HPV genotypes distribution in *Chlamydia trachomatis* co-infection in a large cohort of women from north-east Italy

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Human papillomavirus (HPV) and *Chlamydia trachomatis* are pathogens with oncogenic potential associated with persistent infections. Epidemiological data on *C. trachomatis* infection status, *C. trachomatis*/HPV co-infection and the relationship between HPV genotypes in Italian women are only preliminary. The aim of the present study was to characterize the relationship between HPV genotypes and *C. trachomatis* in an extending cohort of asymptomatic immunocompetent women from an area of north-east Italy. A retrospective study was conducted using Luminex technology on cervical swabs from asymptomatic immunocompetent women, comprising 921 attending the prevention centre for the Cervical Cancer Program and 6214 who had been referred to the Sexually Transmitted Infections Center, with clinical indications of HPV and *C. trachomatis* infections. A quantitative real-time PCR was performed to assess chronic *C. trachomatis* infection by heat-shock protein 60 (*Hsp60*) gene expression. The overall prevalence of the investigated pathogens was 39 % (359/921) for HPV and 4 % (251/6214) for *C. trachomatis*. The *Hsp60* gene was detected in 57 % of the women infected with *C. trachomatis*. HPV co-infection was present in 58 % of *C. trachomatis*-infected women. A high prevalence of co-infection was found in women with chronic *C. trachomatis* infection (68 %, *P* = 0.0002), especially in women < 25 years (72 %) where HPV multiple infections were found in 78 % (*P* = 0.022). HPV genotype distribution showed that uncommon low-risk genotypes were associated with *C. trachomatis*. These results indicate a high frequency of co-detection of multiple HPV genotypes in chronically infected young women and suggest that the expression of the *C. trachomatis Hsp60* gene may favour HPV infection.

INTRODUCTION

*Chlamydia trachomatis* and human papillomavirus (HPV) cause two of the main sexually transmitted infections, with potentially oncogenic characteristics linked to persistence of infection. Although HPV infections are often transient and asymptomatic, an unresolved infection, defined as the persisting presence of HPV DNA in the cervix, represents a pre-requisite for cervical cancer, one of the leading cancers in women worldwide (Bosch *et al.*, 2013). In fact, 20 % of women with persistent carcinogenic HPV infection at 1 year develop cervical intraepithelial neoplasia or cervical cancer within the subsequent 5 years (Oakeshott *et al.*, 2012).

Among the HPV types that have been isolated and sequenced, 12 HPV genotypes (HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58 and -59) have been classified as ‘carcinogenic to humans’ (group 1), one genotype (HPV68) as ‘probably carcinogenic’ (group 2A) and another seven (HPV26, -53, -66, -67, -70, -73 and -82) as ‘possibly carcinogenic’ (group 2B) (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012). Based on their oncogenic potential, HPV genotypes have been associated with different degrees of cervical lesions. High-risk HPV (HR-HPV) genotypes have been associated with pre-malignant or malignant lesions of the cervix, although recent studies have demonstrated their

Abbreviations: *Ct*, cycle threshold; HPV, human papillomavirus; HR-HPV, high-risk human papillomavirus; LR-HPV, low-risk human papillomavirus; MFI, median fluorescence intensity; prHR-HPV, presumable high-risk human papillomavirus.
involvement in other genital cancers, including cancer of the vulva, vagina and anus. HPV16 and -18 are the most frequently detected genotypes in cervical cancer worldwide, accounting for 70% of squamous cell carcinoma of the cervix (Tommasino, 2014).

Development of cervical cancer depends on the oncogenic potential of HPV, its genotype distribution in different geographical areas and host co-factors, including cervical infections with other sexually transmitted pathogens, all of which contribute to the final outcome (Ault, 2006; de Sanjose et al., 2007; Silva et al., 2013, 2014).

Genital C. trachomatis infection is a common sexually transmitted disease associated with promiscuous sexual behaviour (Paavonen, 2012; Silva et al., 2014). It is associated with cervical dysplasia and with an eightfold increase in the risk of an unhealthy cervix (Bhatla et al., 2013). C. trachomatis infection has been identified as a major health concern associated with adverse effects on women’s reproductive status (Ljubin-Sternak & Meštrović, 2014). C. trachomatis infection has also been associated with cervical hypertrophy and induction of squamous metaplasia, indicating a possible relationship with HPV infection (Jensen et al., 2014; Ljubin-Sternak, & Meštrović, 2014). The biological effects of C. trachomatis infection may include damage of the mucosal barrier, thus improving HPV cell entry, and the chronic status of the C. trachomatis infection seems to impair the immune response, favouring the persistence of HPV infection.

