Genotyping of the prevalent *Chlamydia trachomatis* strains involved in cervical infections in women in Ahvaz, Iran

Behrouz Taheri Beni, Hossein Motamedi and Mohammad Roayaei Ardakani

To determine the prevalence of cervical *Chlamydia trachomatis* genotypes in Iran for the first time and their association with three clinical symptoms/signs, i.e. abnormal vaginal discharge, lower abdominal pain (LAP) and swab-induced bleeding, and patient age, 620 cervical specimens were obtained from women with symptomatic genital infection referred to gynaecological clinics and 108 *C. trachomatis*-positive specimens were genotyped by direct *omp1* gene PCR-RFLP analysis. Eight genotypes were identified. The most prevalent genotype was E (31.5 %), followed by F (23.1 %), D/Da (13 %), K (9.2 %), I (8.3 %), G (7.5 %), H (5.5 %) and J (1.9 %). For analysing the association of *C. trachomatis* genotypes with symptoms/signs and age, *P*-values were separately evaluated for genogroups and genotypes. The analysis of genogroups showed that women infected with genogroup F/G manifested the symptom of LAP significantly more often than those infected with the other genogroups (*P* = 0.02), while the analysis of genotypes revealed that women infected with genotype F reported LAP slightly more often than women infected with the other genotypes (*P* = 0.08). No significant correlation between genogroups and age was found; however, genotype E was somewhat less prevalent among women aged 25–34 years than among other age groups (*P* = 0.08).

INTRODUCTION

*Chlamydia trachomatis* causes various diseases and syndromes, including trachoma, urogenital infection and lymphogranuloma venereum, depending on the serovar involved. Currently, 19 serovars and numerous variants of genotypes (A, B/Ba, C, D/Da, E, F, G, Ga, H, I/Ia, J, K, L1, L2, L2a and L3) have been identified by using polyclonal and monoclonal antibodies against the major outer-membrane protein (Hsu et al., 2006; Ngandjio et al., 2004). Serovars A–C are mostly associated with trachoma, serovars D–K are mostly associated with urogenital infections and serovars L1–L3 are commonly associated with lymphogranuloma venereum (Morré et al., 1998; Sturm-Ramirez et al., 2000; Yuan et al., 1989). Urogenital *C. trachomatis* infections are the most prevalent sexually transmitted diseases (STDs) with approximately 92 million new cases throughout the world annually (WHO, 2001).

Serological typing methods have a limitation in that newly emerging types may be missed and culturing of clinical isolates is usually required (Eckert et al., 2000), whereas genotyping methods are more sensitive and specific and could be performed directly with crude cells of clinical specimens without culturing (Lan et al., 1993). Genital *C. trachomatis* infections have a broad spectrum of clinical manifestations. Serovar-specific differences in virulence might be responsible for such diverse clinical manifestations of chlamydial infection (van de Laar et al., 1996).

Determining the epidemiological relationship between *C. trachomatis* strains from different areas of Iran and other countries could be a suitable guideline for designing epidemiological programmes to control chlamydial infections and consequently control STDs in Iran, because genital *C. trachomatis* is currently the most prevalent sexually transmitted pathogen. In spite of the importance of this issue, there has not been any research done on the typing of *C. trachomatis* in Iran until now. Considering this lack of information, the aim of this study was to determine the prevalence of *C. trachomatis* genotypes in symptomatic cervical infections in Iranian women for the first time. Furthermore, this study introduced a direct *omp1* PCR-RFLP genotyping method, which could promote similar studies in other areas of Iran to obtain more epidemiological information about *C. trachomatis* and improve epidemiological management of STDs. Since there are no STD clinics in Iran, it seems that such research is important.
and necessary. Another aim was to evaluate the statistical association of genogroups/genotypes with three clinical symptoms/signs and age.

