Am J Cancer Res 2016;6(6):1371-1383
www.ajcr.us /ISSN:2156-6976/ajcr0027886

Original Article

Association of human papillomavirus, *Neisseria gonorrhoeae* and *Chlamydia trachomatis* co-infections on the risk of high-grade squamous intraepithelial cervical lesion

André LP de Abreu[1,5], Natália Malaguti[1], Raquel P Souza[1], Nelson S Uchimura[2], Érika C Ferreira[3], Monalisa W Pereira[1], Maria DB Carvalho[2], Sandra M Pelloso[4], Marcelo G Bonini[5], Fabrícia Gimenes[1], Marcia EL Consolaro[1]

[1]Clinical Cytology Laboratory, State University of Maringá (UEM), Maringá, Paraná, Brazil; [2]Department of Medicine, State University of Maringá, Paraná, Brazil; [3]Department of Statistics, State University of Maringá, Paraná, Brazil; [4]Department of Nursing, State University of Maringá, Paraná, Brazil; [5]Department of Pharmacology, University of Illinois at Chicago, Illinois, USA

Received March 10, 2016; Accepted March 13, 2016; Epub June 1, 2016; Published June 15, 2016

Abstract: The link between high-risk human *Papillomavirus* (HR-HPV) and other sexually transmitted diseases (STDs) in the risk of developing cervical cancer still unclear. Thus, in this report we investigated the rates of co-infections between HPV and other important non-HPV STDs in different cervical findings using a multiplex polymerase chain reaction (M-PCR) to simultaneously detect *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, *Trichomonas vaginalis*, HSV-1 and -2, and *Treponema pallidum.* A total of 838 women aged 18 to 68 years were screened using Papanicolaou smears for cervical abnormalities, HPV and non-HPV STDs using PCR and M-PCR methods. A total of 614 (73.3%) of the women had normal cytology (NILM) and 224 (26.7%) women exhibited abnormal cytology (≥ ASC-US). HPV-DNA prevalence was 33.9%, and HPV-16 was the most prevalent genotype in women with NILM and ≥ ASC-US cytology. Non-HPV STDs were detected in 30.4% women and *T. vaginalis* was the most prevalent one (11.6%). A higher increased risk of ≥ ASC-US and HSIL occurred in co-infections of HR-HPV with *C. trachomatis* and *N. gonorrhoeae*. Co-infections of HPV-DNA and HR-HPV with HSV-2 exhibited a similar increased risk but only with ≥ ASC-US. Co-infections of HPV-DNA and HR-HPV with *T. vaginalis* demonstrated a similar increased risk of ≥ ASC-US and HSIL. We found that *C. trachomatis* and *N. gonorrhoeae* were the primary pathogens associated with HR-HPV for the increased risk for all grades of cervical abnormalities but mainly for HSIL, suggesting a possible synergistic action in cervical lesions progression. Our results reinforce the hypothesis that some non-HPV STDs might play a role as co-factors in HPV-mediated cervical carcinogenesis. These data improve our understanding of the etiology of SCC and may also be useful for disease prevention.

Keywords: Cervical cancer, HPV, co-factors, sexually transmitted diseases, progression risk, HSIL

Introduction

Cervical cancer is the fourth leading cause of cancer in women worldwide, despite the existence of highly effective prevention and screening methods [1, 2]. Persistent high-risk human *Papillomavirus* (HR-HPV) infection is the central factor in the development of squamous cell cervical carcinoma (SCC) and HR-HPV is a prerequisite for progression to high-grade squamous intraepithelial lesions (HSIL) [1-3]. HR-HPV infections are most eventually cleared, but a few progress to HSIL (10% of HPV infections) and SCC (< 1% of HPV infections) [1-5]. The reasons for this variable natural history are poorly understood, but it is generally assumed that other causes or co-factors are important for the development of neoplasia in HPV-infected women [4-6].

Several possible risk factors were associated with persistent HR-HPV in the last two decades and contribute to cervical carcinogenesis [7-14]. Co-factors related to individual's life-

Table 1. Baseline features of the study cohort in overall and according to cytologic findings

Characteristics	Cytological findings		
	Overall women (N=838)	NILM (N=614)	Abnormal (N=224)
	n (%)	n (%)	n (%)
Age ranges (years)			
Mean (SD)	40.03 ± 10.0	41.36 ± 9.6	36.4 ± 10.0
< 25	84 (10.0)	45 (7.3)	39 (17.4)
25-34	223 (26.6)	147 (24.0)	76 (33.9)
35-44	238 (28.4)	181 (29.5)	57 (22.5)
45-54	168 (20.0)	140 (22.8)	28 (12.5)
55-64	101 (12.1)	85 (13.8)	16 (7.1)
≥ 65	24 (2.9)	16 (2.6)	8 (3.6)
Age of first sexual intercourse (years)			
≤ 13	40 (4.8)	27 (4.4)	13 (5.8)
14-16	290 (34.6)	197 (32.1)	93 (41.5)
17-19	297 (35.4)	234 (38.1)	63 (28.1)
≥ 20	211 (25.2)	156 (25.4)	55 (24.5)
Sexual partners during life (n)			
1	312 (37.2)	246 (40.1)	66 (29.5)
2-5	387 (46.2)	258 (42.0)	129 (57.6)
6-10	76 (9.1)	54 (8.8)	22 (9.8)
> 10	63 (7.5)	56 (9.1)	7 (3.2)
Pregnancies to term (n)			
0	103 (12.3)	60 (9.8)	43 (19.2)
1-2	424 (50.6)	320 (52.1)	104 (46.4)
3-4	251 (30.0)	195 (31.8)	56 (26.2)
≥ 5	60 (7.2)	39 (6.4)	21 (9.4)
Use of hormonal contraceptive			
No	579 (69.1)	451 (73.5)	128 (57.1)
Yes	259 (30.9)	163 (26.5)	96 (42.9)
Use of other contraceptive type			
No	640 (76.4)	487 (79.3)	153 (68.3)
Yes	198 (23.6)	127 (20.7)	71 (31.7)

Baseline characteristics in overall women included in the study and according to cytologic findings. Mean (SD), mean ± standard deviation. NILM, negative for intraepithelial lesion or malignancy; Abnormal, included atypical glandular cells (AGC), squamous intraepithelial lesions (SIL) of low (LSIL) or high (HSIL) grade, and atypical squamous cells (ASC) of undetermined significance (ASC-US) or not possible exclude HSIL (ASC-H).

style, such as the use of tobacco and hormonal contraceptives, early sexual activity and multiple sex partners were associated with SCC [5-8]. Host genetic variability, intratype HPV variations, multi-infection with multiple HPV genotypes and co-infection with other agents are associated with the persistence and progression of a premalignant condition to SCC [6, 9-14]. This association has been studied since 1989 when Schmauz et al., [15] published that multiple sexually transmitted diseases (STDs) were a risk factor for SCC and suggested that additional non-HPV STDs may act a s HPV co-factors [16, 17]. The non-HPV STDs herpes simplex virus-2 (HSV-2) and *Chlamydia trachomatis* [11, 12, 16, 18] have demonstrated the most consistent evidence of an association with HPV. However, there is no consensus on the impact of other genital pathogens in association with HR-HPV on disease progression.

