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Coinfection of bacterial endocervicitis and human papillomavirus in women of reproductive age

Coinfecção de endocervicites bacterianas e papilomavírus humano em mulheres em idade reprodutiva

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ABSTRACT

Aim: To evaluate the coinfection rate between bacterial sexually transmitted infection (STI) and Human Papillomavirus (HPV) infection among reproductive-age women and identify risk factors associated with these infections. **Methods:** Five hundred and thirty reproductive-age women, HPV positive, who participated in a previous project about the persistence of Human Papillomavirus infection and cytokines profile were enrolled in this study. Vaginal samples were taken to classify vaginal microbiota by microscopy, according to appropriated scores. Endocervical samples were collected to assess the presence of infection by HPV. For HPV testing we used a manufacturing kit (Roche), then amplified a region of the L1 gene. The remaining endocervical secretion was used to determine *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Mycoplasma genitalium* infection rates. **Results:** Prevalence of HPV coinfection with *C. trachomatis*, *N. gonorrhoeae*, and *M. genitalium* was 14.2%, 1.2% and 1.4% respectively. About 42% of women had more than one type of HPV, 1.7% of women presented High-grade Squamous Intraepithelial Lesions (HSIL), and 5.3% presented Low-grade Squamous Intraepithelial Lesions (LSIL). Overall vaginal dysbiosis was 46%, and 34.9% of those had bacterial vaginosis. Abnormal vaginal microbiota was associated with *C. trachomatis* infection or high-risk HPV. **Discussion/ Conclusions:** A thorough assessment is crucial to provide a better comprehension of bacterial STIs in the presence of HPV. The overall HPV coinfection with *C. trachomatis* is high. The association of abnormal vaginal microbiota reinforces the importance of maintaining a dominated *Lactobacilli spp*. vaginal microbiota to protect against the acquisition of STIs, especially in HPV-infected women.

Keywords: Coinfection, dysbiosis, HPV, sexually transmitted infection.

RESUMO

Objetivo: Avaliar a taxa de coinfecção entre infecções sexualmente transmissíveis (ISTs) bacterianas e infecção por Papilomavírus Humano (HPV) entre mulheres em idade reprodutiva e identificar fatores de risco associados a essas infecções. **Metodologia:** Quinhentas e trinta mulheres em idade reprodutiva, HPV positivas, incluídas em projeto anterior sobre persistência da infecção por HPV, foram incluídas neste estudo. Amostras vaginais foram coletadas para classificar a microbiota vaginal por meio de microscopia. Amostras endocervicais foram avaliadas para a presença de HPV. Para o teste de HPV, utilizamos um kit de fabricação (Roche) e, em seguida, amplificamos uma região do gene L1. As secreções endocervicais restantes foram utilizadas para determinar as taxas de infecção por *Chlamydia trachomatis*, *Neisseria gonorrhoeae* e *Mycoplasma genitalium*. **Resultados:** A prevalência de coinfecção por HPV com *C. trachomatis*, *N. gonorrhoeae* e *M. genitalium* foi de 14,2%, 1,2% e 1,4%, respectivamente. Aproximadamente 42% das mulheres tinha mais de um tipo de HPV, 1,7% apresentaram lesões intraepiteliais escamosas de alto grau (HSIL) e 5,3% apresentaram lesões intraepiteliais escamosas de baixo grau (LSIL). A disbiose vaginal geral foi de 46%, e 34,9% dessas tinham vaginose bacteriana. A presença de disbiose vaginal foi associada à infecção por *C. trachomatis* ou HPV de alto risco. **Discussão/ Conclusão:** Uma avaliação minuciosa é crucial para proporcionar melhor compreensão das ISTs bacterianas e HPV. A coinfecção geral por HPV com *C. trachomatis* é alta. A associação da microbiota vaginal anormal reforça a importância de manter uma microbiota vaginal dominada por *Lactobacillus spp*. para proteger contra ISTs, especialmente mulheres infectadas por HPV.

Palavras-chave: Coinfecção, disbiose, HPV, infecção sexualmente transmissível.

