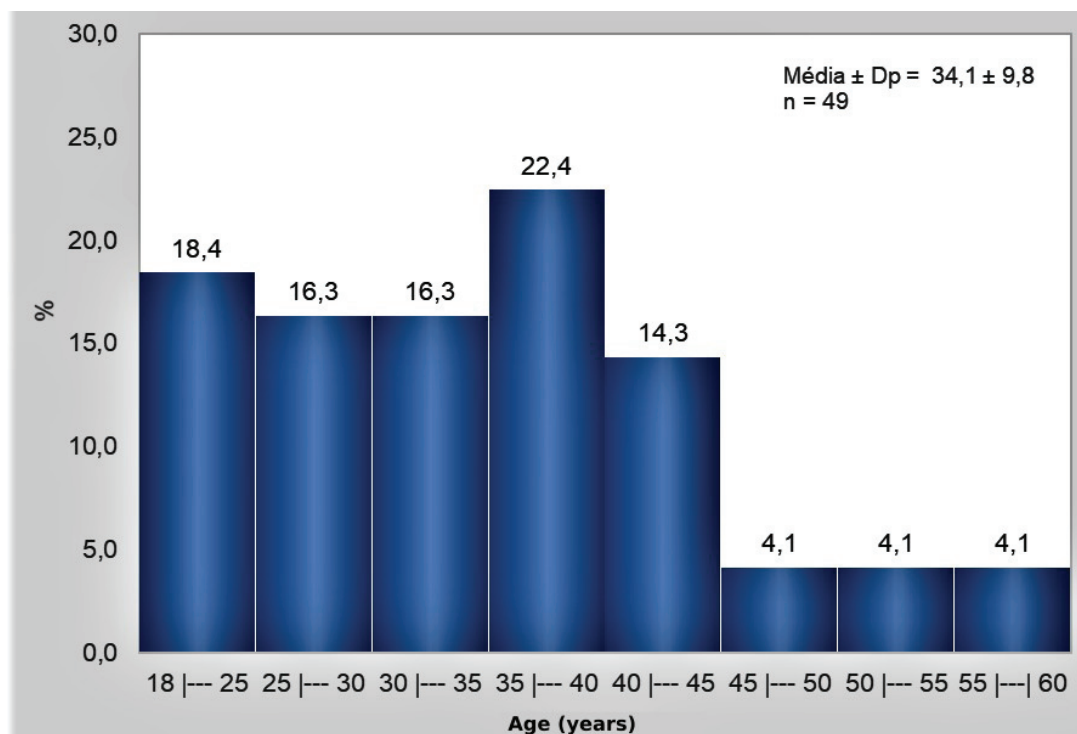


Figure 1. Age distribution of the analyzed patients.

The use of molecular methods for detecting deoxyribonucleic acid (DNA) and HPV genotyping, such as in situ hybridization, hybrid capture and polymerase chain reaction (PCR) in cervical specimens, in addition to cytology may be an important strategy for identifying patients predisposed to malignant processes, contributing to reduce morbidity and mortality from cervical cancer (Carvalho et al., 2010; Queiroz, Rocha, Filho, & Santos, 2015; Quintero et al., 2013).

In this study, positive HPV DNA was detected in all (100%) of the 49 cases analyzed, which was expected, since all patients had abnormal Pap and/or colposcopy test results. Furthermore, the samples were collected at specialized care centers that concentrate the majority of these cases. Duarte et al., (2017) also emphasized that the majority of research reported in the literature identifies infection rates and HPV subtypes in women with invasive or noninvasive cervical lesions or women referred for a Pap smear, where there is a tendency to have higher rates of HPV infection and cervical lesions associated with viral infection.

Several studies carried out by different authors in the last decade have analyzed samples from the uterine cervix and, with the aid of molecular techniques, have found the prevalence of positive HPV DNA in the 90%-100% range (Bosch & de Sanjose, 2007; Chang et al., 2005; Fedrizzi, Laureano, Schlup, Campos, & Menezes, 2008). Oliveira, Rodrigues, Lopes, Fernandez, and Cavalcanti (2003) conducted studies with 43 women and found the presence of HPV DNA in 95% (41/43) of the cases, where HPV 16 was the most prevalent subtype (60.4%) in single infections.

Human papillomavirus 16 and 18 were identified in 29 of these cases (59.2%) (Figure 2B-C). Therefore, 20 (40.8%) women whose DNA-HPV was detected by GP05/06 were infected by other subtypes of HPV not included in the study, (because, for the detection of each subtype of HPV there is a need for specific primers). Human papillomavirus 16 was present in 29 cases (59.2%). There was co-infection with HPV 18 in 4 (8.2%) of these cases. However, there were no cases with only HPV 18 infection in the present study (Tables 1 and 2).

Data from several studies also revealed that HPV-16 and HPV-18 are the most prevalent subtypes and can be associated or not with other subtypes (Oliveira, Rodrigues, Lopes, Fernandez, & Cavalcanti, 2003). In a study by Andersson, Mints, Sällström, and Wilander (2005), 215 samples were evaluated, of these, 45 contained invasive squamous cell carcinomas, with the most common subtypes detected being HPV-16 and HPV-18 (76% of the cases).