The real prevalence of HR-HPV and non-HPV STDs co-infections in cervical samples is not clear. The overall prevalence of STDs is an under-recognized condition in several populations despite its clinical importance, which is likely because of difficulties in diagnoses that depend on traditional methods, such as cultures and enzyme immunoassays [17, 19]. The recent development and introduction of cultivation/serological-independent molecular-based techniques, such as polymerase chain reaction (PCR), revolutionized the diagnosis of genital tract infections [19-21]. Multiplex PCR (M-PCR) allows the simultaneous detection of multiple pathogens in symptomatic and asymptomatic patients, including viral infections [19, 21]. This simultaneous detection furthers our understanding of the true prevalence of co-infections between HR-HPV and non-HPV STDs in different cervical findings.

The present report investigated the rates of co-infections between HPV and other important

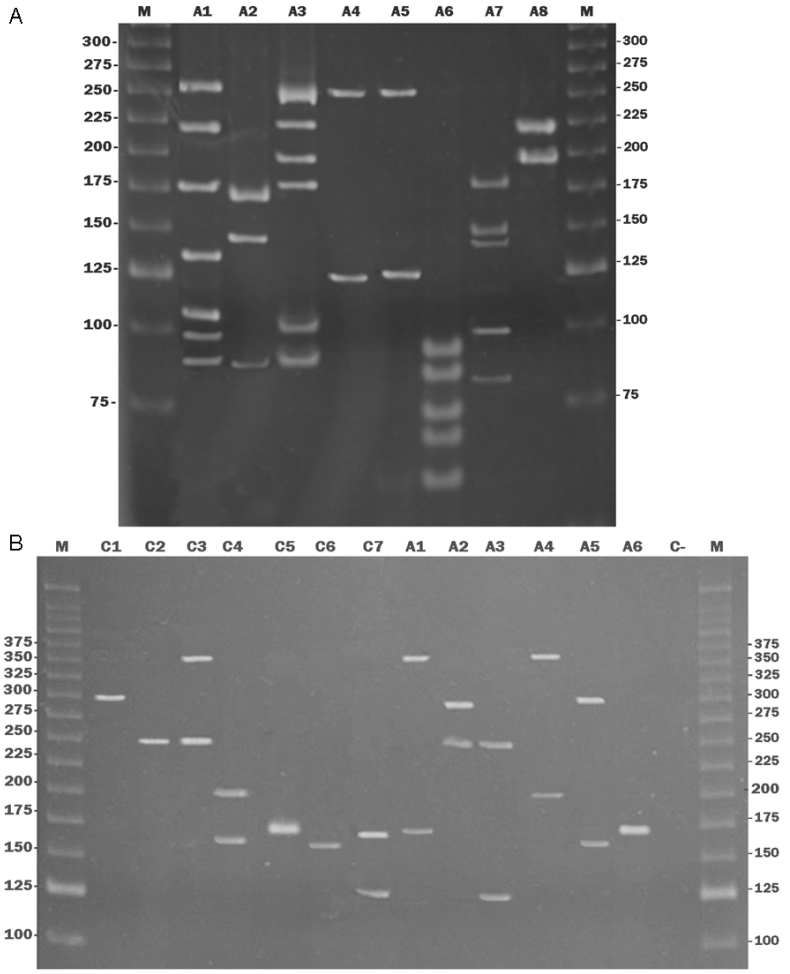

Figure 1. Electrophoretic analysis of HPV genotyping by Polimerase Chain Reaction (PCR)-restriction fragment length polymorphism analysis (PCR-RFLP) using *Hpy*CH4V and *Nla*III (A) and of amplified fragments by using a multiplex-PCR (B) for non-HPV sexually transmitted diseases (STDs), both in 8% polyacrylamide gel stained with ethidium bromide. (A) Sample A1, genotypes 31, 59 and 82 in HPV co-infection (253, 216, 174, 127, 112, 94 and 91 base pairs-bp); A2, genotype 72 in single HPV infection (171, 137 and 90 bp); A3, genotypes 16, 31 and 59 in HPV co-infection (253, 216, 191, 174, 94 and 91 bp); A4 and A5, genotype 56 in single HPV infection (244 and 121 bp); A6, genotype -66 in single HPV infection (114, 91, 72, 66 and 58 bp); A7, genotypes 18 and 53 in double HPV infection (174, 151, 144, 100 and 83 bp); A8, genotype 16 in single HPV infection (216 and 191 bp); M, molecular weight marker (25 bp). (B) Lane C_1: positive control of *T. pallidum* (291 bp); lane C_2: control of HSV-2 (249 bp); lane C_3: control of *C. trachomatis* and HSV-2 (361 and 249 bp); lane C_4: control of *M. genitalium* and *N. gonorrhoeae* (193 and 162 bp); lane C_5: control of *T. vaginalis* (170 bp); lane C_6: control of *N. gonorrhoeae* (162 bp); lane C_7: control of *T. vaginalis* and HSV-1 (170 and 123 bp); lane A_1: positive sample of *C. trachomatis* and *T. vaginalis* (361 and 170 bp); lane A_2: positive sample of *T. pallidum* and HSV-2 (291 and 249 bp); lane A_3: positive sample of HSV-2 and HSV-1 (249 and 123 bp); lane A_4: positive sample of *C. trachomatis* and *M. genitalium*(361 and 193 bp); lane A_5: positive sample of *T. pallidum* and *N. gonorrhoeae* (291 and 162 bp); lane A6: positive sample of *T. vaginalis* (170 bp); lane C-: negative control; M: molecular weight marker (25 bp).

sexual pathogens in different cytological cervical findings using M-PCR to simultaneously detect *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, *Trichomonas vaginalis*, herpes simplex virus (HSV)-1, HSV-2 and *Treponema pallidum.* We found that *C. trachomatis* and *N. gonorrhoeae* were the primary pathogens associated with HR-HPV for the increased risk for all grades of cervical abnormalities, mainly for HSIL, suggesting a possible synergistic action in cervical lesions progression.

Materials and methods

Study population

This transversal study enrolled women living in Maringá city/Paraná State/Brazil attending basic health units (BHU) of the Public Health System for cervical cancer screening consultations and reference services for colposcopy (Zona Sul Clinic) from August 2012 to March 2013. Women were excluded because of any of the following factors: pregnancy; postpartum; previous hysterectomy; vaginal bleeding; previous history of cancer; without history of sexual activity; recent treatment for any pathology of the urogenital tract; ablative or excisional therapy to the cervix within the previous 12 months; and no observation and/or collection sample of squamous columnar junction (SCJ). All participants voluntarily agreed to provide a sample for Pap screening and DNA detection of HPV and non-HPV STDs, and signed an informed consent before enrollment. This study was approved by the Committee for Ethics in Research Involving Humans at the State University of Maringá/UEM/Brazil

Table 2. Rates of HPV and non-HPV STDs in overall women included in the study and according to cytological findings.