METHODS

Clinical samples and processing. Six hundred and fifty cervical specimens were obtained from 650 women with symptomatic genital infection at 11 gynaecology and obstetric clinics (distributed mainly in the western and eastern parts of urban areas of Ahvaz, Iran, and probably covering about one-quarter of the population) from October 2007 to December 2008. Thirty specimens were excluded from the investigation due to shortage of specimen fluid and the absence of information about the manifestations of infection. For sampling, symptomatically infected women were defined as those presenting with one or more clinical symptoms of genitourinary infection including abnormal vaginal discharge (AVD), lower abdominal pain (LAP), postcoital bleeding, dysuria, spotting, dysmenorrhea and dyspareunia (Morré et al., 2000). From these, the two symptoms of AVD and LAP and the one sign of swab-induced bleeding (SIB) were statistically analysed. Specimens were collected with sterile cotton swabs from the endocervix in such a way that the swab had the most contact with the epithelium, so that SIB would occur in patients prone to bleeding. Swabs were then placed into tubes containing 1 ml 1× PBS, transported on ice to the laboratory and stored at −20 °C prior to processing. For DNA extraction, the sample tube was vortexed vigorously, the swab was discarded, 400 μl of the specimen was centrifuged at 13,000 r.p.m. for 30 min, and the pellet was suspended in 100 μl 10 mM Tris/HCl (pH 7.5), stored at −70 °C for 48 h and then thawed. This freeze–thawed suspension was boiled for 10 min. Thereafter, the suspension was centrifuged at 10,000 r.p.m. for 2 min and the supernatant was stored at −20 °C until PCR.

Screening of specimens by C. trachomatis plasmid PCR. The primers used for amplifying a 201 bp fragment of C. trachomatis cryptic plasmid were CTPl (forward strand: 5’-TAGTAAACTGCCC-ACCTCTATCA-3’) and CTp2 (reverse strand: 5’-TCCCCCTGATTAA-TTCTGTTTC-3’). Ten microlitres of extracted DNA was used as template in the PCR. The final volume of the PCR mixture was 50 μl and final concentrations of ingredients were 50 mM KCl, 1.5 mM MgCl2, 10 mM Tris/HCl (pH 8.3), 200 μM each deoxynucleoside triphosphate (dATP, dTTP, dGTP and dCTP), 25 pmol of each primer (CT1 and CT5) and 1 U Taq DNA polymerase. The thermal PCR program consisted of DNA denaturation at 95 °C for 5 min followed by 35 cycles of amplification, each cycle consisting of a denaturation step at 95 °C for 1 min, annealing at 55 °C for 1 min and elongation at 72 °C for 1.5 min and a final elongation step at 72 °C for 4 min. The semi-nested PCR was carried out as follows. One microlitre of the primary PCR product as DNA template was added to a prepared PCR mixture containing primer PCTM3 located 22 bp downstream of CT1 and previous primer CT5. CT1 and PCTM3 are at bp 34–56 and bp 55–84, respectively. The amplification conditions for semi-nested PCR were the same as the conditions for the primary PCR. The amplification products were visualized after electrophoresis through a 1% agarose gel containing ethidium bromide. The omp1 semi-nested PCR products were digested with restriction enzymes and RFLP analysis was performed based on restriction fragments with sizes larger than 100 bp reported in a previous study (Rodriguez et al., 1991). Restriction digestion was performed in two ways: (i) single digestion with the restriction enzyme Alul; and (ii) triple digestion with the enzymes HpaII, EcoRI and HinfI. The single digestion differentiates 10 genotypes, A, C, E, F, G, I, J, K, L1 and L2, while B, Ba and D, and H and L3, exhibited similar patterns. The triple digestion differentiates 11 genotypes, D, E, F, G, H, I, J, K, L1, L2 and L3, but genotypes A and C, and B and Ba, have similar patterns. Therefore, the single digestion was first carried out for all samples then the triple digestion was performed for differentiating genotype B/Ba from D and genotype H from L3 and for confirming the other genotypes that were identified by Alul digestion. The differences between genotypes H and Ia in the two digestions were minor; DdeI digestion was used for confirming genotype H (Rodriguez et al., 1993). In this study, genotype D was not differentiated from Da and genotype G was not differentiated from Ga. Analysis of digestion products was performed by 7% PAGE. The gels were stained with 15 μg ethidium bromide ml−1 for 30 min and visualized under UV light.

