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Sexually Transmitted Infections Detected by Multiplex Real Time PCR in Asymptomatic Women and Association with Cervical Intraepithelial Neoplasia

Infecções sexualmente transmissíveis detectadas por PCR multiplex em tempo real em mulheres assintomáticas e associação com neoplasia intraepitelial cervical

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Abstract

Objective   To determine the frequency of sexually transmitted infections (STIs) in asymptomatic women and the association of STIs with cervical intraepithelial neoplasia (CIN).

Methods   A cross-sectional study was performed, enrolling women examined in a general gynecology clinic and in a colposcopy referral center from October 2014 to October 2015. The colposcopy group consisted of 71 women, and the general gynecology group consisted of 55 women. Cervical samples were collected for cervical cytology and a multiplex real-time polymerase chain reaction (PCR) was developed to detect human papillomavirus (HPV) and the STIs caused by the following microorganisms: *Chlamydia trachomatis, Mycoplasma hominis, Mycoplasma genitalium, Ureaplasma urealyticum*, and *Neisseria gonorrhoeae*. A multivariate analysis was performed by logistic regression, considering the significance level of 0.05.

Results   The general frequency of STIs was: 46.8% (HPV); 27.8% (*C. trachomatis*); 28.6% (*M. genitalium*); 0.8% (*M. hominis*); 4.8% (*U. urealyticum*); and 4.8% (*N. gonorrhoeae*). The significant risk factors for CIN were: HPV infection (odds ratio [OR] = 2.53; $p = 0.024$); *C. trachomatis* (OR = 3.04; $p = 0.009$); *M. genitalium* (OR = 2.37; $p = 0.04$); and HPV and *C. trachomatis* coinfection (OR = 3.11; $p = 0.023$). After the multivariate analysis, a significant association was found between HPV and CIN (OR = 2.48; 95% confidence interval [95%CI]: 1.04–5.92; $p = 0.04$); and between *C. trachomatis* and CIN (OR = 2.69; 95%CI: 1.11–6.53; $p = 0.028$).

Keywords
► papillomavirus infections
► chlamydia
► sexually transmitted diseases
► polymerase chain reaction
► cervical intraepithelial neoplasia

Conclusion   The frequency of STIs was high in asymptomatic patients. Infections by HPV and *C. trachomatis* were independently associated with the presence of CIN. The high frequency of STIs in asymptomatic women suggests the need for routine screening of these infections.

Resumo

Objetivo   Determinar a frequência de infecções sexualmente transmissíveis (ISTs) em mulheres assintomáticas e a associação destas infecções com a neoplasia intraepitelial cervical (NIC).

Métodos   Foi realizado um estudo transversal recrutando mulheres atendidas em uma clínica ginecológica geral e em um centro de referência para colposcopia, de outubro de 2014 a outubro de 2015. O grupo de colposcopia consistiu de 71 mulheres, e o grupo de ginecologia geral consistiu de 55 mulheres. Amostras cervicais foram coletadas para citologia cervical e uma reação em cadeia de polimerase (RCP) multiplex em tempo real para detecção do vírus do papiloma humano (HPV) e das ISTs provocadas pelos seguintes micro-organismos: *Chlamydia trachomatis, Mycoplasma hominis, Mycoplasma genitalium, Ureaplasma urealyticum* e *Neisseria gonorrhoeae*. Foi realizada uma análise multivariada por regressão logística, considerando-se o nível de significância de 0,05.

Palavras-chave
► infecções por papilomavírus
► clamídia
► doenças sexualmente transmissíveis
► reação em cadeia de polimerase
► neoplasia intraepitelial cervical

Resultados   A frequência geral de ISTs foi: 46,8% (HPV); 27,8% (*C. trachomatis*); 28,6% (*M. genitalium*); 0,8% (*M. hominis*); 4,8% (*U. urealyticum*); e 4,8% (*N. gonorrhoeae*). Os fatores de risco significantes para NIC foram: infecção pelo HPV (razão de probabilidades [RP] = 2,53; $p = 0,024$); *C. trachomatis* (RP = 3,04; $p = 0,009$); *M. genitalium* (RP = 2,37; $p = 0,04$); e coinfecção por HPV e *C. trachomatis* (RP = 3,11; $p = 0,023$). Após a análise multivariada, foi encontrada uma associação significante entre HPV e NIC (RP = 2.48; intervalo de confiança de 95% [IC95%]: 1,04–5,92; $p = 0,04$) e entre *C. trachomatis* e NIC (RP = 2,69; IC95%: 1,11–6,53; $p = 0,028$).