HPV and non-HPV STDs	Cytological findings			RR (95% CI)	P	Cytological findings	RR (95% CI)	P
	Overall (N=838)	NILM (N=614)	≥ ASC-US (N=224)			HSIL (N=71)		
	n (%)	*n (%)*	*n (%)*			*n (%)*		
HPV-DNA	284 (33.9)	101 (16.4)	183 (81.7)	4.966 (3.727-6.619)	< 0.0001	71 (100.0)	6.079 (4.113-8.984)	< 0.0001
HR-HPV	230 (27.4)	66 (10.7)	164 (73.2)	6.811 (4.925-9.419)	< 0.0001	71 (100.0)	9.303 (6.139-14.10)	< 0.0001
HPV-16	95 (11.3)	14 (2.3)	81 (36.2)	15.86 (8.811-28.54)	< 0.0001	44 (62.0)	27.18 (14.19-52.05)	< 0.0001
LR-HPV	112 (13.4)	63 (10.3)	49 (21.9)	2.132 (1.424-3.192)	0.0003	10 (14.0)	1.373 (0.6741-2.795)	0.4231
HPV-multiple infections	101 (12.0)	44 (7.1)	57 (25.4)	3.551 (2.328-5.416)	< 0.0001	18 (25.3)	3.538 (1.940-6.453)	0.0001
non-HPV STDs	255 (30.4)	193 (31.4)	62 (27.7)	0.8806 (0.6365-1.218)	0.4649	20 (28.2)	0.8962 (0.5317-1.510)	0.7950
C. trachomatis	86 (10.3)	61 (9.9)	25 (11.2)	1.123 (0.6882-1.834)	0.7019	06 (8.4)	0.8506 (0.3549-2.039)	0.8352
N. gonorrhoeae	39 (4.7)	24 (3.9)	15 (6.7)	1.713 (0.8827-3.325)	0.1372	06 (8.4)	2.162 (0.8548-5.468)	0.1229
M. genitalium	29 (3.5)	26 (4.2)	3 (1.3)	0.3163 (0.09477-1.055)	0.0529	01 (1.4)	0.3326 (0.04444-2.489)	0.5087
T. vaginallis	97 (11.6)	78 (12.7)	19 (8.5)	0.6677 (0.3952-1.128)	0.1430	08 (11.3)	0.8870 (0.4114-1.912)	0.8525
HSV-1	9 (1.1)	8 (1.3)	1 (0.4)	0.3426 (0.04259-2.756)	0.4579	00 (0.0)	0.5056 (0.2885-8.858)	1.0000
HSV-2	33 (3.9)	20 (3.3)	13 (5.8)	1.782 (0.8716-3.642)	0.1138	02 (2.8)	0.8648 (0.1979-3.778)	1.0000
T. pallidum	14 (1.7)	13 (2.1)	1 (0.4)	0.2109 (0.02741-1.622)	0.1296	00 (0.0)	0.3183 (0.01871-5.415)	0.3821

NILM, negative for intraepithelial lesion or malignancy; ≥ ASC-US, atypical glandular cells (AGC), squamous intraepithelial lesions (SIL) of low (LSIL) or high (HSIL) grade, and atypical squamous cells (ASC) of undetermined significance (ASC-US) or not possible exclude HSIL (ASC-H); RR (95% CI), crude odds ratio (relative risk) with 95% confidence interval; HPV-DNA, human *Papillomavirus* deoxyribonucleic acid; HR-HPV, high-risk HPV; HSV, herpes simplex virus. non-HPV STDs: *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, *Trichomonas vaginalis*, herpes simplex virus (HSV)-1, HSV-2 and *Treponema pallidum.* non-HPV STDs co-infections: infections with two or more non-STD pathogens in the same sample. *P* value < 0.05 was considered significant.

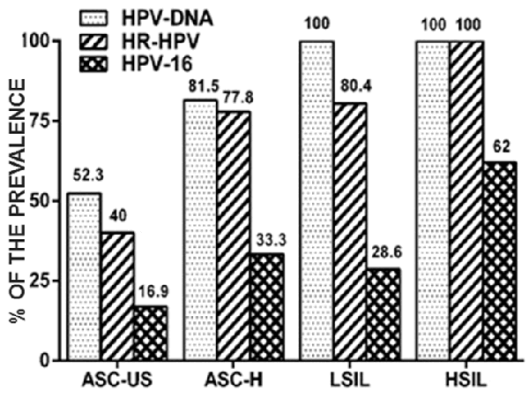

Figure 2. Prevalence of HPV-DNA, HR-HPV and HPV-16 by different abnormal cytological findings. Multiple infections were counted several times. HR-HPV, high-risk human *Papillomavirus*; ASC-US, atypical squamous cells of undetermined significance; ASC-H, atypical squamous cells not possible exclude HSIL; LSIL, low grade squamous intraepithelial lesions; HSIL, high grade squamous intraepithelial lesions.

and registered in the National Commission for Research Ethics (CONEP)/Health Ministry of Brazil (n° 489/2010 and n° 083/2011).

Table 1 outlines the baseline features of the study cohort. A total of 838 women were included in the study with an age range from 18 to 68 years and mean (SD) age was 40.3 ± 10.0 years.

Study procedures

Demographic and baseline characteristics were obtained through from the standard registration form for each woman. Cytological samples were obtained from women who were referred for colposcopy because of abnormal previous cytology findings, attended reference services (Zona Sul Clinic) and women without a history of cervical abnormalities on Papanicolaou (Pap) screenings who attended BHU for their cervical cancer screening consult.

Vaginal, cervical and endocervical samples were collected using an Ayre's spatula and cytobrush for Pap smear collection. Samples for HPV and STDs DNA analyses were immediately suspended in 1.0 mL of a sterile 0.9% NaCl solution and frozen at -80°C until analysis.

Cytology and histopathology

Cytology was conducted without knowledge of HPV status at an accredited clinical laboratory (Clinical Cytology Laboratory of UEM), and the results are reported according to Bethesda System 2001 [22]. The following cytological findings were reported: negative for intraepithelial lesion or malignancy (NILM); atypical glandular cells (AGC); squamous intraepithelial lesions (SIL) of low (LSIL) or high (HSIL) grade; atypical squamous cells (ASC) of undetermined significance (ASC-US) or not possible exclude HSIL (ASC-H); and SCC. A threshold of ≥ ASC-US was used to define all abnormal cytological findings as a group. All cases of ASC-H, HSIL and SCC were analyzed using colposcopy and histology and properly treated and/or screened at follow-up. A panel of 3 pathologists blinded to all participants determined histology results.

HPV detection and genotyping

An AxyPrep^{Tm} Body Fluid Viral DNA/RNA Miniprep Kit (Axygen, Union City, CA, USA) was used for DNA extraction according to the manufacturer's instructions. The quality and quantity of purified DNA were measured using spectrophotometry (NanoDrop 2000 Spectrophotometer, Thermo Scientific, Wilmington, IL, USA).

A single-target PCR (sPCR) method has been in use for HPV detection in our laboratory for several years, and it consists of HPV-PCR amplification performed using primers MY09 (5'CGTCCMAARGGAWACTGATC-3') and MY11 (5'-GCMCAGGGWCATAAYAATGG-3'). The reaction consisted of 2.5 mM of each dNTP, 1 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA), 0.6 mM of $MgCl_2$, 25 mM of each primer and 50 ng of extracted DNA for a final volume of 15 µL. Co-amplification of the human β-globin gene was performed as an internal control using primers GH20 (5'-GAAGAGCCAAGGACAGGTAC-3') and PC04 (5'-CAACTTCATCCACGTTCACC-3') under the same conditions as the HPV-PCR. Two types of controls were also included in each reaction: 'no-DNA' (negative control) and 'HPV-positive DNA' (positive control). PCR products were electrophoresed in 1.0% agarose gel, stained with 1.0 µg/mL ethidium bromide, and photodocumented under UV light.