Statistical analysis. C. trachomatis serovars were classified in three groups on the basis of their genetic relationship and serological cross-reactivity. The B-complex contains the serovars B, Ba, D, E, L1 and L2; the intermediate F/G group contains the serovars F and G; and the C-complex contains serovars A, C, H, I, J, K and L3 (van de Laar et al., 1986). In this research, association of both genotypes and genogroups with three symptoms/signs (AVD, LAP and SIB) and age were separately evaluated by using the spss 15.0 statistical software. Statistical analysis of the data was performed using the chi-square test. A P-value <0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

The 201 bp fragment was amplified in 112 of 620 specimens (18.1%). Table 1 shows the overall prevalence of infection in different age groups. The omp1 sequence was successfully amplified by primary and semi-nested PCR in 109 of 112 plasmid PCR-positive samples but not in three samples. Our investigation showed that semi-nested PCR could increase the sensitivity of omp1 amplification, because omp1 was amplified in 92 of 112 plasmid PCR-positive samples by primary PCR and in 17 of 20 omp1 primary PCR-negative samples by semi-nested PCR. This strategy is useful, particularly for direct PCR on crude suspensions of samples that have low copy numbers of Chlamydia. One of the samples that was very weakly semi-nested PCR-positive after single and triple digestion
produced no band on a polyacrylamide gel; amplification by semi-nested PCR was repeated but the previous result was not improved, probably because of low copy numbers of omp1 amplification products.

PCR-RFLP is the reference method for typing C. trachomatis worldwide and so this method was used in this study. RFLP patterns of omp1 semi-nested PCR products of eight genotypes after single and triple digestion are shown in Fig. 1. Separation of genotypes was performed based on restriction fragments larger than 100 bp produced from the CT1–CT5 sequence of the omp1 gene for the 15 C. trachomatis genotypes evaluated in a previous study (Rodríguez et al., 1991). However, single and triple digestion of the PCTM3–CT5 sequence produced the same restriction fragments larger than 100 bp because, based on BLAST searching at NCBI, the first restriction sites for AluI (AGCT) and HinfI (GAATC) are located at bp 63–66 and bp 78–82 of the omp1 gene, respectively. Both sites are apparent on the PCTM3 primer. Therefore, RFLP patterns of primary PCR and semi-nested PCR products after single digestion are different at a 64 bp fragment and after triple digestion at a 78 bp fragment, which are smaller than 100 bp. Single and triple digestion products of one sample produced no bands on polyacrylamide gel and this sample was not genotyped. In total, 108 of 112 plasmid PCR-positive samples were genotyped. Statistical analysis was performed for these samples and four non-amplified and non-genotyped samples were excluded from the analysis. Table 2 shows the distribution of three genogroups and eight genotypes in age groups; P-values were evaluated by the chi-square test for the association of age with each genogroup and genotype. No statistically significant differences in distribution of genotypes and genogroups in the age groups were found. However, genotype E was found slightly less often among women aged between 25 and 34 years old than among the other age groups (P=0.08). Of the 108 infected women, 64 reported AVD, 22 LAP and 37 of them manifested SIB (Table 3). In the analysis of genogroups, women infected with the F/G genogroup more often manifested LAP than those infected with the other genogroups (P=0.02); and in the analysis of genotypes, women infected with genotype F manifested this symptom somewhat more often than those infected with the other genotypes (P=0.08). No significant relationship between other symptoms/signs and genogroups/genotypes was found.

