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Short Report: Molecular Detection of HPV and *Chlamydia trachomatis* Infections in Brazilian Women with Abnormal Cervical Cytology

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Abstract. The question of whether *Chlamydia trachomatis* (Ct) is a cofactor for human *Papillomavirus* (HPV) in cervical carcinogenesis is still controversial. We conducted a molecular detection study of both infections in 622 Brazilian women, including 252 women with different grades of abnormal cervical cytology and cervical cancer (CC; cases) and 370 women with normal cytology (controls). Although Ct infection did not seem related to CC carcinogenicity, women with abnormal cytology had a significant high rate of Ct infection. Therefore, it is important to adopt protocols for diagnosis and treatment of this bacterium in conjunction with screening for CC in this population.

At present, cervical cancer (CC) is the second leading cause of cancer affecting women,[1,2] preceded only by breast cancer; it develops over a long period through precursor lesions that can be detected by cytological screening.[3] Its frequency varies in different regions of the world, and it is much higher in underdeveloped or developing than developed countries.[1] In Brazil, CC is the third most common cancer among women, with 17,000–18,000 new cases annually.[4] It is well-established that persistent infection by high-risk (HR) human *Papillomavirus* (HPV) is a necessary but not sufficient cause of CC.[5] Considering that a small proportion of HR-HPV–infected women actually develop CC,[6] additional risk factors that may be involved in the development of the disease have been investigated.

Among other sexually transmitted infections (STIs) that can increase the risk of CC, *Chlamydia trachomatis* (Ct) is among the leading factors. Some investigators have proposed that Ct infection could affect HPV acquisition and persistence, increase the rate of transformation to early precursor lesions, and also increase the likelihood that precursor lesions lead to CC.[7] However, the association of Ct as a cofactor for HPV in cervical carcinogenesis is still controversial.[8] This information directly affects the adoption of efforts to control CC, because a Ct–HPV association would require not only HPV vaccination but also adoption of protocols for diagnosis and treatment of the bacteria. Considering that, in Brazil and Latin America in general, few studies have examined this possibility, we conducted a molecular detection study of HPV and Ct infections in Brazilian women with different grades of abnormal cervical cytology and CC in an attempt to contribute to elucidating the involvement of Ct in cervical carcinogenesis.

Six hundred twenty-two women of low socioeconomic status, ages 15–83 years, were recruited between August 1, 2009 and March 31, 2012 in three cities of the state of Paraná, southern Brazil: Maringá, Paiçandú, and União da Vitória. The epidemiological characteristics were obtained through the analysis of data from a standard registration form for each woman. Each woman involved signed the authorization, and the execution of this project was approved by the Committee for Ethics in Research Involving Humans at the State University of Maringá/Paraná, Brazil.

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The cervical and endocervical material was collected with the aid of an Ayre spatula and a cytobrush for cervical smears and PCR (polymerase chain reaction) amplification (suspended in 1 mL 0.9% NaCl solution and stored at –20°C until analysis). The cytological smears were sent to the Public Health System (SUS) Laboratory, and they were evaluated and reported according to the Bethesda System.[3] Of the total number examined, 370 women showed normal cytology with no history or signs of infection (NILM; controls). The remaining 252 women showed abnormal cervical cytology and CC (cases) distributed as 6 atypical glandular cells (AGCs), 93 atypical squamous cells of undetermined significance (ASC-US), 38 atypical squamous cells (ASC-H; cannot exclude high-grade squamous intraepithelial cervical lesion [HSIL]), 55 low-grade squamous intraepithelial cervical lesion (LSIL), 42 HSIL, and 18 CC (squamous cervical cancer).

Briefly, genomic DNA was extracted using DNAzol (Invitrogen, Carlsbad, CA). HPV-PCR amplification for HPV was carried out using MY09 (5′-CGTCCMAARGG AWACTGATC-3′)/MY11(5′-GCMCAGGGWCATAAYA ATGG-3′) and genotyping by PCR-based restriction fragment-length polymorphism analysis using *Hpy*CH4V.[9] For Ct, the PCR technique was also used, with P1 (5′-TCTTTTT AAACCTCCGGAACCCACTT-3′)/P2 (5′-GGATGGCATC GCATAGCATTCTTTG-3′) primers. For HPV and Ct PCR, coamplification of the human β-globin gene was performed as an internal control using primers GH20 (5′-GAAGAGC CAAGGACAGGTAC-3′)/PC04 (5′-CAACTTCATCCACG TTCACC-3′) under the same conditions as HPV or Ct PCR.

The statistical analysis was performed using the STATISTICA 8.0 software, and all variables were expressed as absolute and relative frequencies. The frequency rates of HPV and Ct were evaluated between different groups by nonparametric Z test. The crude odds ratio (OR) was also calculated with a 95% confidence interval (CI). A P value < 0.05 was considered significant.