HPV-positive samples were genotyped by PCR-RFLP (Restriction Fragment Length Polymorphism) as described previously [23, 24]. For initial RFLP, ten microliters of each sample were digested in a final volume of 15 µL with the restriction enzyme *Hpy*CH4V (New England

Table 3. Rates and risk of HPV and non-HPV STDs co-infections in overall women included in the study and according to cytological findings

Co-infections of non-HPV STDs and HPV	Overall (N=838)	Cytological findings		RR (95% CI)	P	Cytological finding	OR (95% CI)	P
		NILM (N=614)	≥ ASC-US (N=224)			HSIL (N=71)		
	n (%)	*n (%)*	*n (%)*			*n (%)*		
C. trachomatis								
HPV-DNA	38 (4.5)	15 (2.4)	23 (10.3)	4.203 (2.154-8.200)	< 0.0001	6 (8.4)	3.459 (1.300-9.202)	0.0197
HR-HPV	28 (3.3)	9 (1.4)	19 (8.4)	5.787 (2.580-12.98)	< 0.0001	6 (8.4)	5.765 (1.993-16.68)	0.0033
N. gonorrhoeae								
HPV-DNA	17 (2.0)	4 (0.6)	13 (5.8)	8.908 (2.874-27.61)	< 0.0001	6 (8.4)	12.97 (3.574-47.08)	0.0002
HR-HPV	15 (1.7)	3 (0.4)	12 (5.3)	10.96 (3.065-39.23)	< 0.0001	6 (8.4)	17.30 (4.232-70.69)	< 0.0001
M. genitalium								
HPV-DNA	6 (0.7)	3 (0.5)	3 (1.3)	2.741 (0.5490-13.69)	0.3515	1 (1.4)	2.883 (0.2957-28.10)	0.3576
HR-HPV	6 (0.7)	3 (0.4)	3 (1.3)	2.741 (0.5490-13.69)	0.3515	1 (1.4)	2.883 (0.2957-28.10)	0.3576
T. vaginallis								
HPV-DNA	32 (3.8)	16 (2.6)	16 (7.1)	2.741 (1.348-5.574)	0.0075	8 (11.2)	4.324 (1.787-10.46)	0.0028
HR-HPV	26 (3.1)	12 (1.9)	14 (6.2)	3.198 (1.457-7.020)	0.0058	8 (11.2)	5.765 (2.279-14.58)	0.0007
HSV-1								
HPV-DNA	2 (0.2)	1 (0.1)	1 (0.4)	2.741 (0.1706-44.04)	0.4642	0 (0)	2.865 (0.1155-71.04)	1.0000
HR-HPV	2 (0.2)	1 (0.1)	1 (0.4)	2.741 (0.1706-44.04)	0.4642	0 (0)	2.865 (0.1155-71.04)	1.0000
HSV-2								
HPV-DNA	14 (1.7)	3 (0.4)	11 (4.9)	10.05 (2.778-36.37)	< 0.0001	2 (2.8)	5.765 (0.9469-35.10)	0.0894
HR-HPV	14 (1.6)	3 (0.4)	11 (4.9)	10.05 (2.778-36.37)	< 0.0001	2 (2.8)	5.765 (0.9469-35.10)	0.0894
T. pallidum								
HPV-DNA	6 (0.7)	5 (0.8)	1 (0.4)	0.548 (0.0636-4.72)	1.0000	0 (0)	0.7813 (0.04273-14.29)	1.0000
HR-HPV	6 (0.7)	5 (0.8)	1 (0.4)	0.548 (0.0636-4.72)	1.0000	0 (0)	0.7813 (0.04273-14.29)	1.0000

NILM, negative for intraepithelial lesion or malignancy; ≥ ASC-US, atypical glandular cells (AGC), squamous intraepithelial lesions (SIL) of low (LSIL) or high (HSIL) grade, and atypical squamous cells (ASC) of undetermined significance (ASC-US) or not possible exclude HSIL (ASC-H); RR (95% CI), crude odds ratio (relative risk) with 95% confidence interval; HPV-DNA, human *Papillomavirus* deoxyribonucleic acid; HR-HPV, high-risk HPV; HSV, herpes simplex virus. non-HPV STDs: *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, *Trichomonas vaginalis*, herpes simplex virus (HSV)-1, HSV-2 and *Treponema pallidum*. non-HPV STDs co-infections: infections with two or more non-STD pathogens in the same sample. *P* value < 0.05 was considered significant.

Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions [23]. To better distinguish some HPV genotypes such as HPV 11/30, 18/68, 44/55, and 61/83/84 which present similar RFLP patterns, was used the same protocol with a second enzyme *Nla*III (New England Biolabs, Ipswich, MA, USA) [24]. Restriction fragments were resolved in 8% polyacrylamide gels. HPV genotypes were determined by analyzing each band with Labimage 1D software (Loccus Biotechnology, São Paulo, Brazil), and comparison of the molecular weights for HPV genotypes determination. A total of 39 individual HPV-DNA genotypes (17 genotypes considered to be either high-risk or potentially high-risk, 22 low-risk genotypes not associated with carcinogenesis, and 1 genotype with undetermined-risk for carcinogenesis) can be determined by PCR-RFLP method used as follows: high-risk (HR) (genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82); low-risk (LR) (6, 11, 30, 34, 40, 42, 43, 44, 54, 55, 61, 62, 64, 67, 69, 70, 72, 74, 81, 83, 84 and 91); and undetermined-risk (UR) (26) (3, 23-25). **Figure 1A** shows the sPCR amplification fragments of positive samples for HPV using 8% polyacrylamide gel.

Non-HPV STDs detection using multiplex-PCR (M-PCR)

M-PCR assay was realized as described previously [19] for simultaneously detect: *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*, *T. vaginalis*, HSV-1, HSV-2 and *T. pallidum*. Briefly, a reaction mixture consisted of 25 μL containing 2.5 mm of each dNTP, 0.6 mm of $MgCl_2$, 25 mm of each primer, 5 μL of extracted DNA (50 ng of total sample) and 1 U of Platinum Taq DNA polymerase (Invitrogen, Grand Island, NY, USA). The conditions were comprised of 35 amplification cycles of denaturation for 10 min at 94°C, annealing for 1 min at 62°C, extension for 1 min at 72°C and final extension for 10 min at 72°C (Thermal cycler, Biosystem, CA, USA). M-PCR products were electrophoresed in 8% polyacrylamide gel. Positive controls for all STDs studied were derived from positive clinical samples that were detected using reference methods, including culture and/or sPCR. All clinical samples were also tested using human β-globin-specific primers GH20/PC04 as an internal control for amplification and DNA integrity under the same conditions as the M-PCR reactions. **Figure 1B** shows the M-PCR amplification fragments of positive samples in 8% polyacrylamide gel.

