**Abstract**

**Background:** Co-infection by *Chlamydia trachomatis* (CT) in women with Human Papillomavirus (HPV) infection has been shown to increase the risk of developing cervical intraepithelial neoplasia (CIN). The present study was designed to analyze the association between HPV and CT co-infection and the severity of cervical neoplasia.

**Methods:** Two hundred fifty-one women with PCR-confirmed HPV infection were tested for CT co-infection by PCR, prior to cervical conizations due to CIN. Prevalence rates of CT and HPV types were reported for the histological diagnosis categories.

**Results:** The prevalence of *Chlamydia trachomatis* was 15.1% (38/251). CT negative women showed a significant association between age ≥ 30 years and CIN 2 or worse diagnosis; this association was not found in CT positive women. In women < 29 years of age, negative for CT, the infection by HPV 16/18 were detected in 50% of the women with CIN 2 or worse diagnosis and in 19.5% of women with CIN 1 or negative (OR=5.83; 95%CI: 2.19-15.57).

**Conclusion:** No association with CIN 2 or worse diagnosis was observed for *Chlamydia trachomatis* positive women for all age groups. These data can suggest that HPV type and no CT infection may correlate with risk for severity of histological diagnoses in younger women.

**Keywords:** HPV; *Chlamydia trachomatis*; PCR; Uterine cervical neoplasms

**Introduction**

Human papillomavirus (HPV), a sexually transmitted deoxyribonucleic acid (DNA) virus, is widely accepted as the cause of cervical cancer [1]. HPV and *Chlamydia trachomatis* (CT) cause most common sexually transmitted infections worldwide [2-4]. The prevalence of both infections is higher in young women [5,6] and factors that are associated with its acquisition are also shared [7]. Co-infection with CT and HPV may increase a woman's risk of developing cervical neoplasia [8-12].

Paba et al. [7] suggested that CT infection may facilitate the entry and persistence of multiple high-risk HPV (HR-HPV) types in the cervical epithelium. This in turn could lead to viral integration, inhibition of apoptosis and overexpression of E6/E7 oncoproteins, and could eventually result in cell transformation. In addition, Barros et al. [9], indicated that positivity for HPV, particularly HPV types 16 and/or 18, combined with seropositivity for CT was significantly associated with a diagnosis of high-grade neoplasia. Nevertheless, other studies have failed to find any association between these infections and the severity of cervical neoplasia [6,11]. Saafaeian et al. [6] found no association between CT status, as assessed by DNA or serology, and the risk of cervical pre-malignancy after controlling for carcinogenic HPV-positive status.

With these controversies in mind, the present study was designed to analyze the association between HPV and CT co-infection and the severity of cervical neoplasia. Women testing positive for high-risk HPV who had been submitted to excision of the transformation zone were admitted to the study, considering that these women are more likely to have cervical neoplasia.

**Methods**

**Study design and ethical standards**

This was a cross-sectional study on 251 consecutive subjects who underwent cervical conization due to CIN and who signed an informed consent form. The study was approved by the Institution's ethics review board.

**Subjects and Samples**

Immediately before conization, ecto and endocervical sampling was carried out, and the residual material was rinsed and stored in 1.0 mL
of Universal Collection Medium (UCM) (QIAGEN Sample and Assay Technologies, QIAGEN Biotechnology Brazil Ltda) for HPV and CT DNA testing. Women with diagnosis or suspicion of CIN2 or worse lesion were submitted to excision of transformation zone or cervical conization according to the current guideline. Of the 290 women tested, 251 (86.6%) were infected by high-risk HPV and were thus included in the analyses.

