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Prevalence and Serovar Distribution of Asymptomatic Cervical *Chlamydia trachomatis* Infections as Determined by Highly Sensitive PCR

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The prevalence rates and serovar distributions of *Chlamydia trachomatis* cervical infections were investigated in two different groups of women. Group I consisted of 393 asymptomatic young women (aged 17 to 30 years) who were invited to participate in a *C. trachomatis* screening program. Group II consisted of 734 randomly selected patients (aged 17 to 68 years) attending an inner-city gynecological outpatient clinic. *C. trachomatis* was detected in cervical scrapes by PCR specific for endogenous plasmid. These plasmid PCR-positive samples were subsequently subjected to genotyping by *C. trachomatis*-specific *omp1* PCR-based restriction fragment length polymorphism analysis (J. Lan, J. M. M. Walboomers, R. Roosendaal, G. J. van Doornum, D. M. MacLaren, C. J. L. M. Meijer, and A. J. C. van den Brule, J. Clin. Microbiol. 31:1060–1065, 1993). The overall prevalence rates of *C. trachomatis* found in patients younger than 30 years were 9.2 and 11.8% in groups I and II, respectively. A clear age dependency was seen in group II, with the highest prevalence rate (20%) found in patients younger than 20 years, while the rate declined significantly after 30 years of age (5.9%). In women younger than 30 years, the genotyping results showed that serovars E, I, and D (in decreasing order) were frequent in group I, while serovars F, E, and G (in decreasing order) were predominantly found in group II. The study shows that *C. trachomatis* infections are highly prevalent in asymptomatic young women. The different serovar distributions found most likely reflect the different compositions of the study groups, but additional analysis of the case histories of individual patients suggests that certain serovars might be associated with symptomatic (i.e., serovar G) or asymptomatic (i.e., serovars D and I) infections.

Chlamydia trachomatis is one of the most frequent causes of sexually transmitted diseases. Currently, 15 serotypes have been identified, i.e., serotypes A to K and L1 to L3. These are extended by serovariants and genovariants, such as Ba, Da, D^-, Ia, and L2a. Serovars D to K are urogenital pathogens and are also responsible for neonatal conjunctivitis and pneumonia. *C. trachomatis* infections may lead to pelvic inflammatory disease (PID), resulting in tubal infertility, ectopic pregnancy, and chronic pain (3, 17, 24). A substantial number of chlamydial infections have an asymptomatic course, and the published prevalence varies between 1.6 and 19%, depending on the population studied and the techniques used (3, 13, 20, 21, 24). It is assumed that asymptomatic *C. trachomatis* infection can also spread to the upper genital tract, causing PID and long-term sequelae such as tubal factor infertility and increased risk of ectopic pregnancy. Therefore, the detection of asymptomatic *C. trachomatis* infections is important. To date, data about serovar distributions in asymptomatic *C. trachomatis* infection are lacking.

Recently, the PCR method has been used to detect *C. trachomatis* infections. PCR has proved to be more sensitive and specific than the conventional microbiological assays (4, 9, 15, 16, 18, 22, 23, 25). This highly sensitive PCR is therefore a valuable tool for studying the prevalence of asymptomatic *C. trachomatis* infections, especially since these infections are often associated with low copy numbers of *C. trachomatis* (20). To facilitate large prevalence studies, we have successfully detected *C. trachomatis* directly in crude cell suspensions of cervical scrapes using a PCR specific for the endogenous plasmid, thus omitting the laborious DNA purification step (9, 10). This method enabled the detection of low copy numbers of chlamydiae in infections which are not detected by cell culture (9, 10). In addition, for serovar differentiation, the PCR has been successfully applied to the amplification of the *omp1* gene and then restriction fragment length polymorphism (RFLP) analysis as a convenient alternative for conventional serotyping (6, 9, 19). By this PCR-based genotyping, which is directly performed in crude cell suspensions, typing of *C. trachomatis* serovars can be easily performed for a large study group (9).

In the present study, the prevalence rate of cervical *C. trachomatis* infections was investigated in an asymptomatic group of young women and in a group of women visiting an outpatient gynecology clinic for a variety of complaints. In addition, the distribution of *C. trachomatis* serovars in these groups of women was studied.

MATERIALS AND METHODS

Populations. Two different populations of women were investigated for the prevalence of *C. trachomatis* cervical infections. Group I consisted of young women (n = 393; aged 17 to 30 years) who were invited to participate in a *C. trachomatis* screening study in and around Amsterdam. They were confirmed as being asymptomatic after extensive interviewing and careful physical examination. Group II consisted of randomly selected patients (n = 734; aged 17 to 68 years) attending an inner-city gynecology outpatient clinic (OLVG Hospital,

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Amsterdam) for various gynecological reasons. In both groups, only women with normal cervical cytology were admitted into the study.