INTRODUCTION

Globally human papillomavirus (HPV) infection is one of the most prevalent sexually transmitted infections (STIs)[1,2]. HPVs are small DNA viruses with a simple DNA double-stranded DNA genome that infects squamous epithelial or cells with the potential for squamous maturation. These viruses are classified by genotype and can be separated in high or low risk, depending on their oncogenic potential. Most women in the world will be infected with genital HPVs at an unspecified time, with a lifetime risk of infection of 50–80%[3]. Most HPV infections resolve as a consequence of the development of cell-mediated immunity accompanied by seroconversion and antibody to the major coat protein L1. However, not all women can make a successful cell mediated immune response, this means they remain HPV DNA positive[4,5].

Persistence of high–risk HPV is the major risk for the further development of high-grade intraepithelial disease and cervical cancer, but also there is evidence that high–risk HPV infection per se is not enough for the subsequent outcomes: squamous cell or adenocarcinoma of the cervix. Generally, in immunocompetent individuals, the progression to high-grade precancerous lesions is slow, yet so, cervical cancer represents a major health issue worldwide; in Brazil, the incidence of new cases remains high. At least 15 genotypes of high–risk HPV have already been identified, still, not all women who present these types will develop cancer[5]. Apart from identifying HPV genotypes, it is of paramount importance to consider other risk factors that transcend sexual behavior, age or smoking habits. The high burden of the persistence of HPV can be enhanced by the presence of other sexually transmitted infections and have an impact on the natural history of HPV[6].

It is widely known that *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Mycoplasma genitalium* are three of the most common bacterial STIs of gynecological relevance, which individually trigger inflammatory processes. Their influence as cofactors regarding the persistence of HPV is known to a small extent[7-11]. Infections by these bacteria share mutual risk factors like short-term relationships, precocious intercourse age, and lack of protection barrier usage. These etiological agents colonize the lower genital tract causing endocervicitis[12-15], but about 70% of the cases are asymptomatic, which represents a major sexual and reproductive problem, as these patients do not get diagnosed or treated. Long-term lower genital tract infections can cause serious consequences like pelvic inflammatory disease (PID), which leads to chronic pain and can evolve to tubal factor infertility and ectopic pregnancy[11].

Also, it is becoming evident that cervicovaginal microbiota is engaged in the persistence of HPV infection. Bacterial vaginosis (BV) is a condition characterized by the replacement of *Lactobacilli* by other bacterial species, mostly anaerobes, and is the most frequent vaginal dysbiosis in women of reproductive age. The association of bacterial vaginosis with STIs is well described in the literature[16-19]. Worldwide, the prevalence of BV varies from 9%-30%[16-18], and in Brazil it is around 30%[18].

Women present different patterns of the vaginal microbiota, meaning that the response against genital tract infections varies among them. The specific immune response to infection is regulated by T lymphocytes and two subclasses determine whether the response will be antibody-mediated or cell-mediated (Th1 and Th2). There is a pivotal need to characterize the endocervical environment in this complex dynamic to better understand the mechanisms implicated in the process of establishment of the human papillomavirus virus in immunocompetent women[18,20-21].

Therefore, the goal of this study was to determine the endocervical coinfection rate by *C. trachomatis*, *N. gonorrhoeae*, and *M. genitalium* in reproductive-age women with human papillomavirus infection seen at Primary Health Care Units in Botucatu, São Paulo, and to identify certain risk factors associated with these infections.

PATIENTS AND METHODS

Patients recruitment and sample collection

In 2012 our research group performed an epidemiological study entitled "Influence of endocervicitis by *Chlamydia trachomatis* in the persistence of Human Papillomavirus infection (HPV) and profile of cytokines produced in the

cervical secretion of immunocompetent women" to assess the influence of *Chlamydia trachomatis* as a cofactor for the risk of persistent HPV infection in 1640 women seen at Primary Health Care Units in Botucatu. For this study, the eligibility criteria were 72 hours of sexual abstinence, no use of antibiotics for the past 30 days, not pregnant, and not at menopause. All patients signed written informed consent. The collection of samples was approved by the Ethics Research Committee of Botucatu Medical School, UNESP (protocol 893.258–2011, FMB, UNESP).