Statistical analysis

Different variables were evaluated for comparisons between normal cytology (NILM) and abnormal cytology (≥ ASC-US: all abnormal cytological findings) or HSIL (only HSIL findings) for analytical calculations. Two-sided Fisher's exact test for 2 x 2 contingency table was used to evaluate the statistical significance between different groups. Crude odds ratios (RR-relative risk) with 95% confidence intervals (CI) were calculated to estimate the associations of HPV and non-HPV STD infections with different cervical cytological findings. 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 SAS 9.2 software (SAS Institute, Cary, NC, USA) and R-statistics 3.1.2 software.

Results

Baseline features

A total of 614 (73.3%) women had NILM, and 224 (26.7%) women exhibited the following abnormal cytology: 5 AGC (0.6%), 65 ASC-US (7.7%), 27 ASC-H (3.2%), 56 LSIL (6.7%), and 71 HSIL (8.5%). Women with abnormal cytology were significantly younger (36.4 ± 10.0 years) than women with NILM cytology (41.3 ± 9.6 years) (P=0.0001).

Women with HPV-DNA were 37.7 ± 10.3 years old, which was younger than negative women (41.2 ± 9.6 years; $P < 0.0001$). Women with HR-HPV were 37.7 ± 13.2 years old and younger than negative women (40.9 ± 11.7 years; P=0.0006). A similar trend occurred for women with HPV genotype co-infections who were 37.6 ± 12.4 years old and younger than negative women (40.4 ± 12.1 years; P=0.0300). However, women with LR-HPV were 38.7 ± 12.4 years old, which was similar to negative women (40.3 ± 12.1 years; P=0.2006).

HPV genotypes in the overall population and by cytology status

Overall population: The prevalence of HPV-DNA was 33.9% (n=284). A total of 39 HPV geno-

types were detected: 17 HR-HPV, 21 LR-HPV and 1 UR-HPV. HPV-16 was the most prevalent in overall population and women with NILM or ≥ ASC-US cytology (n=95, 11.3%; n=14, 2.3%; and n=81, 36.2%, respectively) (**Table 2**). The HPV genotypes with the next highest prevalence were HPV-31 (n=28, 3.3%), HPV-66 (n=26, 3.1%), HPV-58 (n=24, 2.6%) (all HR) and HPV-72 (n=20, 2.4%) (LR). The other genotypes exhibited a prevalence < 2%.

LR-HPV was detected in 13.4% (n=112) of the women studied, most of whom exhibited NILM cytology (n=63, 10.3%) (P=0003). UR-HPV was detected in 0.6% (n=5) of women, all with NILM. HPV genotype co-infections were detected in 101 women (12.0%) (**Table 2**). Ninety-two (91.1%) of these women had two HPV genotypes, and 9 (8.9%) women exhibited three HPV genotypes. HR-HPV was involved in a total of 85.1% of HPV genotypes co-infections (n=86).

NILM cytology X ≥ ASC-US or HSIL cytology: HPV-72 was the second most-detected genotype in women with NILM cytology (n=12, 2.0%), followed by genotypes 31, 66, 61 and 67 (1.8%, 1.6%, 1.3%, and 1.0%, respectively). The other genotypes exhibited a prevalence < 1%.

HPV 58 (n=19, 8.5%) was the second most common genotype in women with cytology ≥ ASC-US, followed by genotypes 31, 66, 33, 18 and 72 (7.6%, 7.1%, 5.4%, 4.9%, 4.5%, and 3.6%, respectively). The other genotypes exhibited a prevalence < 3%.

By different abnormal cytological findings: **Figure 2** shows the prevalence of HPV-DNA, HR-HPV and HPV-16 by different abnormal cytological findings. No cases of AGC were positive for HPV. HPV-DNA and HR-HPV were associated with ASC-H (P=0.0042 and P=0.0067, respectively), LSIL and HSIL (P < 0.0001 for both), but not with ASC-US (P=0.0559 and P=0.5321, respectively). HPV-16 was associated with ASC-US (P > 0.0382) and HSIL (P < 0.0001), but not with ASC-H (P=1.000) or LSIL (P=0.7817).

HPV and relative risk of abnormal cytology

Women with HPV-DNA exhibited an increased risk for ≥ ASC-US and HSIL cytology (relative risk*:* 4.966, 95% CI: 3.727-6.619, P < 0.0001, and relative risk: 6.079, 95% CI: 4.113-8.984, P < 0.0001, respectively). Similar trends were observed in women with multiple HPV infections (relative risk: 3.551, 95% CI: 2.328-5.416, P < 0.0001 for ≥ ASC-US, and relative risk: 3.538, 95% CI: 1.940-6.453, P < 0.0001 for HSIL). The risk for abnormal cytology rose in women with HR-HPV for ≥ ASC-US and HSIL (relative risk: 6.811; 95% CI: 4.925-9.419, P < 0.0001, and relative risk: 9.303; 95% CI: 6.139-14.10, P < 0.0001, respectively). The greatest risk for abnormal cytology occurred in women with HPV-16 (relative risk: 15.86, 95% CI: 8.811-28.54, P < 0.0001, for ≥ ASC-US, and relative risk: 27.18; 95% CI: 14.19-52.05, P < 0.0001 for HSIL). Women with LR-HPV exhibited an increased risk of ≥ ASC-US cytology (relative risk: 2.132; 95% CI: 1.424-3.192, P=0.003) but not HSIL (**Table 2**).

Non-HPV STDs in the overall population and by cytology status

The prevalence of non-HPV STDs in the overall population was 30.4% (n=255), who exhibited 1 (n=209, 82.0%) or more (n=47, 18.0%) pathogens. *T. vaginalis* was the most prevalent pathogen (11.6%) followed by *C. trachomatis* (10.3%), *N. gonorrhoeae* (4.7%), HSV-2 (3.9%), *M. genitalium* (3.5%), *T. pallidum* (1.7%), and HSV-1 (1.1%). Non-HPV STDs were detected in 31.7% (n=91) of HPV-DNA-positive women. None of the non-HPV STDs was associated with NILM, ≥ ASC-US or HSIL cytology, which was similar to the overall population (**Table 2**).

Co-infections between HPV and non-HPV STDs and risk for abnormal cytology

Table 3 presents co-infections between HPV and non-HPV STDs and risk for abnormal cytology. Co-infections *C. trachomatis* plus HPV-DNA increased the risk for ≥ ASC-US cytology (relative risk: 4.203, 95% CI: 2.154-8.200, P < 0.0001). Co-infections *C. trachomatis* plus HPV-HR showed a higher increased risk for ≥ ASC-US cytology (relative risk: 5.787, 95% CI: 2.580-12.98, P < 0.0001) and HSIL (relative risk: 5.765, 95% CI: 1.993-16.68, P=0.0033).

Co-infections *N. gonorrhoeae* plus HPV-DNA increased the risk of ≥ ASC-US cytology (relative risk: 8.908; 95% CI: 2.874-27.61, P < 0.0001) and HSIL (relative risk: 12.97, 95% CI: 3.574-47.08, P=0.0002). Co-infections *N. gonorrhoeae* plus HPV-HR showed a higher increased risk of ≥ ASC-US cytology (relative

risk: 10.96, 95% CI: 3.065-39.23, P < 0.0001) and HSIL (relative risk: 17.30; 95% CI: 4.232-70.69, P < 0.0001).