Sample collection and processing. Cervical scrapes were collected with cervix brushes (Rover B.V., Oss, The Netherlands), placed in 5 ml of phosphate-buffered saline, and transported to the laboratory. The brushes were removed after thorough vortexing. The samples from group I were centrifuged at 14,000 $\times$ *g* for 30 min in order to additionally spin down extracellular elementary bodies of *C. trachomatis*. The pellets were resuspended in 1 ml of 10 mM Tris-HCl (pH 7.5) and were stored at −80°C. The samples from women in group II had been collected and processed in the past for another study and were stored at −80°C until use. The processing of these samples was identical to samples from group I, except that the samples were centrifuged at 4,000 $\times$ *g* for 10 min. Ten microliters of the freeze-thawed samples were used for PCR detection.

PCR detection of *C. trachomatis*. To examine whether either amplifiable DNA or PCR inhibitors were present, the samples were first screened by a PCR specific for the human β-globin gene (11). Samples positive in the β-globin PCR were used for *C. trachomatis* detection by using a plasmid PCR performed as described previously (7, 9). The amplification conditions were the same for these PCRs. Briefly, 10 μl of the freeze-thawed samples was boiled for 10 min and chilled on ice. The primers (flanking a region of 209 bp) used for the human β-globin PCR were BGPCO3-1 (+5′-ACACAACTGTGTTCACTAGC-3′) and BGPCO5 (−5′-GAAACCCAAGAGTCTTCTCT-3′) (11). The primers (flanking a region of 201 bp) used for the *C. trachomatis* plasmid PCR were CTP1 (+5′-TAGTAACTGCCACTTCATCA-3′) and CTP2 (−5′-TTCCCCTTGTAA TTCGTTGC-3′) (7, 9). PCR was performed in a final volume of 50 μl containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 200 μM (each) deoxynucleoside triphosphate (dATP, dTTP, dGTP, and dCTP), 50 pmol of each primer, and 1 U of *Taq* polymerase (Amplitaq; Perkin-Elmer, Branchburg, N.J.). The reaction mixture was overlaid with a few drops of liquid paraffin to prevent evaporation. *C. trachomatis* serovar L2 DNA was used as a positive control. Samples containing distilled water instead of DNA were used as negative controls. These negative controls were also placed after each five samples in order to check for cross-contamination between samples during the performance of the PCR. The amplification was performed with a thermocycler (Biomed, Theres, Germany) and started with 4 min of denaturation at 95°C; this was followed by 40 cycles of amplification. Each cycle consisted of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and chain elongation at 72°C for 1.5 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis, and the chlamydial plasmid PCR products were subsequently analyzed by Southern blot hybridization with a specific internal oligonucleotide probe (plus strand; 5′-ATCTCATTA CCATGCATTAGCAGCTATCCA-3′) end labeled with [γ-^{32}P]ATP (9, 10).

Genotyping of *C. trachomatis*. Samples that were found to be positive by plasmid PCR were subjected to *omp1* PCR-based RFLP genotyping. As described previously (9, 10), the primers used for generating an approximately 1.1-kb fragment of the *omp1* gene were SERO1A (+5′-ATGAAAAAAC TCTTGAAATCGG-3′) and SERO2A (−5′-TTTCTAGA[T/C]TTCAT[T/C]TT GTT-3′). The PCR amplification was started with 6 min of denaturation at 95°C and continued with 49 cycles of amplification. Each cycle consisted of a denaturation step at 95°C for 1 min, an annealing step at 45°C for 3 min, and a chain elongation step at 72°C for 3 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis. In case of negative amplification by *omp1* PCR, an *omp1* nested PCR was performed in order to improve the sensitivity (9). This nested PCR was performed with an outer primer set consisting of NLO (+5′-ATGAAAAAACTCTTGAAATCG-3′) and NRO (−5′-CTCAACTGTAAC TGCGTATTT-3′); this was followed by a PCR with the nested primers PCTM3 (+5′-TCCTTGCAAGCTCTGCCTGTGGGGAATCCT-3′) and SERO2A. For nested PCR, 1 μl of the NLO and NRO primer-directed primary PCR product was pipetted into a prepared nested PCR mixture by using a filter-plugged tip. The amplification condition was identical to that of the *omp1* PCR. When the amplified fragments after either the primary PCR or the nested PCR were visible at the gel level, the products were subjected to RFLP analysis as described previously (9, 10). Briefly, the PCR products were firstly digested with *Alu*I to differentiate serovars B, Ba, D, E, F, G, and K and serovars of the C complex (C, J, H, I, and L3). Serovars belonging to the C complex were further typed by RFLP analysis with *Hin*fI and the combination of *Eco*RI and *Dde*I. Serovar D was further differentiated into D, D^{-}, or Da by restriction site analysis with *Cfo*I. In addition, when the nested PCR products were not visible at the gel level, the digested PCR products were further examined by additional Southern blot hybridization to improve the sensitivity of RFLP analysis. The probe used consisted of *omp1* PCR products derived from 15 *C. trachomatis* reference strains; the PCR products were random primer labelled with [α-^{32}P]dCTP (10).