In the present study, genotypes E, F and D were the most prevalent (68%). In other studies, these genotypes were also responsible for most genital C. trachomatis infections (Geisler et al., 2003; Molano et al., 2005; Mørre et al., 2000; Persson & Osser, 1993). No statistically significant differences in distribution of genotypes and genogroups in the age groups were found. However, genotype E was found slightly less often among women aged between 25 and 34 years old than among women in the other age groups (P=0.08). In this study, genotype Ia was not found in any of the samples, which

---

**Table 1.** Prevalence of cervical Chlamydia trachomatis infection as determined by plasmid PCR

<table>
<thead>
<tr>
<th>Age group (year)</th>
<th>No. PCR-positive (%)</th>
<th>No. PCR-negative (%)</th>
<th>Total no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;25</td>
<td>33 (19.7)</td>
<td>134 (21.6)</td>
<td>167 (26.9)</td>
</tr>
<tr>
<td>25–34</td>
<td>59 (19.8)</td>
<td>239 (38.4)</td>
<td>298 (48.1)</td>
</tr>
<tr>
<td>&gt;34</td>
<td>20 (12.9)</td>
<td>135 (21.7)</td>
<td>155 (25)</td>
</tr>
<tr>
<td>Total</td>
<td>112 (18.1)</td>
<td>508 (81.9)</td>
<td>620 (100)</td>
</tr>
</tbody>
</table>

---

**Fig. 1.** RFLP patterns of the PCTM3–CT5 sequence of omp1 for eight C. trachomatis-positive clinical samples cleaved in two ways: single digestion with AluI endonuclease (lane 1) and triple digestion with HpaII–EcoRI–HinfI endonucleases (lane 2). Lane M is pBR322 digested with HaeIII as molecular size marker.
were all collected from symptomatic infections. These results are in agreement with another study reporting that serovar Ia was found in asymptomatic women only (Morré et al., 2000). In our study, the association of *C. trachomatis* types with symptoms/signs and age was analysed for genogroups and genotypes separately. There was no significant association between AVD and genogroups/genotypes, whereas in other studies, this symptom correlated with serovar K (Gao et al., 2007; Morré et al., 2000). Analysis of genogroups showed that infections caused by F/G groups were associated more often with LAP than those caused by B-complex and C-complex (P < 0.02) groups, whereas the evaluated P-values for comparing genotypes showed that F genotypes were slightly more associated with this symptom than other genotypes, but this was not statistically significant (P = 0.08). These results indicate that to make a careful decision about the association of *C. trachomatis* types with each factor, separate evaluation of the P-value for genogroups and genotypes in relation to that factor is necessary. Two studies have also found a significant correlation between F/G serovars and LAP (Geisler et al., 2003; van Duynhoven et al., 1998). In this research, there was no significant relationship between SIB and genogroups/genotypes; however, in other research, SIB was found somewhat less frequently among women infected with the F/G group (Workowski et al., 1994). In other studies, there were no significant correlations between clinical manifestations and different serovars (Barnes et al., 1987; Persson & Osser, 1993). Contradictory results in the analysis of the association between clinical manifestations and serovars can be explained by various factors such as geographical variation, differences in sample size, population composition and methodological differences (Morré et al., 2000; van de Laar et al., 1996). Furthermore, host variation and co-infection status might also influence the clinical course of infection.

As mentioned earlier, typing of *C. trachomatis* was performed in the present study for the first time in Iran to our knowledge. Most of the studies in Iran have been