In addition, it has been reported that C. trachomatis-induced chronic inflammation produces a local immune perturbation that decreases the number of antigen-presenting cells involved in the clearance of HPV (Malinverni, 1996; Silva et al., 2014; Simonetti et al., 2009; Soper, 2010; Vidhani et al., 2005). Nevertheless, although several epidemiological studies have found a positive association between C. trachomatis- and HPV-related cervical lesions and cervical cancer, the specific relationship between infections by these micro-organisms and cervical neoplasia has not as yet been fully clarified. Moreover, the detection of C. trachomatis in HPV-positive samples has yielded contrasting results (Castle et al., 2003; de Paula et al., 2007; Lehtinen et al., 2011; Luostarinen et al., 2013; Naucler et al., 2007).

In a recent pilot study carried out in north-east Italy, we described a high prevalence of C. trachomatis/HPV co-infection in young asymptomatic women with cytological abnormalities. Chronic C. trachomatis infection characterized the majority of the co-infections (Seraceni et al., 2014).

The aim of the present study was to explore the relationship between HPV genotypes and C. trachomatis status in single versus multiple infections, in an extending cohort of asymptomatic women from the same geographical area.

METHODS

Clinical specimens. A retrospective study was conducted on 7135 cervical samples collected between January 2009 and December 2014 from asymptomatic outpatient immunocompetent women, who had not been vaccinated against HPV infection. This series comprised 921 women (mean age 43 ± 10 years) attending the prevention centre for the Cervical Cancer Program and 6214 (mean age 34 ± 10 years) women, attending the Sexually Transmitted Infections Center, with clinical indications of HPV and C. trachomatis infections.

The cervical samples were collected using a 200 mm polyethylene Cervex brush device (Rovers Medical Devices) and suspended in 1.5 ml TE buffer. Each sample was divided into three 500 μl aliquots and stored at -80 °C until analysis.

No informed consent or other action on the part of the patients was required for this study as the patients’ anonymity was guaranteed. The analysis on this series of samples was conducted as a blind study.

Isolation of DNA from cervical cells. DNA isolation was performed within 24 h of sample collection. After specimen centrifugation, 500 μl each sample was treated with the NucliSENS EasyMAG automated system for total nucleic acid extraction (bioMérieux), according to the manufacturer’s instructions.

C. trachomatis and HPV detection and genotyping. C. trachomatis and HPV were detected simultaneously in cervical samples by bead-based Luminex suspension array technology (Luminex Corp.). To amplify C. trachomatis DNA, a gene encoding a conserved hypothetical virulence plasmid protein was used, with 10 copies as the detection limit of the assay. HPV genotyping was performed using a type-specific E7 PCR bead-based multiplex assay (TS-E7-MPG; IARC), which is able to identify 27 HPV types [HR-HPV types: HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, -68, -73 and -cp108; presumable high-risk (pHR)-HPV types: HPV26, -53, -67, -69 and -82; and low-risk (LR)-HPV types: HPV6, -11, -55, -81, -83 and -84] and the β-globin gene as an internal positive control. Viral genomes detected by the assay ranged from 10 to 1000 copies (Bellaminutti et al., 2014; Comar et al., 2012). Briefly, HPV genotypes were detected as the median fluorescence intensity (MFI) of at least 100 beads per bead set. The background value for each probe was the MFI value resulting from the hybridization mixture without the addition of the PCR product. The cut-off was computed by adding 5 MFI to 1.1, the median background value.

An additional set of HPV types comprising LR-HPV40, -42, -43, -44, -54, -61 and -70 was identified by an Amplicx II HPV Detection assay (Seegene) using the CFX96 Real-time PCR System (Bio-Rad) as indicated by the supplier. A human housekeeping gene was used as an endogenous internal control to ensure DNA purification, and PCR and specimen quality.

C. trachomatis Hsp60 real-time PCR detection. Total RNA was extracted from 500 μl of the stored aliquot using a RNeasy Mini kit (Qiagen). In order to avoid genomic DNA contamination, the RNA was treated with DNase I (RNase-Free DNase Set; Qiagen) and eluted in 50 μl distilled water. cDNA was synthesized using a SuperScript VILO cDNA Synthesis kit (Invitrogen).

Quantitative real-time PCR was performed to assess the transcriptional level of the Hsp60 gene (C1604), as described previously (Contini & Seraceni, 2012; Seraceni et al., 2014). To avoid false-positive results, a serial standard curve, a negative control with PCR-grade water and a positive control (C. trachomatis strain TW-3) were included in the assay.