In the previous study, a questionnaire was applied to the participants to obtain sociodemographic information. In addition, during the physical exam for routine papsmear screening, vaginal samples were taken to classify vaginal microbiota by microscopy, according to Nugent et al. (1991), Cibley & Cibley (1991), and Donders et al. (2002)[22-24]. Endocervical secretion samples were collected to investigate the presence of infection by HPV. For the present study, we included all women diagnosed with human papillomavirus infection and processed all samples for *C. trachomatis*, *N. gonorrhoeae*, and *M. genitalium* infection. We excluded in this study women who presented *Trichomonas vaginalis* infection, syphilis, human immunodeficiency virus, and *Herpes simplex* virus infection, totalizing therefore 530 participants.

Detection of HPV

DNA from cervical samples was extracted using an AmpliLute Liquid Media Extraction DNA Kit (Roche Molecular Systems, Inc.) per the manufacturer´s instructions. HPV genotyping was performed using Linear Array Genotyping Test (Roche Molecular Systems, Inc.), which was used to differentiate 37 HPV types, highlighting the high-risk HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59. Positive and negative controls were included in each assay. The human β-globin target was co-amplified to determine the sample adequacy.

Detection of *Chlamydia trachomatis*

Chlamydia trachomatis screening was performed by conventional Polymerase Chain Reaction (PCR). The reaction was composed of 10μL Go Taq Green Master Mix, 2X (Promega, Madison, WI); 1 μL MgCl2; 0.2μL of each primer (CTP1, CTP2, PL61, and PL62), 4.2μL of autoclaved Milli-Q water (Milli Q Plus, Millipore) and 4μL of DNA sample, in a final volume of 20μL. Incubations were performed in Eppendorf Mastercycler Personal thermocycler (Applied Biosystems, Foster City, CA) employing the following parameters: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of 95°C for 1 minute for denaturation, 55°C for 1 minute for annealing and 72ºC for 1 minute for extension, with a final extension at 72°C for 5 minutes. For all reactions, negative (water) and positive (DNA extracted from *C. trachomatis-infected* McCoy cells) controls were used. For visualization of the amplified products, 1.5% agarose gel electrophoresis was performed and visualized under UV transillumination. The primers used in the reaction amplified two fragments of 201 and 130 base pairs.

Detection of *Neisseria gonorrhoeae*

It was performed a duplex PCR targeting the gonococcal *por*A pseudogene and multicopy *opa* gene. The reaction mix consisted of 10μL of 2x QuantiNova Probe PCR Master Mix (including ROX dye); 20x primer probe mix of 1μ/L of pAp and *opa* genes and 4μL of H2O Rnase free, in all reactions we included a negative control (water) and a 5-points standard curve positive control. The limit of detection was 1 copies-equivalent. Amplification and detection were performed on the *Line-* gene K, with an initial hold of 95ºC for 2 minutes followed by 45 cycles at 95ºC for 5 seconds and 60ºC for the 30s. To validate true positive results, we performed a conventional PCR reaction of *cppB* and *pgi* genes, nonetheless to confirm successful DNA extraction we performed a conventional PCR for detection of β-globin.

Detection of *Mycoplasma genitalium*

For the detection of *M. genitalium*, we performed Real-time PCR targeting the *MgPa* gene that codes an important adhesion protein, using the primers MgPa355F-GAGAAATACCTGATGGTCAGCA and MgPa432R-GTTAATATCATA T A A A G C T C T A C C G T T G T T A T C The reaction consisted of 12.50μL of 2x SYBR green; 0.75μL of each primer; 6μL of autoclaved Milli-Q water (Milli Q Plus, Millipore), and 5μL of DNA sample, totalizing 25μL of the final volume, also for all reactions we included a negative (water) and a 5-points standard curve using the positive control

(DNA) provided by Dr. Jorge Timenetsky of the University of São Paulo. The limit of detection was 10 copies-equivalent. Amplification and detection were made on StepOne Plus (Applied Biosystems) with the following condition: 95ºC of initial hold 95ºC for 10 minutes, followed by 40 cycles at 95ºC for 1 minute, and 60ºC for 1 second.