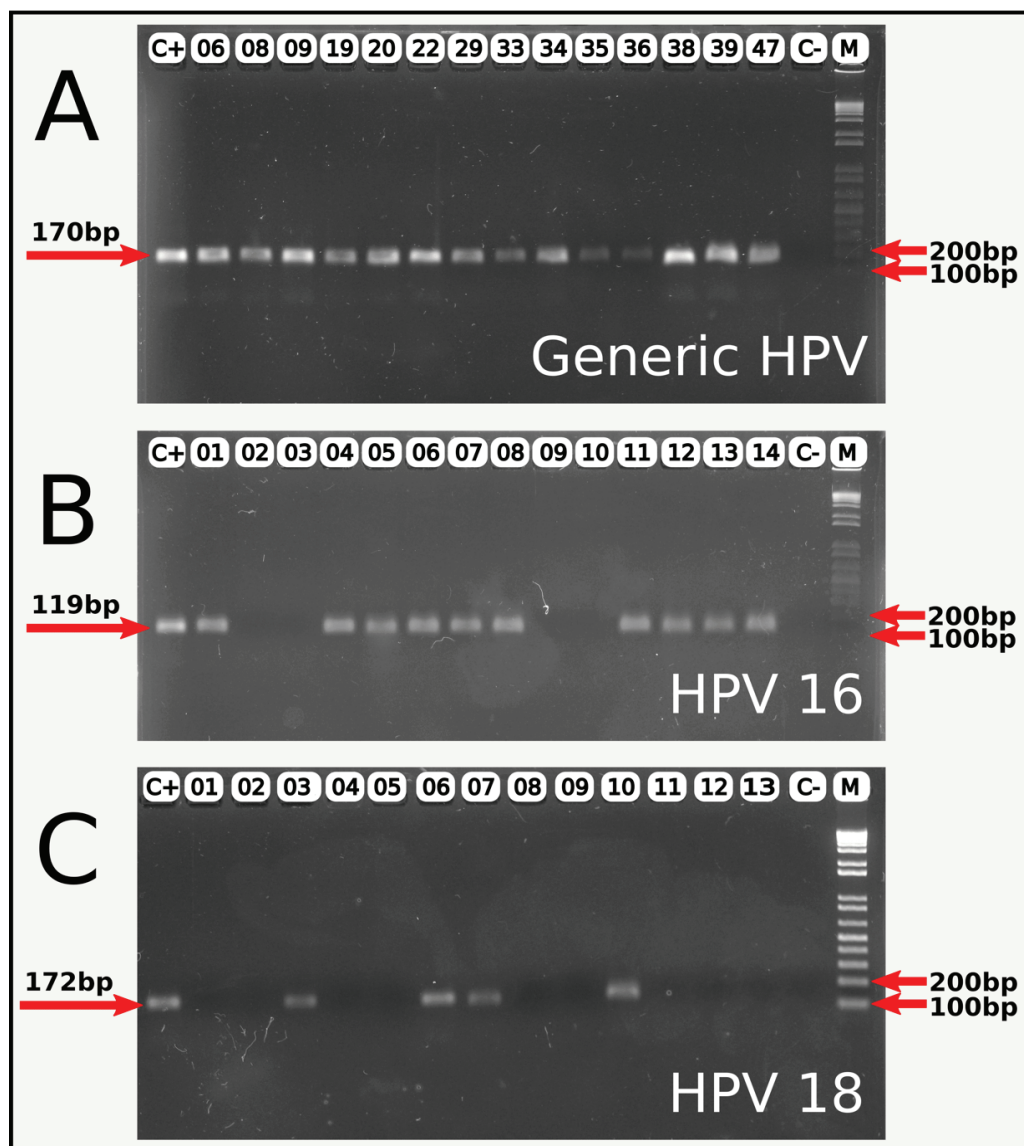


Figure 2. Electrophoretic profile of PCR amplified products. (A) HPV generic primers; (B) specific primers for HPV 16; and (C) specific primers for HPV 18. C +: positive control; 01-47: samples numbers; C -: negative control; M: Invitrogen 100 bp DNA Marker

In a literature review using qualitative studies, Rosa et al. (2009) reported the HPV subtypes prevalent in several countries and found that in Brazil HPV 16 was predominant in cervical cancers in all regions, with the following frequencies: Southern (52%), Central Western (57%), Northeastern (59%), Northern (43.5%) and Southeastern (52%). The HPV-18 subtype ranked second, except in the Central Western and Northeastern regions where subtypes HPV-33 and HPV-31, respectively, were prevalent.

Table 1. Distribution according to molecular diagnostic results of samples from patients in two referral units, Manaus – AM.

Variables (n = 49)	f _i	%	95% CI
HPV 16	29	59.2	44.2 – 73.0
HPV 18	4	8.2	2.3 – 19.6

f_i = simple absolute frequency; 95% CI = 95% confidence interval.