The concentration of unknown clinical samples was determined based on their cycle threshold (Ct) values using analytical software (sds 2.4; Applied Biosystems) (Branikatsch et al., 2012), while the specificity of the PCR was assessed by analysing the melting curve temperature (Tm) of the amplified products (Contini et al., 2005), and by agarose and PAGE analysis for the expected amplification product of 161 bp.
The sensitivity of each run was defined as the lowest dilution of DNA ($2 \times 10^{-4}$ ng/ml, corresponding to 1 genome copies/μl) detected by the 7900HT instrument (Applied Biosystems) (Contini et al., 2011). Standard curve equations were used to calculate the absolute number of gene mRNA copies. Results were expressed as mRNA C. trachomatis genome copies/μl of sample.

**Statistical analysis.** All statistical analyses were performed using IBM SPSS Statistics 20. A $\chi^2$ test was used to compare the frequencies of discrete variables. Fisher’s exact test was applied when necessary. The comparison between groups was carried out using a Mann–Whitney test. A P value of $\leq 0.05$ was considered the threshold of statistical significance for all tests.

**RESULTS**

**HPV and C. trachomatis infections**

In the group of 921 women at risk for HPV, the prevalence of infection was 39 % (359/921) (Table 1). The trends of infection, analysed using the cut-off age of 25 years in accordance with the Italian Cervical Screening Program (Comparetto & Borruto, 2015), showed similar frequencies in the two age groups [38.6 % (59/153) in women $\leq$ 25 years and 39 % (300/768) in women $>25$ years, $P=0.93$ respectively]. Sixty per cent (216/359) of the HPV infections were sustained by a single genotype, while multiple genotypes were detected in the remaining 40 % (143/359) of the samples. The frequencies that showed no statistically significant difference ($P=0.19$) between the two age groups are included in Table 1. Notably, all these women tested negative for C. trachomatis.

In Table 2, the overall prevalence of C. trachomatis infection in the 6124 at risk women was 4 % (251/6124). The age distribution analysis based on the cut-off age of 25 years, which is considered the most important predictor factor for C. trachomatis infection (Eggleston et al., 2011), showed a prevalence of 14 % (127/885; $P<0.0001$) in women aged $\leq 25$ years and of 2 % (124/5329) in older women. Chronic C. trachomatis infection was diagnosed in 57 % (144/251) of the total infected women, showing the highest prevalence [69 % (88/127); $P<0.0001$] in women aged $\leq 25$ years.

**C. trachomatis and HPV co-infection**

**C. trachomatis Hsp60 chronic infection.** mRNA expression of the C. trachomatis Hsp60 gene was more frequent in women co-infected with HPV [68 % (98/144); $P=0.0002$], but the difference was not statistically significant when adjusted for age [72 % (63/88); $P=0.28$ (Table 3).}

HPV single infections were detected in 31 % (30/98) of women, while multiple infections accounted for the remaining 69 % (68/98). In particular, the prevalence of multiple infections was 78 % (49/63) in younger women, compared with 54 % (19/35) in older women ($P=0.0221$). In contrast, the prevalence of HPV single infections was higher in older women [46 % (16/35)].

**HPV distribution in women co-infected with C. trachomatis and in women infected with HPV alone**

**Overall distribution of HPV genotypes in the two groups.** In Fig. 1 the overall distribution of HPV genotypes is shown in detail. High-, intermediate-and low-risk genotypes that are uncommon in this geographical area were detected more frequently in women with C. trachomatis infection (i.e. HPV40, -42, -43, -44, -45, -66

<table>
<thead>
<tr>
<th>Table 1. Overall prevalence of HPV in women with clinical indications of HPV infection, distribution of HPV infection (single vs multiple) and C. trachomatis status in women who tested positive for HPV, according to age cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age group</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>$\leq 25$ ($n=153$, 17 %)</td>
</tr>
<tr>
<td>$&gt;25$ ($n=768$, 83 %)</td>
</tr>
<tr>
<td>Total ($n=921$)</td>
</tr>
</tbody>
</table>

* $P=0.93$.  
† $P=0.19$. 

The clinical cut-off was 25 years. The difference between the groups was considered statistically significant for $P \leq 0.05$.  