RESULTS

All samples tested in the study were suitable for *C. trachomatis* plasmid PCR, as shown by the successful amplification of 209 bp of the human β-globin gene. The overall prevalence rates of *C. trachomatis* in cervical scrapes determined by plasmid PCR were 9.2 and 7.9% in groups I and II, respectively (Table 1). However, when the samples within corresponding age groups (<30 years of age) were analyzed, the prevalence rates became 9.2 and 11.8% in groups I and II, respectively. The prevalence rates in women in their early 20s (20 to 24 years) and late 20s (25 to 29 years) were similar, with prevalence rates of 8.4 and 9.1%, respectively, in group I, and 9.1 and 10.6%, respectively, in group II. In group II a clear age dependency was observed, showing that the *C. trachomatis* prevalence rate was high among patients younger than 30 years, with the highest rate (approximately 20%) found in women younger than 20 years. The *C. trachomatis* prevalence rate declined significantly to an average of 5.9% in women older than 30 years. Table 1 summarizes the rates of prevalence of *C. trachomatis* in cervical scrapes in relation to age.

TABLE 1. Prevalence of cervical *C. trachomatis* infection in relation to age in two different populations determined by PCR[a]

Age (yr)	Group I			Group II		
	No. of samples tested	No. CT+ by gel/by SB[b]	Prevalence (%)	No. of samples tested	No. CT+ by gel/by SB[b]	Prevalence (%)
<20	18	2/2	11.1	45	6/9	20
20–24	154	9/13	8.4	88	5/8	9.1
25–29	231	14/21	9.1	113	7/12	10.6
Total	393	25/36	9.2	246	18/29	11.8
30–34				95	3/7	7.4
35–39				100	3/5	5.0
40–44				100	0/6	6.0
45–49				110	2/8	7.3
>50				83	0/3	3.6
Total				734	26/58	7.9

[a] Group I consisted of asymptomatic young women who were invited to participate in a *C. trachomatis* screening. Group II consisted of patients attending an outpatient clinic, OLVG Hospital, in the inner city of Amsterdam.

[b] CT+, *C. trachomatis* positive. The organism was detected by *C. trachomatis* plasmid PCR after gel (gel) and Southern blot (SB) analyses.

The intensities of PCR products, as analyzed at the gel level and after Southern blot analyses, differed between those derived from young women and those derived from older women. In group I, 25 samples were found to be *C. trachomatis* positive by PCR, as detected at the gel level, while another 11 samples were positive only after Southern blot analysis. In group II, 26 samples were positive by PCR, as detected at the gel level, and another 32 samples were positive after Southern blot analysis. The number of samples with strong PCR signals decreased when the age of the women increased (Table 1).

The *C. trachomatis*-positive scrapes detected by plasmid PCR were further examined by *omp1* PCR for additional genotyping, and the results are summarized in Table 2. By using chlamydial *omp1* PCR, 21 of 36 (58%) plasmid PCR-positive samples in group I and 30 of 58 (52%) samples in group II were positive and were genotyped by RFLP analysis. Three of the 21 samples of group I were positive after the nested PCR. In group II, 5 of the 30 samples were positive after the nested PCR. As shown by the corresponding intensity of the PCR products as detected either by gel electrophoresis or by Southern blot hybridization, the positivity of the *omp1* PCR correlated well with the positivity of the plasmid PCR. Of the samples weakly positive by plasmid PCR, as detected by additional hybridization, only a few were positive by nested *omp1* PCR. Among women younger than 30 years, serovars E, I, D, and F were found in decreasing order in group I, whereas serovars F, E, and G were found in decreasing order in group II. The

TABLE 2. *C. trachomatis* serovar distributions in relation to age in different populations determined by *omp1* PCR-based RFLP genotyping