Conclusões   A frequência de ISTs foi alta em mulheres assintomáticas. Infecções por HPV e *C. trachomatis* foram independentemente associadas com a presença de NIC. A alta frequência de ISTs em mulheres assintomáticas sugere a necessidade de rastreamento rotineiro dessas infecções.

Introduction

Cervical carcinoma is the third most common cancer among women worldwide, and the highest number of cases is being detected in women from developing countries. The carcinogenic human papillomavirus (HPV), a highly prevalent sexually transmitted infection (STI), is a necessary cause to the development of cervical intraepithelial neoplasia (CIN), and it has a central role in the etiology of cervical carcinoma.[1] However, it has been suggested that other factors may also modulate the risk of the progression of a cervical infection to malignancy.[2,3] Moreover, only part of the women infected with high-risk HPV will develop CIN/cervical carcinoma, suggesting that the carcinogenic process following HPV infection and CIN are likely influenced by other biological cofactors.[4]

Many studies consider *Chlamydia trachomatis* as a cofactor for the development of CIN, as well as other significant cell abnormalities in women with a history of HPV infection.[2,3,5] Currently, STIs are a major worldwide public health problem. The most prevalent STIs caused by viruses and bacteria are HPV and *C. trachomatis*. The incidence rate is increasing in developed countries, and according to the World Health Organization (WHO), more than 1 million STIs are contracted every day worldwide.[1] Untreated genital infection in women may lead to pelvic inflammatory disease, salpingitis, ovarian tube abscess, or perihepatitis, and may present with or without additional complications, such as chronic pelvic pain, ectopic pregnancy, and infertility.[5]

It is estimated that each year 131 million people are infected with *C. trachomatis*, and 78 million people with *Neisseria gonorrhoeae*. The high prevalence of *C. trachomatis* and *N. gonorrhoeae*, including significant rates of asymptomatic infections (50–80% of men and women), and the potential for severe complications are important factors that justify the routine screening of these bacteria.[6,7]

The widespread prevalence of STIs, and the public health costs associated with the difficult diagnosis of these infections have led to the development of fast and reliable methods for diagnosing STIs.[8] In addition, STIs often go undiagnosed and, when associated with antibiotic resistance, they are also becoming increasingly difficult to treat. Nevertheless, as a great number of pathogens are associated with STIs, and because STIs can be caused by polymicrobial infections, methods to identify multiple pathogens in a single sample are more interesting.[9,10] Infections such as those caused by *C. trachomatis*, *N.*

gonorrhoeae, *Ureaplasma urealyticum*, *Mycoplasma hominis* and *Mycoplasma genitalium* are frequently asymptomatic.[4] Epidemiological studies on the impact of these infections on HPV contraction and on the development of CIN or cervical carcinoma have yielded equivocal results due to the difficulty in separating biological from behavioral effects.[3,4]

The objective of the present study was to identify the frequency of STIs, associated or not with HPV, and to determine their possible association with CIN among asymptomatic women.

Methods

Ethical Aspects
The study was approved by the Ethics in Research Committee of Instituto de Educação e Pesquisa da Santa Casa de Belo Horizonte, Brazil (CAAE-0544.0.000), and registered in the National Ethics in Research Commission (CONEP, in the Portuguese acronym; CAAE-22053313.5.0000.5138).