HPV and C. trachomatis co-infection was present in 58 % (145/251) of the C. trachomatis positive samples and was most frequent in women $\leq 25$ years [68 % (86/127); $P=0.0014$]. In these series, HPV was present as a single infection in 32 % (47/145) of the samples, while multiple infections were found in the remaining 68 % (98/145). Interestingly, multiple HPV infections were frequent in the younger subcohort [77 % (66/86); $P=0.0065$ (Table 2).}
Specific HPV genotypes in women with C. trachomatis

Table 2. Overall prevalence of C. trachomatis infection and chlamydial status in women with clinical indications of C. trachomatis infection, according to age cohort, and distribution of HPV infection (single vs multiple) in women who tested positive for C. trachomatis

The clinical cut-off was 25 years. The difference between the groups was considered statistically significant for \( P \leq 0.05 \).

<table>
<thead>
<tr>
<th>Age group</th>
<th>C. trachomatis (^+) [n (%)]</th>
<th>C. trachomatis Hsp60 (^+) [n (%)]</th>
<th>HPV infection [n (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \leq 25 ) ((n=885, 14 %))</td>
<td>127 (14)*</td>
<td>88 (69)*</td>
<td>86 (68)(^\dagger)</td>
</tr>
<tr>
<td>&gt;25 ((n=5329, 86 %))</td>
<td>124 (2)</td>
<td>56 (45)</td>
<td>59 (48)</td>
</tr>
<tr>
<td>Total ((n=6214))</td>
<td>251/6214 (4)</td>
<td>144/251 (57)</td>
<td>145/251 (58)</td>
</tr>
</tbody>
</table>

\( ^* P<0.0001. \)
\( ^\dagger P=0.0014. \)
\( ^\ddagger P=0.0065. \)

and -73). It is worth noting that the HPV43 and -44 LR types were detected only in women with co-infection. In the group of women infected with HPV alone, HR-HPV16 was the most frequently represented genotype (33 %) followed by HPV31 (16 %) and HPV62 (12 %). Interestingly, the genotypes HPV55, -61, -62, -81, -82, -83, -84 and -cp108 were found exclusively in this group.

HPV genotype distribution in single and multiple infections. Fig. 2 shows the distribution of HPV genotypes in relation to single and multiple HPV infections in women with C. trachomatis co-infection and in women infected with HPV alone. In the group of women diagnosed with HPV, HR-HPV16 was the most represented genotype and was highly detected in women with a single infection (21 %) (Fig. 2a). Notably, the genotypes HPV61 and -62 were detected more frequently (8 and 10 %, respectively) in multiple infections compared with single infections, while HPV52 (6 %) was present only in multiple infections (Fig. 2b).

In co-infected women, the frequency of each HPV genotype was higher in women affected by multiple infections. HPV31 (23 %), HPV42 (19 %), HPV56 and -66 (15 %), HPV6 and -51 (14 %), HPV73 (13 %), HPV16 and -59 (12 %) were the main genotypes identified in this group (Fig. 2b). In particular, in multiple infections, HPV6, -51, -59 and -66 were detected together in 19 % of the cases, while HPV31 and -56 and HPV51 and -66 were detected together in 8 and 6 %, respectively.

DISCUSSION

A large body of epidemiological data suggests that C. trachomatis may play a role as a co-factor in cervical carcinogenesis (Silins et al., 2005), although the question of how C. trachomatis interacts with HPV in the transformation process is still a matter of debate (Idahl et al., 2010; Paavonen, 2012; Silva et al., 2014; Tavares et al., 2014). Tavares et al. (2014) suggested that C. trachomatis infection could influence the natural history of HPV, proposing that C. trachomatis and HPV might mutually interact with each other, rather than sharing a common way of transmission.

In our previous pilot study, we detected a high prevalence of C. trachomatis/HPV co-infection, especially in young women diagnosed with pre-cancer cervical lesions.

In the current large study, we confirmed the same trend of co-infection prevalence (58 %), with a peak in women aged \( \leq 25 \) years (68 %), sustained mainly by a chronic C. trachomatis status and by multiple HPV genotypes. This finding seems to be in contrast to a recent Italian survey that reported a prevalence of C. trachomatis/HPV co-infection of 2.7 % in young women (Panatto et al., 2015).

Table 3. Prevalence of chronic C. trachomatis chronic infection in HPV co-infected women and type of HPV infection (single vs multiple), according to age cohorts (clinical cut-off 25 years)

The clinical cut-off was 25 years. The difference between the groups was considered statistically significant for \( P \leq 0.05 \).