Co-infections *T. vaginalis* plus HPV-DNA increased the risk of ≥ ASC-US cytology (relative risk: 2.741, 95% CI: 1.348-5.574, P=0.0075) and HSIL (relative risk: 4.324, 95% CI: 1.787-10.46, P=0.0028). Co-infections *T. vaginalis* plus HPV-HR showed similar findings with HPV-DNA, with an increased risk of ≥ ASC-US cytology (relative risk: 3.198, 95% CI: 1.457-7.020, P=0058) and HSIL (relative risk: 5.765, 95% CI: 2.279-14.58, P=0.0007).

Co-infections HSV-2 plus HPV-DNA and HR-HPV had the same increased risk of ≥ ASC-US cytology (relative risk: 10.05, 95% CI: 2.778-36.37, P < 0.0001).

Discussion

This study investigated the rates of co-infections between HPV and other important sexual pathogens in different cytological cervical findings using M-PCR assay to simultaneously detect *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*, *T. vaginalis*, HSV-1, HSV-2, and *T. pallidum.* Our main findings were a higher increased risk of ≥ ASC-US and mainly for HSIL in women with co-infections of HR-HPV and *C. trachomatis* or *N. gonorrhoeae*, suggesting a possible synergistic action in cervical lesions progression. Meanwhile, we found that co-infections of HR-HPV with HSV-2 exhibited an increased risk of ≥ ASC-US cytology only, which suggests that the virus is necessary for the initial transformation of the cells but not for SCC progression. Additionally, co-infections of HR-HPV with *T. vaginalis* demonstrated a similar increased risk of ≥ ASC-US and HSIL suggesting that the agent was a common concomitant non-HPV STD in abnormal cytology, but not as a co-factor of HR-HPV in cervical lesions progression.

HPV is necessary, but not sufficient, to cause SCC, which supports the influence of additional factors in carcinogenesis associated with HR-HPV [1-5]. Our results are consistent with the hypothesis that non-HPV STDs exert a synergistic action with HR-HPV and act as co-factors in disease progression [4-6]. These findings are very important because the identification of co-factors for HR-HPV may be useful for disease prevention [16] although cervical screening offers some protection against SCC because of the early detection of premalignant lesions [1, 2]. However, our results also suggest that differences exist in the potential to function as a co-factor for HR-HPV between different STDs agents what are discussed below.

The rates of HPV-DNA and HR-HPV and their association with abnormal cytology of different grades are consistent with several previous reports [1-5, 10, 12, 17]. The prevalence of HPV genotypes is also consistent with several previous reports [1-5, 10, 12, 17]. HPV-16 was the most prevalent pathogen in normal and abnormal cytological findings, including HSIL. Similar trends were observed for the epidemiological characteristics of the women of our sample [2-6, 14, 17].

Epidemiological studies suggested that concomitant infections with pathogens that are also transmitted through sexual activity contribute to an increased risk of SCC [6, 9-14]. Non-HPV STDs are commonly associated with changes in epithelial cells, which facilitate the entry of HPV virions and changes in the immunological response pathways that may decrease the host's ability to resolve HPV infection [11, 13, 17]. Cervical inflammation (e.g., cervicitis) is associated with prolonged HPV infection [26] and dysplasia [27]. *N. gonorrhoeae*, *C. trachomatis*, HSV, *T. vaginalis*, and *M. genitalium* [28, 29] may primarily cause infectious cervicitis.

Co-infections of HR-HPV and *C. trachomatis* exhibited a five times higher increased risk of HSIL in our study. These data are consistent with previous studies [12, 14, 19] and reinforce the hypothesis discussed below. *C. trachomatis* is primarily a non-HPV STD studied as a co-factor to HPV in cervical carcinogenesis [30]. *C. trachomatis* infection may increase the susceptibility to HPV via the production of micro-abrasions or alterations in epithelial cells, which facilitates the entry of virions [13]. Some investigators proposed that concurrent *C. trachomatis* infection reduced the host's ability to resolve HPV infection. Chronic cervical inflammation influences HPV persistence via a raised production of free radicals and reduction of host cell-mediated immunity [13, 31]. *C. trachomatis* infection induces a shift in the immune response, and the unresolved infections are associated with the humoral (T helper cell type/

Th2) immune response, but the cellular (Th1) immune response is important for the clearance of HPV lesions. Therefore, modulation of the cervical immune response by *C. trachomatis* may influence the clearance of HPV lesions [31]. We recently analyzed the state of matrix metalloproteinases (MMP-9) and its physiological inhibitor, RECK protein (Reversion-inducing Cysteine-rich protein with Kazal motifs) axis in cervical carcinogenesis. MMPs are important enzymes in the tumor microenvironment that are associated with the progression of cervical lesions towards SCC. We found that an MMP-9/RECK imbalance in cervical smears was significantly associated with HSIL and infection by alpha-9 HPV and *C. trachomatis*. These data suggested that *C. trachomatis* induced MMP-9/RECK imbalance during cervical inflammation, and this imbalance played a role in HPV-mediated cervical carcinogenesis [12].

Notably, co-infection of HR-HPV and *N. gonorrhoeae* was the non-HPV STD studied that demonstrated the greatest risk of ≥ ASC-US (relative risk: 10.96) and HSIL (relative risk: 17.3). Few studies investigated the feasibility of this bacterium as an HR-HPV co-factor in the progression of cervical lesions. Previous studies revealed no association between this bacterium, HR-HPV and abnormal cytology [13, 32]. However, *N. gonorrhoeae* was detected using different methodologies (e.g., conventional microscopy and/or culture methods and PCR-based methods), which may at least partially explain the differences with our results. The possibility that bacteria and HR-HPV act synergistically to influence the progression of cervical lesions is plausible because this infection presents a greater incidence worldwide, and it causes cervicitis [28]. Therefore, the mechanisms are similar to *C. trachomatis* for the persistence and elimination of HPV. Our results highlight the need for further studies evaluating the real impact of the co-infection of HR-HPV and *N. gonorrhoeae* and the increased risk of cervical lesions of different grades.

Various studies of HSV-2 on viral load, serological status and co-infection with HPV were performed [16, 33, 34]. However, only a few studies explored the relationship between HSV-DNA and SCC using PCR-based methods, with variable results [35, 36]. All cases of co-infection of HPV and HSV-2 in the present study occurred specifically with HR-HPV. Therefore, co-infection of HPV-DNA and HR-HPV with HSV-2 exhibited a ten times higher increased risk of ≥ ASC-US cytology, but no increased risk to HSIL was observed. These results support the "hit-and-run" mechanism, which states that HSV-2 participates in some initial phases of cervical carcinogenesis but does not require its retention. Therefore, HSV-2 is not detected consistently in all cervical biopsies of cervical lesions/SCC, which suggests that HSV is necessary for the initial transformation of cells but not for SCC progression [33, 37].