Study Population
This is a cross-sectional study that involved 126 women who live in Belo Horizonte, state of Minas Gerais, Brazil, during the period between March 1st, 2014, and September 30th, 2015. The patients were divided into 2 groups: Group 1, composed of 55 women who presented at a general gynecology outpatient clinic (low risk of STI) at Centro de Especialidades Médicas, Santa Casa de Belo Horizonte; and Group 2, composed of 71 women that were referred to a colposcopy clinic (high risk of STI) at Ambulatório de Patologia Cervical of the Centro de Especialidades Médicas, Santa Casa de Belo Horizonte. Among the 71 patients evaluated in the colposcopy clinic, 59 (84%) had abnormal cytology, 41 (59%) underwent a biopsy, 33 (46.5%) presented CIN, and 19 (27%) were CIN 2-3. All patients were asymptomatic for condyloma, abdominal pain, and abnormal vaginal discharge. The exclusion criteria were: pregnancy or postpartum periods, previous hysterectomy, and vaginal bleeding. All of the participants voluntarily agreed to provide a sample for a Pap smear, and for HPV DNA and STIs detection, and signed an informed consent form.

Clinical Sample Collection
Cervical cytology was performed in all patients. Samples were collected using an Ayre spatula and cytobrush (Cytobrush Kolplast, Ci Ltda, Itapeva, São Paulo, Brazil) for Pap smear collection. The results were reported according to the 2001 Bethesda System.[11] The scrape samples for the DNA analyses of HPV and STIs were immediately suspended in specimen transport medium and stored at -80°C until the analysis. Women attending the colposcopy referral clinic were submitted to colposcopy and biopsy, when indicated. The CIN diagnosis was considered after the histopathological analysis.

DNA Extraction
The DNA was extracted from clinical samples as described by Sambrook and Gething.[12] The concentration and quality of the DNA was determined by spectrophotometry (Nanovue, GE Healthcare, Chicago, IL, US). The DNA extracts were stored at -70°C until used.

HPV and STI Detection
The detection of HPV was performed by multiplex real-time polymerase chain reaction (M-qPCR) using the general primers GP5 + /GP6+ (de Roda Husman et al)[13] and PCO3/PCO4 as internal control following the conditions described by Silva et al.[14]

The detection of others STIs was performed by M-qPCR with TaqMan (Applied Biosystems, Foster, CA, US) probes using specific assays to detect simultaneously *N. gonorrhoeae*, *C. trachomatis*, *U. urealyticum*, *M. hominis*, and *M. genitalium*. The *β-actin* gene was used as an internal control to ensure the quality of the extracted DNA. Positive STI controls were used in all M-qPCRs, as well as a negative control. All reactions were performed in universal cycling conditions with TaqMan Universal Master Mix buffer in the 7500 Real-Time PCR System (Applied Biosystems, Foster, CA, US). The assays were performed in duplicate for all genes, and the results were analyzed using the 7500 Fast Software, version 2.1 (Applied Biosystems, Foster, CA, US). The samples were analyzed at Instituto de Educação e Pesquisa da Santa Casa de Belo Horizonte, in a partnership with Laboratório Hermes Pardini.

Statistical Analysis
The two-sided Fisher exact test for the 2×2 contingency table was used to evaluate the statistical significance between different groups (general outpatients, colposcopy referral clinic, and patients with or without CIN). Odds ratios (ORs) with 95% confidence intervals (95%CIs) were calculated to estimate the association of HPV, non-HPV, and STIs with CIN. Variables that exhibited statistical significance at the 0.2 level were included in the multivariate analysis using a logistic regression model adjusted by potential confounders. Statistical significance was defined as $p < 0.05$. All statistical analyses were performed using SPSS Statistics for Windows, version 17.0 (SPSS Inc., Chicago, IL, US).

Results

The characteristics of the patients in each group, such as age, number of partners, smoking habits, early sexual activity, and contraception are shown in ►**Table 1**. The mean age of the patients included in the present study was 35 years old. The analyzed variables were not significantly different when the groups were compared.

The frequency of STIs, independently of CIN occurrence, were: HPV, 46.8%; *M. genitalium*, 28.6%; *C. trachomatis*, 27.8%; *U. urealyticum*, 4.8%; *N. gonorrhoeae*, 4.8%; and *M. hominis*, 0.8%. ►**Table 2** shows the frequency of the STIs among women according to their groups. Infection by HPV was significantly higher among the women in group 2 (colposcopy) (OR = 2.98; 95%CI: 1.43–6.22; $p = 0.003$). No statistical differences between the analyzed groups were observed with the other STIs.