<table>
<thead>
<tr>
<th>Age group</th>
<th>C. trachomatis Hsp60 (^+) [n (%)]</th>
<th>HPV infection [n (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \leq 25 ) ((n=88, 61 %))</td>
<td>63/88 (72)(^\dagger)</td>
<td>14/63 (22)</td>
</tr>
<tr>
<td>&gt;25 ((n=56, 39 %))</td>
<td>35/56 (63)</td>
<td>16/35 (46)</td>
</tr>
<tr>
<td>Total ((n=144))</td>
<td>98/144 (68)*</td>
<td>30/98 (31)</td>
</tr>
</tbody>
</table>

\( ^* P=0.0002. \)
\( ^\dagger P=0.28. \)
\( ^\ddagger P=0.022. \)
**Fig. 1.** Prevalence (%) of HPV genotype distribution in *C. trachomatis*/HPV co-infected and HPV-infected women.

**Fig. 2.** Prevalence (%) of HPV single (a) and multiple (b) infections in *C. trachomatis*/HPV co-infected and HPV-infected women.
This discrepancy could be due to the more sensitive detection technique employed in our study (Comar et al., 2012) and to the adoption of a different method to calculate prevalence, using the number of women infected with C. trachomatis as the denominator. Our results seem to confirm a role for C. trachomatis in enhancing susceptibility to multiple HPVs or in type-specific HPV redetection, as a result of the reactivation of low-level persistent HPV (Shew et al., 2013).

Regarding the characteristics of HPV, single and multiple infections displayed interesting distribution patterns in our cohort. HPV single infections were detected mostly in women who were negative for C. trachomatis, while multiple HPV infections were found to be prevalent in C. trachomatis/HPV co-infected women. Within this pattern, the distribution of HPV genotypes revealed the presence of single genotypes and of particular genotype associations, rarely detected in this geographical area, when compared with the Italian HPV screening data (Carozzi et al., 2014; Giorgi Rossi et al., 2011). In fact, we either found a high percentage of LR genotypes (HPV40, -42, -43 and -44) or particular associations of LR and HR genotypes (HPV6, -51, -59 and -66; HPV31 and -56; HPV51 and -66) only in women with C. trachomatis/HPV multiple infections.

Although the present study provides no evidence of an association between genotypes and clinical outcome, the detection of HR-HPV56 and HR-HPV51 in this series confirms the recent Italian data that described these genotypes as the most frequent types associated with multiple infections (Agarossi et al., 2009; Carozzi et al., 2014) in which HPV51 has been shown to play a relevant role in the pathogenesis of invasive cervical cancer (Piana et al., 2013).

A number of recent Italian studies have described HPV16 (Bianchi et al., 2013; Carozzi et al., 2014; Giorgi Rossi et al., 2011) and HPVs belonging to groups 1, 2A or 2B as the most prevalent types among subjects with C. trachomatis/HPV co-infection (Panatto et al., 2015). It seems that Chlamydia enhances HPV16 protein expression in low-grade squamous lesions, suggesting that the activity of the virus may be modified by C. trachomatis infection. Conversely, in our study, HPV16 was the most represented genotype only in women without C. trachomatis co-infection, while different HPV genotypes with oncogenic potential were most prevalent in C. trachomatis women.

Regarding the possible mechanism of C. trachomatis/HPV interaction, even if C. trachomatis is not present in cervical adenocarcinomas (Quint et al., 2009), its ability to trigger a local inflammatory process in the upper genital tract seems to facilitate HPV cell transformation during carcinogenesis. The inflammatory micro-environment caused by a chronic C. trachomatis infection increases the release of oxidative stress proteins that may influence HPV cell entry, replication and virus integration by enhancing cellular DNA breaks (Deluca et al., 2011; Silva et al., 2014; Tavares et al., 2014). It has also been suggested that the presence of HR-HPV oncoproteins during Hsp60 expression may induce apoptotic stimuli, uncontrolled proliferation and, eventually, neoplastic transformation (Cappello et al., 2009; Dean et al., 2008).

Confirming our previous data, in this study, low levels of Hsp60 gene expression were found in HPV co-infected women, suggesting that the maintenance of a steady-state transcript level could favour a balance between a pro-inflammatory micro-environment and HPV (Silva et al., 2014; Vriend et al., 2015). HPV genotypes with low oncogenic potential have been associated more frequently with this chronic status, deserving further consideration in C. trachomatis/HPV relationship and cervical abnormalities.

In conclusion, the data from this study emphasize the need for a C. trachomatis screening programme targeted at young women. The high frequency of concomitant C. trachomatis and multiple HPV infections detected in this group suggests that early prophylactic HPV vaccination together with a screening programme for C. trachomatis could select asymptomatic women at high risk for associated diseases.

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