T. vaginalis was the most prevalent non-HPV STD detected in our study. Co-infections of HPV-DNA and HR-HPV with *T. vaginalis* demonstrated a similar increased risk of ≥ ASC-US and HSIL. These findings are consistent with another recent study that suggested that *T. vaginalis* was a concomitant non-HPV STD in abnormal cytology, but it was not thought to be a co-factor of HR-HPV in cervical lesion progression to SCC [38].

Others non-HPV STD studied, including *M. genitalium,* HSV-1 and *T. pallidum*, were neither associated with HPV-DNA nor HR-HPV in different cytological findings. The results of previous studies about the association of *M. genitalium* with HPV in samples with abnormal cytology are controversial [17, 39]. Therefore, the role of *M. genitalium* as a risk factor in cervical lesion progression has not been completely elucidated and requires further investigation. The few studies of co-infections between HPV and HSV-1 reported similar results to ours, demonstrating that HSV-1 was not involved in SCC development [33]. However, our study is the first study, to our knowledge, to evaluate whether *T. pallidum* was related to HPV in cervical disease. We found no associations, but that real influence as a co-factor still not clear.

The present study had no data on the history of HPV and STD infections in the women prior to enrollment in the study or in the follow-up, which limits our interpretations of the influence on HPV persistence. Other non-HPV STD pathogens were not included in the study, and we cannot extend our results to all non-HPV STDs. It is important to note that our study did not aim to assess the carcinogenicity mechanisms of the synergistic action between HR-HPV and

non-HPV STDs, but to assess the possible occurrence of this synergistic action.

In conclusion, this study provided an opportunity to determine the rates of co-infection between HPV and other important STDs in different cytological cervical findings using an M-PCR assay to detect simultaneously pathogens. *C. trachomatis* and *N. gonorrhoeae* were the primary pathogens associated with HR-HPV for the increased risk for all grades of cervical abnormalities, mainly for HSIL, suggesting a possible synergistic action in cervical lesions progression. Our results reinforce the hypothesis that some non-HPV STDs might play a role as co-factors in HPV-mediated cervical carcinogenesis. These data improve our understanding of the etiology of SCC and may also be useful for disease prevention.

Aknowledgements

This work was supported by grants from the from Fundação Araucária de Apoio ao Desenvolvimento Científico e Tecnológico, Paraná State Government, Project n° 37635/2013, and Coordenação de Aperfeiçoamento de Pessoal de Nível superior (CAPES), Brazilian Government Project PVE A109-2013.

Disclosure of conflict of interest

None.

Address correspondence to: Marcia EL Consolaro, Department of Clinical Analysis and Biomedicine, Clinical Cytology Laboratory, State University of Maringá, Paraná, CEP 87020-900, Brazil. Tel: (+55 44) 3011-4795; Fax: (+55 44) 3011-4850; E-mail: melconsolaro@gmail.com

References

[1] Koh WJ, Greer BE, Abu-Rustum NR, Apte SM, Campos SM, Cho KR, Chu C, Cohn D, Crispens MA, Dorigo O, Eifel PJ, Fisher CM, Frederick P, Gaffney DK, Han E, Huh WK, Lurain JR 3rd, Mutch D, Fader AN, Remmenga SW, Reynolds RK, Teng N, Tillmanns T, Valea FA, Yashar CM, McMillian NR, Scavone JL. Cervical Cancer, Version 2.2015. J Nat Comp CancNetw 2015; 13: 395-404.

[2] Kjaer SK, Frederiksen K, Munk C, Iftner T. Long-term absolute risk of cervical intraepithelial neoplasia grade 3 or worse following human papillomavirus infection: role of persistence. J Natl Cancer Inst 2010; 102: 1478-1488.

[3] Monsonego J, Cox JT, Behrens C, Sandri M, Franco EL, Yap PS, Huh W. Prevalence of high-risk human papillomavirus genotypes and associated risk of cervical precancerous lesions in a large U.S. screening population: Data from the ATHENA trial. Gynecol Oncol 2015; 137: 47-54.

[4] Ho GY, Bierman R, Beardsley L, Chang CJ, Burk RD. Natural history of cervicovaginal papillomavirus infection in young women. N Engl J Med 1998; 338: 423-8.

[5] Tota JE, Chevarie-Davis M, Richardson LA, Devries M, Franco EL. Epidemiology and burden of HPV infection and related diseases: implications for prevention strategies. Prev Med 2011; 53; Sup1: S12-S21.

[6] Bodily J, Laimins LA. Persistence of human papillomavirus infection: keys to malignant progression. Trends Microbiol 2011; 19: 33-9.

[7] International Collaboration of Epidemiological Studies of Cervical Cancer, Appleby P, Beral V, Berrington de González A, Colin D, Franceschi S, Goodill A, Green J, Peto J, Plummer M, Sweetland S. Carcinoma of the cervix and tobacco smoking: collaborative reanalysis of individual data on 13,541 women with carcinoma of the cervix and 23,017 women without carcinoma of the cervix from 23 epidemiological studies. Int J Cancer 2006; 118: 1481-95.

[8] Moreno V, Bosch FX, Munoz N, Meijer CJ, Shah KV, Walboomers JM, Herrero R, Franceschi S; International Agency for Research on Cancer. Multicentric Cervical Cancer Study Group. Effect of oral contraceptives on risk of cervical cancer in women with human papillomavirus infection: the IARC multicentric case-control study. Lancet 2002; 359: 1085-92.

[9] Castle PE, Giuliano AR. Chapter 4: Genital tract infections, cervical inflammation, and antioxidant nutrients: assessing their roles as human papillomavirus cofactors. J Natl Cancer Inst Monogr 2003; 29-34.

[10] Carrillo-García A, Ponce-de-León-Rosales S, Cantú-de-León D, Fragoso-Ontiveros V, Martínez-Ramírez I, Orozco-Colín A, Mohar A, Lizano M. Impact of human papillomavirus coinfections on the risk of high-grade squamous intraepithelial lesion and cervical cancer. Gynecol Oncol 2014; 134: 534-9.

[11] Paba P, Bonifacio D, Di Bonito L, Ombres D, Favalli C, Syrjänen K, et al. Coexpression of HSV2 and Chlamydia trachomatis in HPV-positive cervical cancer and cervical intraepithelial neoplasia lesions is associated with aberrations in key intracellular pathways. Intervirology 2008; 51: 230-4.

[12] Discacciati MG, Gimenes F, Pennacchi PC, Faião-Flores F, Zeferino LC, Derchain SM, Teixeira JC, Costa MC, Zonta M, Termini L, Boccardo E, Longatto-Filho A, Consolaro ME, Villa LL, Maria-Engler SS. MMP-9/RECK Imbalance: A Mechanism Associated with High-Grade Cervical Lesions and Genital Infection by Human Papillomavirus and Chlamydia trachomatis. Cancer Epidemiol Biomarkers Prev 2015; 24: 1539-1547.

[13] Verteramo R, Pierangeli A, Mancini E, Calzolari E, Bucci M, Osborn J, Nicosia R, Chiarini F, Antonelli G, Degener AM. Human Papillomaviruses and genital co-infections in gynaecological outpatients. BMC Infect Dis 2009; 9: 16.

[14] Lizano M, Berumen J, García-Carrancá A. HPV-related carcinogenesis: basic concepts, viral types and variants. Arch Med Res 2009; 40: 428-34.