On the other hand, when comparing the incidence of CIN with STIs, a significant difference was found between the presence of HPV and CIN (OR = 2.53; 95%CI: 1.11–5.75;

Table 1 Epidemiological characteristics of the patients according to the groups: general outpatient clinic (Group 1) and colposcopy (Group 2)

Characteristics	Group 1 (n = 56)	Group 2 (n = 71)	Total (n = 126)	p-value*	OR	95%CI
Age (years)#						
< 35	26 (29.2)	39 (70.8)	65	0.261	1.50	0.73–3.04
≥ 35	30 (21.7)	30 (78.3)	61			
Number of sexual partners						
> 5	23 (26.3)	34 (73.7)	57	0.401	1.35	0.66–2.75
< 5	33 (26.1)	36 (73.9)	69			
Early sexual activity						
< 18	28 (30.6)	45 (69.4)	73	0.121	1.76	0.85-3.60
≥ 18	28 (20.8)	25 (79.2)	53			
Smoker						
Yes	9 (25.0)	15 (75.0)	24	0.447	1.42	0.57–3.55
No	47 (26.5)	55 (73.5)	102			
Oral contraception						
Yes	33 (20.3)	36 (79.7)	69	0.401	0.738	0.36-1.50
No	23 (33.3)	34 (66.7)	57			

Abbreviations: 95%CI, 95% confidence interval; OR, odds ratio.
Notes: *Two-sided Fisher exact test; #mean age: 35 years old.

Table 2 Frequency of STIs according to general outpatient clinic (group 1) or colposcopy referral center (group 2)

STIs	Group 1 (n = 55) %	Group 2 (n = 71) %	Total (n = 126)	p-value*	OR	95%CI
HPV						
Yes	18 (30.5)	41 (69.5)	59	0.003	2.98	1.43–6.22
No	38 (56.7)	29 (43.3)	67			
Chlamydia trachomatis						
Yes	13 (37.1)	22 (62.9)	35	0.306	1.51	0.68–3.37
No	43 (47.3)	48 (52.7)	91			
Neisseria gonorrhoeae						
Yes	3 (50.0)	3 (50.0)	6	0.779	0.79	0.15–4.07
No	53 (44.2)	67 (55.8)	120			
Mycoplasma genitalium						
Yes	16 (44.4)	20 (55.6)	36	1.000	1.00	0.45–2.17
No	40 (44.4)	50 (55.6)	90			
Mycoplasma hominis						
Yes	01 (100.0)	0 (0)	1	0.444	2.27	1.86–2.77
No	55 (44.0)	70 (56.0)	125			
Ureaplasma urealyticum						
Yes	3 (50.0)	3 (50.0)	6	0.549	0.79	0.15–4.07
No	53 (44.2)	57 (55.8)	120			

Abbreviations: 95%CI, 95% confidence interval; HPV, human papillomavirus; STI, sexually transmitted infection; OR, odds ratio.
Notes: *Two-sided Fisher exact test; group 1= general outpatient clinic; group 2= colposcopy referral center.

Table 3 Frequency of STIs in patients with (+) and without (-) CIN

STIs	CIN + (n = 33) %	CIN - (n = 93) %	Total (n = 126)	p-value*	OR	95%IC
HPV						
Yes	21 (35.6)	38 (64.4)	59	0.024	2.53	1.11–5.75
No	12 (17.9)	55 (82.1)	67			
Chlamydia trachomatis						
Yes	15 (42.9)	20 (57.1)	35	0.009	3.04	1.30–7.08
No	18 (19.8)	73 (80.2)	91			
Neisseria gonorrhoeae						
Yes	01 (16.7)	5 (83.3)	6	0.503	0.55	0.62–4.88
No	32 (26.7)	88 (73.3)	120			
Mycoplasma genitalium						
Yes	14 (38.9)	22 (61.1)	36	0.040	2.37	1.02–5.50
No	19 (21.1)	71 (78.9)	90			
Mycoplasma hominis						
Yes	0 (0.0)	1 (100)	1	0.738	1.35	1.22–1.50
No	33 (0.0)	92 (73.6)	125			
Ureaplasma urealyticum						
Yes	0 (0.0)	6 (100)	6	0.155	1.37	1.23–1.54
No	33 (27.5)	87 (72.5)	120			

Abbreviations: 95%CI, 95% confidence interval; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; OR, odds ratio; STI, sexually transmitted infection.
Note: *Two-sided Fisher exact test.