For all women studied ($N = 622$), the mean age was 41.0 ± 13.6 years for the controls ($N = 370$), and 35.5 ± 13.9 years for the cases ($N = 252$). Most of the women studied were between 30 and 40 years (26.53%), with histories of menarche at 13–14 years (53.12%) and first sexual intercourse at < 17 years (53.50%), were not using hormonal contraceptives (77.10%), had more than or equal to three children (78.48%), and had more than or equal to two sexual partners (89.97%).

TABLE 1

Positivity to total HPV DNA, HR-HPV, LR-HPV, Ct, total HPV plus Ct, HR-HPV plus Ct, and LR-HPV plus Ct in Brazilian women with cervical abnormalities ($N = 252$) without considering the multiple HPV infection

	HPV positivity *n* (%)			HPV plus Ct positivity *n* (%)			
Cervical cytology	HPV	HR-HPV	LR-HPV	HPV + Ct	HR-HPV + Ct	LR-HPV + Ct	Ct
NILM (controls; $N = 370$)	28 (7.60)	14 (3.80)	13 (3.51)	4 (1.10)	1 (0.27)	3 (0.81)	36 (9.73)
AGC ($N = 6$)	2 (33.33)	2 (33.33)	–	–	–	–	–
ASC-US ($N = 93$)	37 (39.80)*	19 (20.43)	21 (22.60)	8 (8.60)	2 (2.15)	6 (6.45)	17 (18.30)
ASC-H ($N = 38$)	26 (68.42)†	21 (55.30)‡	5 (13.16)	6 (15.80)	5 (13.16)	1 (2.63)	7 (18.42)
LSIL ($N = 55$)	42 (76.40)†	30 (54.55)§	12 (21.82)	8 (14.55)	6 (10.91)	3 (5.45)	9 (16.40)
HSIL ($N = 42$)	42 (100.0)†	36 (85.71)†	11 (26.20)	7 (16.70)	7 (16.70)	1 (2.40)	7 (16.70)
CC ($N = 18$)	18 (100.0)†	18 (100.0)†	6 (33.33)	3 (16.70)	3 (16.70)	2 (11.11)	3 (16.70)
Total ($N = 622$)	195 (31.35)	140 (22.51)	68 (10.93)	36 (5.80)	24 (3.86)	16 (2.60)	79 (12.70)

P value < 0.05 is considered significant. Cytology findings: controls (NILM; $N = 370$); cases: AGCs ($N = 6$), ASC-US ($N = 93$), ASC-H ($N = 38$), LSIL ($N = 55$), HSIL ($N = 42$), and CC ($N = 18$).
* $P = 0.0053$; *P* value compared HPV infection with ASC-US.
† $P = 0.0001$; *P* value compared HPV infection with ASC-H, LSIL, HSIL, and CC and compared HR-HPV with HSIL and CC.
‡ $P = 0.0037$; *P* value compared HR-HPV infection with ASC-H.
§ $P = 0.0025$; *P* value compared HR-HPV infection with LSIL.

The total frequency of women with HPV was 31.35% ($N = 195/622$), and 7.23% ($N = 45$) of these women had multiple HPV infections (two or more HPV genotypes in a single woman). For low-risk (LR)-HPV and HR-HPV, without considering the multiple HPV infections, the frequency was 22.51% ($N = 140$) and 10.93% ($N = 68$), respectively (Table 1). Women with HPV were between 24 and 40 years old, and HPV was associated with the use of hormonal contraceptives ($P = 0.005$), more than or equal to three children ($P = 0.007$), and more than or equal to two sexual partners ($P = 0.000$).

The general frequency of women with Ct was 12.70% ($N = 79/622$), and for simultaneous infections, total HPV plus Ct infections, HR-HPV plus Ct infections, and LR-HPV plus Ct infections, the frequencies were 5.80% ($N = 36$), 3.90% ($N = 24$), and 2.60% ($N = 16$), respectively ($P > 0.05$) (Table 1). Women with Ct were between 23 and 40 years old ($P = 0.000$) and reported more than or equal to two sexual partners ($P = 0.009$).

For the cases ($N = 252$), the rate for HPV infection was significant, with OR = 24 (15.07–38.22; $P < 0.0001$). The frequency rates of total HPV, HR-HPV, and LR-HPV were 66.30% ($N = 167$), 50.00% ($N = 126$), and 21.82% ($N = 55$), respectively ($P > 0.05$). The total HPV was associated with cases ($P = 0.0001$) and the different grades of abnormal

TABLE 2

HPV genotypes detected in all women studied including with normal cytology (controls), with cervical abnormalities (cases) and also with *Chlamydia trachomatis* (Ct) without considered HPV multiple infections