<table>
<thead>
<tr>
<th>Genogroup and genotype</th>
<th>No. (%) of women infected in each age group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;25</td>
<td>25–34</td>
</tr>
<tr>
<td>B-complex</td>
<td>18 (54.5)</td>
<td>19 (34.5)</td>
</tr>
<tr>
<td>D/Da</td>
<td>4 (12.1)</td>
<td>7 (21.8)</td>
</tr>
<tr>
<td>E</td>
<td>14 (42.4)</td>
<td>12 (34.5)</td>
</tr>
<tr>
<td>F/G group</td>
<td>8 (24.2)</td>
<td>20 (36.4)</td>
</tr>
<tr>
<td>F</td>
<td>6 (18.2)</td>
<td>15 (27.3)</td>
</tr>
<tr>
<td>G</td>
<td>2 (6)</td>
<td>5 (9.1)</td>
</tr>
<tr>
<td>C-complex</td>
<td>7 (21.2)</td>
<td>16 (29)</td>
</tr>
<tr>
<td>H</td>
<td>2 (6)</td>
<td>3 (5.4)</td>
</tr>
<tr>
<td>I</td>
<td>3 (9.1)</td>
<td>5 (9.1)</td>
</tr>
<tr>
<td>J</td>
<td>0 (0)</td>
<td>2 (3.6)</td>
</tr>
<tr>
<td>K</td>
<td>2 (6)</td>
<td>6 (11)</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
<td>55</td>
</tr>
</tbody>
</table>

**Table 2.** Distribution of cervical *Chlamydia trachomatis* genogroups and genotypes in relation to age

<table>
<thead>
<tr>
<th>Symptom/sign*</th>
<th>No. (%) of each genogroup or genotype</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-complex</td>
<td>D/Da</td>
<td>E</td>
</tr>
<tr>
<td>AVD</td>
<td>33 (69)</td>
<td>8 (57)</td>
</tr>
<tr>
<td>LAP</td>
<td>7 (14.6)</td>
<td>12 (36.4)</td>
</tr>
<tr>
<td>SIB</td>
<td>14 (29)</td>
<td>5 (36)</td>
</tr>
</tbody>
</table>

*AVD, Abnormal vaginal discharge; LAP, lower abdominal pain; SIB, swab-induced bleeding.
carried out to determine the prevalence of this infection; a number of these are discussed here. One of these studies was carried out to determine the prevalence of chlamydial infection in urethral specimens from 150 symptomatic male patients in Mashhad, Iran. A cell culture diagnostic technique showed that 14 (9.3 %) patients were infected with C. trachomatis (Ghanaat et al., 2008). Another study was performed to determine the prevalence of C. trachomatis infection in women attending Obstetrics and Gynaecology clinics in Tehran, Iran; 12.6 % (133/1052) were positive for this bacterium by PCR analysis. Of these PCR-positive samples, 86 were available for retesting by strand displacement amplification, of which 67 were positive (Chamani-Tabrizi et al., 2007). Results of another study in Iran carried out to determine the frequency of C. trachomatis in 123 women with cervicitis by a PCR-EIA method using primers to amplify a CT-specific plasmid showed that the overall frequency of C. trachomatis infection among women was 17 % (21/123) (Hashemi et al., 2007). In another investigation in Babol, Iran, the prevalence of C. trachomatis infection was determined to be 11.6 % (64/550) using the ELISA test for IgA and IgG in blood samples from 550 symptomatic and asymptomatic women (Bakhtiari & Firoozjahi, 2007). Nazer et al. (2008) studied asymptomatic C. trachomatis infections by omp1 gene-based PCR. They found that 22.1 % (31/140) of women in Tehran, Iran, were infected with this bacterium. In one study in Ahvaz, the prevalence of C. trachomatis infection among females with vaginal discharge was determined. Out of 202 patients, 33 (16.3 %) were positive for this bacterium (Samarbaf-Zadeh et al., 2007).

In conclusion, C. trachomatis genotypes E, F and D were the most prevalent among women with symptomatic infection in Ahvaz, Iran. No significant difference was found in distribution of genogroups/genotypes among patient age groups. Association of genogroups/genotypes with AVD and SIB was not significant; however, women infected with F/G genogroups showed LAP more often than those infected with B-complex and C-complex genotypes.

ACKNOWLEDGEMENTS

The authors wish to thank the vice chancellor for research of Shahid Chamran University for the research grant. We are also thankful to the head of the Biology and Biotechnology Research Centre for providing laboratory facilities.

REFERENCES