$p = 0.024$), *C. trachomatis* and CIN (OR = 3.04; 95% CI: 1.30–7.08; $p = 0.009$), and *M. genitalium* and CIN (OR = 2.37; 95% CI: 1.02–5.50; $p = 0.040$). No significant associations were observed with the other STIs (►**Table 3**).

►**Table 4** shows the frequency of HPV and any other STIs associated with the occurrence of CIN. It can be seen that a significant association was found between CIN and HPV plus any other STI, and between CIN and HPV plus *C. Trachomatis*.

Finally, a multivariate analysis was performed to select the final predictor variables from the univariate model (a *p*-value of 0.15 was considered). A significant association between HPV and CIN (OR = 2.48; 95%CI: 1.04–5.92; $p = 0.04$) and between *C. trachomatis* and CIN (OR = 2.69; 95%CI: 1.11–6.53; $p = 0.028$) was observed.

Discussion

Cervical cancer remains a leading cause of death among women in developing countries, and HPV has its defined role as an etiological cause of cervical cancer. Human papillomavirus is present in 99.7% of women with uterine cervical

Table 4 Frequency of coinfection of HPV + STIs associated with the occurrence of cervical intraepithelial neoplasia

Coinfections	CIN + (n = 33) %	CIN - (n = 93) %	Total	p-value*	OR	95%CI
HPV + STI#						
Yes	13 (44.8)	16 (55.2)	29	0.011	3.12	1.29–7.55
No	20 (20.6)	77 (79.4)	97			
HPV + Chlamydia trachomatis						
Yes	9 (47.4)	10 (52.6)	19	0.023	3.11	1.13–8.53
No	24 (22.4)	83 (77.6)	107			
HPV + Mycoplasma genitalium						
Yes	6 (31.6)	13 (68.4)	19	0.562	1.36	0.47–3.95
No	27 (25.2)	80 (74.8)	107			

Abbreviations: 95%CI, 95% confidence interval; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; OR, odds ratio; STI, sexual transmitted infection.
Notes: *Two-sided Fisher exact test; # any other STI or more than one.

cancer, and it is considered the most common sexually transmitted infection worldwide.[3] However, HPV infections are generally self-limited, and only persistent infections with high-risk HPV are associated with the risk of development of CIN or of invasive cervical cancer. The persistence of the infection has been related to some factors, among them the concomitant infection by *C. trachomatis* and other STIs.[15,16]

The aim of the present study was to evaluate the frequency of STIs in asymptomatic patients, using M-qPCR to identify five genital infections: *C. trachomatis*, *N. gonorrhoeae* and *mollicutes* (*M. genitalium, M. hominis*, and *U. urealyticum*), besides the detection of HPV DNA by conventional PCR.[17] The frequency of these STIs was detected in two groups of women: one high-risk group (cervical pathology clinic), and a low-risk group (general gynecology outpatient clinic)

Molecular tests by M-qPCR represent the first line option for the diagnosis and screening of STIs in developed countries.[5,6,8] The advantages of this methodology are not only related to its high specificity and sensitivity, but also to its ability to identify several etiological agents in a single test. The present study used M-qPCR to detect five STIs, but it is possible to make these analyzes for larger numbers of microorganisms. Choe et al,[18] using M-qPCR for 7 STI agents in 897 urine samples of symptomatic and asymptomatic patients, concluded that the method has high sensitivity and specificity and, above all, enables a rapid simultaneous diagnosis of various STIs.