Detection and genotyping of HPV

HPV DNA was amplified using the PGMY09/11 primers that amplify a 450-bp fragment of the L1 open reading frame. HPV DNA genotyping was performed using a reverse line blot hybridization assay in which the 450-bp PCR amplicon was hybridized to a nylon strip containing immobilized probes [13]. The strip contained 2 levels of β-globin control probes, 18 HR-HPV probes (HR-HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 55, 56, 58, 59, 68, 73, 82 and 83) and 9 low-risk HPV probes (low-risk HPV 6, 11, 40, 53, 54, 57, 66 and 84). The 100 µL final volume of the amplification mixture contained 4 mM of MgCl₂, 50 mM of KCl, 7.5 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Foster City, CA, USA), 200 mM each of deoxyadenosine triphosphate, deoxycytidine triphosphate and deoxyguanosine triphosphate, 600 mM of deoxyuridine triphosphate, 100 pmol of each 5'-biotinylated β-globin primers, 2.5 µL of each biotinylated PGMY09/PMG111 primer pool, and 2.5 µL of the 5'-biotinylated β-globin primers, GH20 and POC04. The amplification profile was: activation of AmpliTaq Gold for 9 minutes at 95°C, denaturation for 1 minute at 95°C, annealing for 1 minute at 55°C and extension at 72°C for 1 minute, for a total of 40 cycles, followed by a 5-minute terminal extension step at 72°C. Amplicons were denatured at 0.4 N NaOH. In a reverse-line blot assay, 40 µL of the denatured product were added to 3 mL of hybridization buffer containing the HPV genotypes and 2 concentrations of the β-globin probes, immobilized on nylon strips. Positive hybridization was precipitated on the membrane at the probe line. In specimens that were considered HPV-negative, the 2 β-globin lines (high and low copies) either appeared at levels comparable with those of positive controls or were repeated until the criteria for globin positivity were achieved.

Histopathology

The specimens were reviewed according to the World Health Organization criteria and were classified as: non-neoplastic diagnosis, CIN 1, CIN 2, CIN 3, invasive squamous cell carcinoma and adenocarcinomas [14].

Chlamydia trachomatis detection

To test the quality of the DNA samples, amplified β-globin was analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining. CT was detected by PCR amplification of a sequence in the cryptic plasmid, generating a fragment of about 512 bp [15]. The reaction mixture contained 2.5 mM MgCl₂, 200 µM dNTPs, 10 pmol of each primer (H1/H2), 1.5 U of Platinum Taq DNA polymerase (Invitrogen Corporation, San Diego, CA, USA) and 2 µL of the sample in a final volume of 20 µL. Reactions were carried out with an initial incubation at 94°C for 5 minutes, followed by 40 cycles of 1 minute at 94°C, 1 minute at 45°C, and 1 minute at 72°C, and a final elongation step of 7 minutes at 72°C. The entire amplified PCR product was analyzed by polyacrylamide gel electrophoresis. Chlamydia trachomatis serovar L2 DNA was used as a positive control.

Statistical analyses

All calculations were performed with Statistical Analysis System (SAS) software, version 8.0, and significance was set to 5% (95% confidence intervals). Prevalence rates of CT and HPV types were reported for the histological diagnosis categories. The association between categorical variables was tested using odds ratios (OR) with their respective 95% confidence intervals. Differences were considered also significant when p value was less than 0.05.

Results

The mean age of the 251 women included in the study was 34.2 years and the median was 32 years (range 17 to 75 years). In the group of women who tested positive for CT mean age was 32.4 years, while in the CT negative group, mean age was 34.6 years, and this difference was not statistically significant (p=0.10). The mean age of the women who tested positive for HPV 16 and/or HPV 18 was 34.6 years, while the mean age of the women infected with other HPV types was 33.8 years, and this difference was also not significant (p=0.62). However, the women with of CIN 2 or worse diagnosis (mean age 35.8 years) were significantly older than those with a diagnosis of CIN 1 or non-neoplastic diagnosis (30.5 years) (p=0.006). The data about women age are not shown in table.

The overall prevalence of CT was 15.1% (38/251). The prevalence of CT in women with a diagnosis of CIN 1/ non-neoplasia was 19.7%; (14/71) and 13.3% (24/180) in women with CIN 2 or worse. CT infection was not associated with the severity of cervical neoplasia in HPV positive women (OR=0.63; 95%CI: 0.29-1.38 p = 0.20) (Table 1).