[15] Schmauz R, Okong P, de Villiers EM, Dennin R, Brade L, Lwanga SK, Owor R. Multiple infections in cases of cervical cancer from a high-incidence area in tropical Africa. Int J Cancer 1989; 43: 805-9.

[16] Zereu M, Zettler CG, Cambruzzi E, Zelmanowicz A. Herpes simplex virus type 2 and Chlamydia trachomatis in adenocarcinoma of the uterine cervix. Gynecol Oncol 2007; 105: 172-5.

[17] Magana-Contreras M, Contreras-Paredes A, Chavez-Blanco A, Lizano M, Cruz-Hernandez YdeL, Cruz-Hernandez EdeL. Prevalence of Sexually Transmitted Pathogens Associated With HPV Infection in Cervical Samples in a Mexican Population. J Med Virol 2015; 87: 2098-105.

[18] Lehtinen M, Ault KA,Lyytikainen E,Dillner J, Garland SM, Ferris DG, Koutsky LA, Sings HL, Lu S, Haupt RM, Paavonen J; FUTURE I and II Study Group. Chlamydia trachomatis infection and risk of cervical intraepithelial neoplasia. Sex Transm Infect 2011; 87: 372-6.

[19] Souza RP, de Abreu AL, Ferreira ÉC, Rocha-Brischiliari SC, de B Carvalho MD, Pelloso SM, Bonini MG, Gimenes F, Consolaro ME. Simultaneous detection of seven sexually transmitted pathogens in human immunodeficiency virus-infected Brazilian women by multiplex polymerase chain reaction. Am J Trop Med Hyg 2013; 89: 1199-202.

[20] Muvunyi CM, Dhont N, Verhelst R, Crucitti T, Reijans M, Mulders B, Simons G, Temmerman M, Claeys G, Padalko E. Evaluation of a new multiplex polymerase chain reaction assay STDFinder for the simultaneous detection of 7 sexually transmitted disease pathogens. Diagn Microbiol Infect Dis 2011; 71: 29-37.

[21] Malaguti N, Bahls LD, Uchimura NS, Gimenes F, Consolaro ME. Sensitive Detection of Thirteen Bacterial Vaginosis-Associated Agents Using Multiplex Polymerase Chain Reaction. Biomed Res Int 2015; 2015: 645853

[22] Solomon D and Nayar R. The Bethesda System for Cervicovaginal Cytology. Definitions, Criteria, and Explanatory Notes. 3Ed; Anticancer Res 2015; 35: 5708.

[23] Santiago E, Camacho L, Junquera ML, Vázquez F. Full HPV typing by a single restriction enzyme. J Clin Virol 2006; 37: 38-46.

[24] Chen L, Watanabe K, Haruyama T, Kobayashi N. Simple and rapid human papillomavirus genotyping method by restriction fragment length polymorphism analysis with two restriction enzymes. J Med Virol 2013; 85: 1229-34.

[25] Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F, Benbrahim-Tallaa L, Guha N, Freeman C, Galichet L, Cogliano V; WHO International Agency for Research on Cancer Monograph Working Group. A review of human carcinogens--Part B: biological agents. Lancet Oncol 2009; 10: 321-2.

[26] Shew ML, Fortenberry JD, Tu W, Juliar BE, Batteiger BE, Qadadri B, Brown DR. Association of condom use, sexual behaviors, and sexually transmitted infections with the duration of genital human papillomavirus infection among adolescent women. Arch Pediatr Adolesc Med 2006; 160: 151-6.

[27] Castle PE, Hillier SL, Rabe LK, Hildesheim A, Herrero R, Bratti MC, Sherman ME, Burk RD, Rodriguez AC, Alfaro M, Hutchinson ML, Morales J, Schiffman M. An association of cervical inflammation with high-grade cervical neoplasia in women infected with oncogenic human papillomavirus (HPV). Cancer Epidemiol Biomarkers Prev 2001; 10: 1021-7.

[28] Marrazzo JM, Martin DH. Management of women with cervicitis. Clin Infect Dis 2007; 44 Suppl 3: S102-10.

[29] George C, Deligeoroglou E. Microbial ecology of the lower genital tract in women with sexually transmitted diseases. J Med Microbiol 2012; 61: 1347-51.

[30] de Abreu AL, Nogara PR, Souza RP, da Silva MC, Uchimura NS, Zanko RL, Ferreira EC, Tognim MC, Teixeira JJ, Gimenes F, Consolaro ME. Molecular detection of HPV and Chlamydia trachomatis infections in Brazilian women with abnormal cervical cytology. Am J Trop Med Hyg 2012; 87: 1149-51.

[31] Samoff E, Koumans EH, Markowitz LE, Sternberg M, Sawyer MK, Swan D, Papp JR, Black CM, Unger ER. Association of Chlamydia trachomatis with persistence of high-risk types of human papillomavirus in a cohort of female adolescents. Am J Epidemiol 2005; 162: 668-75

[32] Lazenby GB, Taylor PT, Badman BS, McHaki E, Korte JE, Soper DE, Young Pierce J. An association between Trichomonas vaginalis and high-risk human papillomavirus in rural Tanzanian women undergoing cervical cancer screening. Clin Ther 2014; 36: 38-45.

[33] Zhao Y, Cao X, Zheng Y, Tang J, Cai W, Wang H, Gao Y, Wang Y. Relationship between cervical disease and infection with human papillomavirus types 16 and 18, and Herpes simplex virus 1 and 2. J Med Virol 2012; 84: 1920-7.

[34] Kalyani Raju. Virus and Cervical Cancer: Role and implication: A review. Biomed Res Ther 2015; 2: 220-230.

[35] Tran-Thanh D, Provencher D, Koushik A, Duarte-Franco E, Kessous A, Drouin P, Wheeler CM, Dubuc-Lissoir J, Gauthier P, Allaire G, Vauclair R, Dipaolo JA, Gravitt P, Franco E, Coutlée F. Herpes simplex virus type II is not a cofactor to human papillomavirus in cancer of the uterine cervix. Am J Obstet Gynecol 2003; 188: 129-34.

[36] Paba P, Bonifacio D, DiBonito L, Ombres D, Favalli C, Syrjanen K, Ciotti M. Co-expression of HSV 2 and Chlamydia trachomatis in HPV-positive cervical cancer and cervical intraepithelial neoplasia lesions is associated with aberrations in key intracellular pathways. Intervirology 2008; 51: 230-4.

[37] Galloway DA, McDougall JK. The oncogenic potential of herpes simplex viruses: Evidence for a "hit-and-run" mechanism. Nature 1983; 302: 21-4.

[38] Donders GG, Depuydt CE, Bogers JP, Vereecken AJ. Association of Trichomonas vaginalis and Cytological Abnormalities of the Cervix in Low Risk Women. PLoS One 2013; 8: e86266.

[39] Biernat-Sudolska M, Szostek S, Rojek-Zakrzewska D, Klimek M, Kosz-Vnenchak M. Concomitant infections with human papillomavirus and various Mycoplasma and Ureaplasma species in women with abnormal cervical cytology. Adv Med Sci 2011; 56: 299-303.