The present study showed that even in asymptomatic women, belonging or not to a high-risk group, there is a high frequency of STIs, especially HPV (46.8%), *M. genitalium* (28.6%) and *C. trachomatis* (27.8%). Among 126 patients, 66% were infected with at least one STI, and some were simultaneously infected by 2, 3, and even 4 pathogens. In similar investigations, a high prevalence of HPV (46%), *C. trachomatis* (21%), *N. gonorrhoeae* (5%) and *Gardnerella vaginalis* (39%) was reported in symptomatic and asymptomatic groups of women, suggesting that, belonging or not to a high-risk, group, all women should be screened and treated for STIs.[17,19] In addition, other results are in line with the findings of the present study, reinforcing the need to screen for STIs in asymptomatic women.[20]

Regarding *C. trachomatis,* a larger frequency of infection was found than the one reported in the current literature (27.8%). In general, women from developing countries present a higher prevalence of *C. trachomatis* infection, but not as high as that found in the present study. Some authors have reported prevalence values of *C. trachomatis* ranging from 5 to 22%, reaching 41% among female adolescents.[3] On the other hand, in developed countries, the prevalence of *C. trachomatis* has presented a frequency ranging from 2 to 10% in the general population, and from 5 to 20% among adolescent females.[21]

Similarly, a high frequency (28.6%) of *M. genitalium* was found, and it was greater than the literature reports.[22] We would like to point that the sexual transmission and the variable prevalence of *M. genitalium* around the world are important in public health, especially in countries where there is no data on the affected population. Currently, there iis a suggestion to include the routine screening for this STI, considering the possible damage for women's health related to the high prevalence in asymptom-

atic women. The association of *M. genitalium* with CIN is yet to be established in the literature because this STI has been investigated more recently with the use of PCR for multiple pathogens. At the same time, studies have shown the association of several STIs with CIN without evaluating the isolated association of *M. genitalium* with the cervical neoplasia.[22,23]

Neisseria gonorrhoeae was found in 4.8% of the women in the present study, a frequency similar to that reported in the literature, with frequencies ranging from 3 to 12%.[17] It is important to highlight that all women in the present study had no genital symptoms. In the literature, $\sim$ 70% of women were asymptomatic, but $\sim$ 10% of the cases could evolve to salpingitis or pelvic inflammatory disease and infertility.[7,10]

It is known that the presence of HPV is necessary and one of the most important factors for the development of cervical cancer. However, it is also recognized that the role of other different risk factors in association with HPV is crucial in cervical carcinogenesis. Several studies have shown that factors such as smoking, multiparity, prolonged use of contraceptives, and other STIs can triple the risk of development of precancerous lesions, or even of cancer, among women infected with oncogenic types of HPV 16 and 18.[24] In the present study, HPV types were not investigated.

Epithelial cells play an important role in the induction of an innate immune response in the female genital tract. These cells lined on the mucosal surfaces not only act as a physical barrier, but also actively participate in the secretion of antimicrobial substances and immune factors such as defensins, lactoferrin, and lysozyme. It is believed that an STI can reduce the resolution of HPV infection, with an induction of a pattern of innate and acquired immune response, especially humoral immunity.[25] In the present study, coinfection by HPV and *C. trachomatis* reached 15% ($p = 0.023$). Some authors have found a high prevalence of coinfection by HPV and *C. trachomatis* in patients with CIN 2-3, suggesting that these infections need to be investigated and treated in young women.[4,26] The effect of *C. trachomatis* in the cervical epithelium may be defined as a gateway, enabling the HPV to acess the basal layer of the epithelium, increasing the viral load, interacting with HPV high-risk types, and modulating the host immune response by inhibiting the apoptosis process.[25,27]

Conclusion

Our study demonstrated a high frequency of STIs in asymptomatic women, confirming the association of HPV with CIN, and an unexpected significant association between *C. trachomatis* and CIN. We suggest a discussion about the opportunity of including these STIs in a routine gynecological screening, using biomolecular methods that could detect multiple infections simultaneously, including HPV infection, to avoid the development of CIN.

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Conflict of Interests
The authors have none to declare.

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