In women with CIN 2 or worse diagnosis, CT was present in 17.9% of those infected with HPV 16 and/or 18 and in 9.8% of those infected with other HPV types, but this difference was not statistically significant (OR = 2.01; 95% CI: 0.84-4.81; p = 0.17). In women with CIN 1 or non-neoplastic diagnosis, CT was present in 28.2% of those infected with HPV 16 and/or 18 and in 9.4% of those infected with other HPV types, but this difference was statistically borderline (OR= 3.79; 95%CI: 0.95-15.07 p=0.09) (Table 2).
showed no statistical significance (OR = 2.03; 95%CI: 0.5-8.23 p=0.2) (Table 3).

Table 2: The association between *Chlamydia trachomatis* (CT) infection status, HPV types and histological outcome in women submitted to excision of the transformation zone.

<table>
<thead>
<tr>
<th>Age Group</th>
<th>CT</th>
<th>CT-positive cases</th>
<th>OR (95%CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN2 or worse</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>14</td>
<td>(100)</td>
<td>2.01 (0.84-4.81)</td>
<td>0.17</td>
</tr>
<tr>
<td>Negative</td>
<td>64</td>
<td>(82.1)</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>78</td>
<td>(100)</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Association between age group and the severity of cervical neoplasia, according to *Chlamydia trachomatis* status in women submitted to excision of the transformation zone.

Table 4: Association between age group, *Chlamydia trachomatis* (CT) status, HPV types and histological diagnosis.

Taking into consideration women ≥ 30 years of age and CT-negative, the prevalence of HPV 16 and/or HPV 18 was 48.2% in those with CIN 2 or worse diagnosis and 43.3% in those with CIN 1 or non-neoplastic diagnosis; this difference was not statistically significant (OR = 1.08; 95%CI: 0.45-2.62 p=0.96). Considering the group of CT-negative women ≤ 29 years of age, the prevalence of HPV 16 and/or HPV 18 infection was 50% in those with CIN 2 or worse diagnosis, and 19.5% in the women with CIN 1 or non-neoplastic diagnosis; in these women, the association between HPV 16 and/or 18 and CIN 2 or worse diagnosis showed OR= of 5.83, (95%CI: 2.19-15.57 p=0.002) (Table 4).

Discussion

The model of this study was designed to enhance the analysis of the association of the effect of CT infection in cervical carcinogenesis HPV-induced, reason by which all women included were HPV positive. Still, the selection of women with indication for conizations aimed to provide a sample with high probability of diagnosis of CIN 2 or more severe lesion in the surgical specimen, as was observed in the results.

This study corroborates the concept that co-infection with HPV and CT is a common event [16] however, no association between CT infection detected by PCR with CIN 2 or worse diagnosis was observed in women with HR-HPV. On the other hand, among younger women without CT infection, HPV 16 and/or HPV 18 infection was associated
with CIN 2 or worse, but this association was not observed for older women.

The association between CT, HPV and the detection of cervical intraepithelial neoplasia or invasive cervical cancer was also reported in some studies in which CT was assessed by PCR or other DNA tests, but there is no consensus [9,17,18]. De Paula et al. [19] reported that although a significant association was found for HPV infection and the precursor lesions of cervical cancer, it was not possible to establish a significant association between these lesions and CT or HPV and/or CT co-infection. According to Safaeian et al. [6] CT could be associated with cervical cancer because it is associated with HPV acquisition by a causal link through common risk factors such as infected partners and sexual behavior or by a causal disruption of the epithelial tissue; these authors believe it is unlikely that CT infection affects HPV persistence and progression to cervical premalignancy. Nevertheless, if CT infection plays any role in the etiology of cervical carcinogenesis, the possible mechanism would be the facilitation of the entry and the promotion of the persistence of HR-HPV as result of chronic inflammation and resistance to apoptosis [6,19]. Considering the long time interval between for the development of cervical neoplasia, it is possible that bacterial infection by CT does not persist enough to be detectable by DNA tests in cervical epithelial neoplasia [6,17,20-22]. In fact, a positive PCR for CT may indicate acute infection or rarely chronic and this may explain some differences found among these studies.