Table 2. Relationship between HPV 16 and HPV 18 results in samples from patients in two referral units of Manaus – AM.

HPV 16	HPV 18				Total
	Positive		Negative		
	f _i	%	f _i	%	
Positive	4	8.2	25	51.0	29
Negative	-	-	20	40.8	20
Total	4	8.2	45	91.8	49

f_i = simple absolute frequency; Agreement observed = 77.6%, with 95% CI (63.4% - 88.2%). Percentages refer to the total samples.

Table 3 shows the prevalence of HPV 16 according to the colposcopy examination, with only one patient (3.4%) diagnosed with ASC-H (atypical squamous cells, cannot exclude a high-grade intraepithelial lesion) and she was also infected by HPV 16. In turn, of the seven women with ASC-US (atypical squamous cells of undetermined significance), only one (3.4%) was infected by HPV 16. Of the 17 patients with LSIL (low-grade squamous intraepithelial lesion), three (10.4%) presented a positive result for HPV 16 and all 24 (82.8%) women with HSIL (high-grade squamous intraepithelial lesion) were also infected by viral subtype 16.

Table 3. Cytology results regarding the detection of HPV 16 in samples from patients in two referral units of Manaus - AM.

Variables	HPV 16				Total
	Positive (n = 29)		Negative (n = 20)		
	f _i	%	f _i	%	
Colposcopy					
ASC-H	1	3.4	-	-	1
ASC-US	1	3.4	6	30.0	7
LSIL	3	10.4	14	70.0	17
HSIL	24	82.8	-	-	24

f_i = simple absolute frequency.

Trottier et al. (2006), analyzing the association of infection with multiple HPV subtypes in samples of 2,462 Brazilian women, found that only 3.2% had multiple infections. In the present study, there was co-infection between the two subtypes (16 and 18) in 4 (8.2%) of the samples used. This is an important finding that corroborates studies that affirm the existence of a relationship between HPV groups of high and low oncogenic potential with cell abnormalities diagnosed in the cytopathological results (zur Hausen & de Villiers, 1994; Regenmortel et al., 2000; Albring, Brentano, & Vargas, 2006).

Table 4 compares the prevalence of HPV 18 according to the colposcopy examination. Only one patient (5.6%) was diagnosed with ASC-H (atypical squamous cells, cannot exclude high-grade intraepithelial lesion), and she was not infected by HPV 18, while, of the seven women with ASC-US (atypical squamous cells of undetermined significance), one was infected by HPV 18. Likewise, the tests of the 17 women with LSIL (low-grade squamous intraepithelial lesion) were negative for HPV 18. However, of the 24 women with HSIL (high-grade squamous intraepithelial lesion), 4 (44.4%) were infected by viral subtype 18.

Table 4. Cytology results regarding the detection of HPV 18 in samples from patients in two referral units of Manaus - AM.

Variables	HPV 18				Total
	Positive (n = 4)		Negative (n = 45)		
	f _i	%	f _i	%	
Colposcopy					
ASC-H	-	-	1	2.2	1
ASC-US	-	-	7	15.6	7
LSIL	-	-	17	37.8	17
HSIL	4	100.0	20	44.4	24

f_i = simple absolute frequency.

Roberts et al. (2006) observed a prevalence of 32.3% of HPV 16 and 6% of HPV 18 in 1,848 cervical biopsies. Regarding the diagnosis of HSIL, HPV 16 was detected in 47.5% of the cases, and both HPV 18 and 16 were found in 5.9% of the cases. In the present study HPV 16 was found at a higher frequency than HPV 18, corroborating the literature data about the most prevalent oncogenic subtypes. In a study conducted in 2009, Rosa and colleagues affirmed that persistent HPV infection leads to the onset of cervical intraepithelial neoplasia and that the presence of HPV 16 and HPV 18 contribute to this persistence, as 70% of cancers are caused by these two subtypes. The authors also emphasized that HPV alone cannot lead to cervical cancer and that the presence of some cofactors such, as age, marital status, an active sex life, sexual promiscuity, multiparity, low socioeconomic status, co-infections with other agents, smoking, low frequency of Pap smears, activation of the immune system, or host genetic makeup also impact cervical oncogenesis.

In the present study, there was only one case in which the cytology diagnosis did not indicate high-grade HPV infection, and PCR showed that this subject was positive for HPV 16. Therefore, HPV DNA of the high-risk group was present even in women with positive cytology for other cell abnormalities, which confirms

the importance of gaining more insight into the mechanisms involved in cervical oncogenesis, to ensure early diagnosis and prevention of cervical cancer, given the presence of high-risk HPV in these cases.

Conclusion

With the use of the generic primers GP05/06 it was possible to identify, through PCR, HPV DNA in 100% (49/49) of the endocervical samples. The HPV 16 subtype was found in 29 (59.2%) samples through PCR with the use of specific primers for the detection of this subtype. Through PCR, the HPV 18 subtype was found in 4 (8.2%) samples, as well as 4 co-infections with the HPV 16 subtype. In the present study, the PCR molecular method proved to be effective in diagnosing the presence of specific HPV subtypes (16 and 18).

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