Diagnosis of abnormal vaginal flora

Vaginal samples were spread onto glass microscope slides to classify the microbiota pattern following Gram stain according to Nugent's scoring system: normal (scores 0-3), intermediate (4-6), and bacterial vaginosis (7-10). Vaginal candidiasis was based on the presence of yeast blastopores or pseudohyphae on the smears with accompanying inflammatory cells in the presence of vaginal discharge or vulvovaginal pruritus. Cytolytic vaginosis was diagnosed by an increased number of lactobacilli, a paucity of white blood cells, evidence of cytolysis, and the presence of discharge[23]. Aerobic vaginitis was diagnosed if smears were deficient in lactobacilli, positive for cocci or coarse bacilli, positive for parabasal epithelial cells, and/or positive for vaginal leucocytes[24].

Cervical Cytology Screening

Pap smears were fixed in absolute ethanol, stained, and read at the Cytology Unit of the Department of Pathology, Botucatu Medical School, São Paulo State University (Unesp) and were classified according to the Bethesda system.

Statistical analysis

Analyses were performed using GraphPad Prism 8. The variables age, years of school and number of partners were represented as median and interquartile values (P25-P75). The categorical variables were represented as total number followed by percentage. The significance level adopted was 5%.

RESULTS

Of 530 HPV-positive women included in this study, full laboratory results were available for all participants regarding *C. trachomatis*, *N. gonorrhoeae*, and *M. genitalium* infection. The median age was 29 years old (23–35), 52.8% did not use condoms, 78.9% were not smokers and the median number of partners was 3 (2 – 5) (Table 1). The prevalence of HPV coinfection with *C. trachomatis*, *N. gonorrhoeae* and *M. genitalium* was 14.2%, 1.2% and 1.4%, respectively. A total of 41.7% of women had more than one type of HPV, 1.7% of women presented High-grade Squamous Intraepithelial Lesions (HSIL), and 5.3% presented Low-grade Squamous Intraepithelial Lesions (LSIL). Overall vaginal dysbiosis was 46%, this included bacterial vaginosis (34.9%), Flora II plus candidiasis (4.0%), and others (7.2%). Altered vaginal microbiota was associated with *C. trachomatis* (p<0.0001) or high-risk HPV (p=0.0002) infection, but it was not associated with having more than one type of HPV (Tables 2 and 3).

Table 1. Sociodemographic and lifestyle data of patients included in this study.

Variables		Cohort (n=530) n (%)
Age Median (P25-P75)		29 (23–35)
Years of school Median (P25-P75)		11 (8-11)
Number of partners Median (P25-P75)		3 (2-5)
Ethnicity		
	White	58.7 (311/530)
	Other	41.3 (219/530)
Marital status		
	Stable union	34.2 (181/530)
	Single	18.7 (99/530)
	Others	47.1 (250/530)
Smoking habits		
	Yes	23.0 (122/530)
	No	78.9 (418/530)
Use of condom		
	Yes	46.6 (247/530)
	No	52.8 (280/530)

P25: percentil 25, P75: percentil 75.

Table 2. HPV, endocervical bacterial infections, and vaginal microbiota.

Variables	Cohort (n=530) n (%)
Vaginal Microbiota	
Normal Flora	286 (53.9)
Bacterial vaginosis	185 (34.9)
Intermediate+ Candidiasis	21 (4.0)
Other	38 (7.2)
Multiple HPV infections	
HSIL	9 (1.7)
LSIL	28 (5.3)
C. trachomatis infection	75 (14.2)
N. gonorrhoeae infection	6 (1.2)
M. genitalium infection	7 (1.4)

HSIL: High-grade Squamous Intraepithelial Lesions; LSIL: Low–grade Squamous Intraepithelial Lesions.

Table 3. HPV infection status regarding vaginal microbiota.

Variables		Vaginal Dysbiosis (n=244)	Healthy microbiota (n=286)	P-value
HPV				
	High-risk	207	197	0.0002
	Low-risk	37	89	
Multiple infections		108 (44.3%)	113 (39.5%)	0.588
C. trachomatis infection		49 (20.1%)	24 (8.4%)	<0. 0001
N. gonorrhoeae infection		2 (0.8%)	5 (1.8%)	0.064

Test performed Chi-square. HPV: human papillomavirus.