A positive association between CT, HPV and the detection of cervical neoplasia or invasive cervical cancer has been reported in majority of studies in which CT was assessed by serology [9,16]. Dahlström et al. [18] conducted a prospective seroepidemiological study and showed that previous exposure to CT, indicated by positive serum antibodies, increased the woman's risk of cervical cancer (OR = 1.9; 95% CI: 1.5-2.3). Considering that serology reflects a previous or chronic infection and the detection of PCR detects an infection present and considering that carcinogenesis is a process that is not acute, it is admissible that the research of CT by serology is most appropriate to assess its association with cervical neoplasia.

Considering the association between HPV 16 and HPV 18 and CIN 2 or worse diagnostic in young women CT negative observed in this study, there are indications that a genotype-specific natural history implicated in the development of cervical cancer precursors: one type, more frequent, HPV16/18 related, developing quickly and early in life; another one, non-16/18 HR-HPV related, developing later, slowly, through low- to high-grade lesions [23]. This hypothesis may explain the results of the present study, which showed a significant association between the severity of cervical neoplasia and HPV 16 and/or HPV 18 infection in CT-negative women < 30 years of age, but failed to find a similar association in the case of women ≥ 30 years of age. Brotherton et al. [24] also showed that HPV 16 is more common in young than old women with high-grade cervical lesions and emphasized that this finding was consistent with all but one out of eighteen studies identified in the literature. In fact, according Sideri et al. [25] the risk to develop CIN2 or worse diagnosis is age and genotype related: it decreases with age in CIN 2 or worse related to genotype 16 and/or 18, while it increases with age in CIN2 or worse not related to HPV16 or 18. In addition, high risk HPV positive, but 16/18 negative CIN patients showed a difference in age trend between lower and higher CIN grades. These authors suggest that HPV HPV 16 and 18 related CIN appear sooner than other high risk HPV genotype related CIN.

A limitation of this study was the few (38/251) positive cases of CT, which may preclude any inference with respect to determining the risk for developing high-grade neoplasia. Other limitation of this study was that the CT genotypes were not analyzed, and they could represent different risk profiles for cervical cancer [22]. Heterogeneous population of CT prevailing in the female population was found with the identification of genotypes D, E, F, and K in a study conducted in Brazil; CT genotyping revealed that genotypes D and E were found most frequently, as observed in other studies on genotypic diversity [15].

In conclusion, this study showed that current CT infection detected by PCR was not associated with the risk of HPV-positive women developing a high-grade lesion. Association was observed between HPV 16 and/or 18 infection and high-grade lesions in younger women, when CT infection was not detected. These data can suggest that HPV type and no CT infection may correlate with risk for severity of histological diagnoses in younger women. The lack of consistent evidence of the association of CT infection and risk of cervical cancer indicates that the treatment of CT is much more relevant for preventing morbidities such as pelvic inflammatory disease and infertility rather than for reducing the risk of a CIN lesion.

Authors contributions

JMCS participated in the construction of study design, case selection, was responsible for the evaluation of CT-PCR assay, was data manager and wrote the manuscript. SHRS participated in the construction of study design, helped in study coordination and helped in manuscript drafting and revising. RRFV and SD participated in the construction of study design and revised the manuscript. DRP and EAC oversaw all laboratory aspects of the study and revised the manuscript. LOZS was responsible for statistical analysis and helped draft the manuscript. LCZ is the project manager and principal investigator of the study, conceptualized the study and was involved in study design, study coordination and revising the manuscript. All authors read and approved this manuscript.

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References


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