	NILM (n = 370)		Cervical abnormalities (n = 252)			Ct (n = 79)		
HPV genotypes	n	%	n	%	**P* value	n	%	***P* value
HR-HPV								
16	5	50.0	62	46.9	0.99	13	59.1	0.99
51	–	–	7	5.3	–	1	4.5	–
45	1	10.0	8	6.1	0.84	4	18.2	0.80
18	1	10.0	7	5.3	0.81	–	–	–
82	–	–	6	4.5	–	2	9.1	–
31	2	20.0	10	7.7	0.60	2	9.1	0.78
59	1	10.0	7	5.3	0.81	–	–	–
35	–	–	6	4.5	–	–	–	–
33	–	–	8	6.1	–	–	–	–
66	–	–	6	4.5	–	–	–	–
52	–	–	3	2.3	–	–	–	–
68	–	–	2	1.5	–	–	–	–
Total	10	100.0	132	100.0	0.99	22	100.0	0.99
LR-HPV								
72	1	12.5	23	36.5	0.50	6	40.0	0.50
42	–	–	6	9.5	–	1	6.7	–
61	2	25.0	1	1.5	0.55	1	6.7	0.67
69	1	12.5	3	4.8	0.77	1	6.7	0.86
81	2	25.0	4	6.3	0.57	–	–	–
67	2	25.0	1	1.5	0.55	1	6.7	0.66
53	–	–	2	3.2	–	2	13.3	–
40	–	–	1	1.5	–	1	6.7	–
11	–	–	3	4.8	–	2	13.3	–
Others[1]			20					
Total	8	100.0	63	100.0	0.99	15	100.0	0.99

P value < 0.05 is considered significant.
Cytology findings: controls (normal –NILM) cases: atypical glandular cells, atypical squamous cells of undetermined significance or -cannot exclude high-grade squamous intraepithelial cervical lesion, low-grade or high-grade squamous intraepithelial cervical lesion, and squamous cervical carcinoma. HPV = HPV positive; HR-HPV = high-risk HPV; LR-HPV = low-risk HPV; Ct = *Chlamydia trachomatis* positive; Others[1] = 6, 13, 39, 54, 58, 62 and 70.
* *P* value compared normal cytology versus cervical abnormalities.
** *P* value compared *Chlamydia* detection versus HPV genotypes.

cervical cytology: ASC-US ($P = 0.0053$), ASC-H ($P = 0.0001$), LSIL ($P = 0.0001$), HSIL ($P = 0.0001$), and CC ($P = 0.0001$). HR-HPV was associated with ASC-H ($P = 0.0037$), LSIL ($P = 0.0025$), HSIL ($P = 0.0001$), and CC ($P = 0.0001$). LR-HPV was not associated with abnormal cervical cytology or CC ($P = 0.1295$). The frequency of total HPV and HR-HPV increased with the severity of the abnormal cervical cytology from ASC-US to CC as well ($P < 0.0001$) (Table 1).

The cases also showed a significant rate for Ct infection, with OR = 1.907 (1.187–3.07; $P = 0.007922$), and simultaneous total HPV and Ct infections, with OR = 15.25 (5.355–43.43; $P = 0.0001$). The frequency of Ct in the cases was 17.06% ($N = 43/252$), and frequencies in total HPV plus Ct infections, HR-HPV plus Ct infections, and LR-HPV plus Ct infections were 12.70% ($N = 32$), 9.13% ($N = 23$), and 5.16% ($N = 13$), respectively ($P > 0.05$). Ct infection was not significantly associated with total HPV ($P = 0.4923$), HR-HPV ($P = 0.5454$), LR-HPV ($P = 0.9531$), abnormal cervical cytology in general, or some of the different grades of these abnormalities or CC ($P = 0.3463$).

In all, 28 different HPV genotypes were detected in the women studied (12 HR-HPV and 16 LR-HPV). HPV-16 was the most common HPV genotype ($N = 67/622$, 10.80%) in the cases as a whole ($N = 62/252$, 24.60%), HSIL and CC ($P < 0.0001$), and simultaneous infections with Ct ($N = 13/79$, 16.45%). HPV-72 (LR) was the second most common genotype ($N = 24/622$, 3.90%) in the cases as a whole (23/252, 9.13%; $P = 0.5005$), and only one LR-HPV was observed in HSIL and CC (always in multiple infections with HR-HPV) (Table 2). The only HPV genotype detected in women with AGC was HPV-18 ($N = 2/6$, 33.33%).

In the present study, we found that Ct was not associated with total HPV, HR-HPV, LR-HPV, or some of the different grades of these lesions or CC. Other similar studies have shown that Ct infection was not associated with CC, because the prevalence of bacteria is very low in this cancer.[10,11] However, a positive or negative association between HPV, Ct, and CC has been found in studies comparing results obtained by different methodologies, such as serological status for Ct and HPV, presence of one or another agent by highly sensitive molecular techniques, and others by serological status.[10–12] Because of these methodological differences, it is difficult to compare results from different studies, and caution is needed in interpreting the possible existence of any linkage between Ct and cervical carcinogenesis.

Our results indicated that women with abnormal cytology had a higher rate of HPV, Ct, or HPV plus Ct infections. This higher rate is well-established for HPV.[5] One possibility for the higher rate of Ct infection is that, although each infection causes a different disease entity, Ct and HPV are common infections in sexually active young women, and therefore, the rate factors for acquiring each of these STIs are similar.[7] Our results are in agreement for age and number of sexual partners with the results described in the work by Miller and others[7] that Ct DNA positivity may simply be a function of high-risk sexual behavior.

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