DISCUSSION

The presence of HPV is a necessary precursor for cervical cancer development, yet not quite sufficient to induce invasive disease. Some of the factors involved are HPV genotype, sustained viral oncogene expression, viral load, viral genome integration, and persistence[25,26]. The factors contributing to these events remain unclear; however, the theoretical framework is based on biological and behavioral determinants[26].

The epidemiological profile of Brazilian population is restricted since sexually transmitted infections, except for HIV, syphilis, and hepatitis, are not mandatory reported, therefore, the access to reliable national data is, to some extent, complex. Despite this, studies carried out across the country exhibit a prevalence of *C. trachomatis* that ranges from 12.2% - 22.2%[27,28] and the prevalence of *N. gonorrhoeae* is about 1.5%[29]. Regarding *M. genitalium*, some studies performed in Brazil indicate infection rates of 0.9% to 28.1%. This discrepancy can be explained by differences among populations and by the site of sample collection; once in the endocervix, the inflammatory response is more robust than in the vagina, expanding

therefore the survival rate of this microorganism[30].

Although the characterization of the endocervical environment is thoroughly approached in the literature, to the best of our knowledge the investigation of human papillomavirus conjointly with three sexually transmitted infections is scarce. Our rates of prevalence for *C. trachomatis*, *N. gonorrhoeae*, and *M. genitalium* were 14.2%, 1.2% and 1.4%, respectively. The pathogenicity of *M. genitalium* was established in the '90s and, consequently, its screening is still scarce. Additionally, in our experience, the standardization of a technique for *M. genitalium* detection proved to be challenging.

Data of the interference of *N. gonorrhoeae* inducing a disruption in the cervical epithelial barrier or another pathway is insufficient in the literature. The detection of this etiological agent by bacterial culture is still considered to be the gold standard; however, this method is starting to be replaced by a less time-consuming method, the NAATS (nucleic acid amplification tests). *N. gonorrhoeae* comprise different subtypes that exhibit different sequences, varying temporally and geographically, thus resulting in different NAATS and, most importantly, in sequence-related false-negative[31]. Our detection protocol targets two genes: multicopy *opa* gene, and *por*A pseudogene, since only one gene might not be sufficiently conserved in a subtype. Therefore, we followed the recommendation of performing two confirmatory assays, to confirm true positives and ensure great sensitivity and specificity[32].

Regarding vaginal microbiota, the frequency of vaginal dysbiosis, and specifically the presence of bacterial vaginosis, in patients who were positive for *C. trachomatis* was high. In addition, we noticed that women with low-risk HPV presented normal patterns of vaginal flora. A study performed in Korea calculated the relative risk between vaginal microbiota and high-risk HPV by their interaction and synergy, and found that women with cervical intraepithelial neoplasia had higher vaginal diversity than healthy controls[33]. We also detected in our cohort a statistically significant association between women who presented vaginal microbiota diversity and high-risk HPV. Once bacterial vaginosis-associated strict anaerobes colonize, they produce enzymes and metabolites, which may compromise the cervical epithelial barrier, facilitating HPV entry to the basal keratinocytes. This, in turn, acts on several cellular pathways that can enable a persistent, productive viral infection and establishment of the disease[33-36]. Conversely, the acidic environment can inhibit the growth of several potentially pathogenic species, like *C. trachomatis*, *N. gonorrhoeae*, and *Gardnerella vaginalis*[37-40], and it creates ideal conditions for the cellular metabolic function of the cervicovaginal environment[41-43]. A study made by our research group showed that proinflammatory cytokines remained unaltered in women with healthy vaginal microbiota even in the presence of endocervicitis by *C. trachomatis*, but bacterial vaginosis was associated with altered inflammatory cytokines, reinforcing the relevance of maintaining a dominated *Lactobacilli* vaginal flora precisely to protect against the acquisition of STIs[44].

CONCLUSION

A thorough assessment is crucial to provide a better comprehension of bacterial STIs in the presence of HPV. The overall HPV coinfection with *C. trachomatis* is high, and the association of abnormal vaginal microbiota reinforces the importance of maintaining a dominated *Lactobacilli* vaginal microbiota to protect against the acquisition of STIs, especially in HPV infected women.

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