Group and age interval (yr)[a]	No. of *C. trachomatis* isolates of serovar:									
	Ba	D	E	F	G	H	I	J	K	Total
Group I										
<20				1						1
20–24	1	2				2	2			7
25–29		2	7	2			2			13
Total	1	4	7	3		2	4			21
Group II										
<20			1	1	1	2		1		6
20–24			3	2	1		1			7
25–29	1	1		3	1				1	7
30–34				2	1		1			4
35–39	1			1		1	1			4
40–44	1									1
45–49				1						1
Total	3	1	4	10	4	3	3	1	1	30

[a] Group I consisted of asymptomatic young women invited to participate in a *C. trachomatis* screening study. Group II consisted of patients attending a gynecologic outpatient clinic.

serovar distributions found in the two populations studied are given in Fig. 1. There was a tendency for serovars D and I to be found more frequently in asymptomatic women and for serovar G to be found more frequently in symptomatic patients.

DISCUSSION

The present study demonstrated that the prevalence of asymptomatic *C. trachomatis* infections in cervical scrapes, as determined by a sensitive PCR assay, is high and age dependent. In the present study, a prevalence rate of 9.2% was found in asymptomatic young women (<30 years of age in group I). Comparison of the gynecological outpatients (group II) with the confirmed asymptomatic women (groups I) in a corresponding age group younger than age 30 years revealed a slightly higher rate of prevalence of *C. trachomatis* infection in the outpatient group (11.8%). This is probably due to the presence of symptomatic women in this population. Indeed, through analyses of the patient files of *C. trachomatis*-positive patients in this group, it was shown that about 47% of these women were clinically symptomatic (abnormal vaginal discharge, abnormal and irregular bleeding, pelvic pain, adnexitis).

The highest *C. trachomatis* prevalence rate (approximately 20%) was found in women younger than 20 years of age (group II), and 80% of these positive women presented with signs or symptoms suggestive of chlamydial infection (see above). However, the prevalence rate in age-matched asymptomatic women (group I) was still 11%. These data are in agreement with previously published reports, showing high *C. trachomatis* prevalence rates (up to 23%) among sexually active adolescent women attending gynecology clinics (7a) and also confirm the high prevalence (7.3 to 12.4%) found among asymptomatic women (20, 21). These asymptomatic young women (and their male partners), at their sexually active age, form an important reservoir for *C. trachomatis* transmission. When untreated, the asymptomatic women are possibly at risk of developing PID, ectopic pregnancy, and tubal infertility. In addition, the finding that women in their late 20s (25 to 29 years of age) had a prevalence rate of chlamydial infections similar to that of women in their early 20s (20 to 25 years of age) was in contrast to other studies showing a decline of the prevalence rates after 25 years of age (20, 21). Besides differences in the population investigated, another explanation might be that in the present study we used a highly sensitive PCR which is capable of detecting infections with low copy numbers of *C. trachomatis*. Indeed, weakly positive samples were more often observed in women older than 25 years than in women younger than 25 years. These weakly positive samples might have been undetected by other microbiological assays (5, 8, 12–14, 21, 28).

Additional *C. trachomatis* genotyping in women younger than age 30 years revealed that the serovar distributions were primarily different in the two groups investigated (Fig. 1). Serovars E, I, and D were more often found in group I than group II, while serovars F and G were more frequently found in group II than in group I. In both groups, about 30% of the samples were weakly positive in the plasmid PCR and were detected after Southern blot analysis. These samples were not amplifiable by the *omp1* PCR. This might be due to DNA fragmentation during storage of the samples in Tris-HCl buffer, probably resulting in decreasing numbers and shorter fragments of target DNA for *omp1* PCR amplification. This was shown by the finding that in a β-globin PCR DNA fragments larger than 500 bp could not be amplified in these samples (data not shown). Nevertheless, the serovar distributions analyzed in the *omp1* PCR-positive samples showed that these were not age dependent. The most striking differences observed were that serovars D and I were most prominent in group I (the asymptomatic population) and that serovar G was found only in group II. Indeed, examination of the case histories of these patients with serovar G infections (Table 2) revealed that they were all symptomatic patients. The other serovars were distributed among symptomatic and asymptomatic women in group II without noticeable differences. Data concerning the different degrees of virulence of different serovars are still limited. Batteiger et al. (2) reported that infection with serovar F strains is often associated with prominent local inflammation in the cervix, whereas infection with serovar D strains is less associated with local inflammation. Moreover, serovar F was found to be associated with fewer symptoms in women with endocervical infections or PID (26). Alternatively, these differences in the *C. trachomatis* serovar distributions that we found might reflect the different compositions of the study groups. Workowski et al. (27) found that there was a higher incidence of serovar Ia (a variant of serovar I) and a lower incidence of serovar D among black Americans than among non-black Americans, suggesting that either behavioral

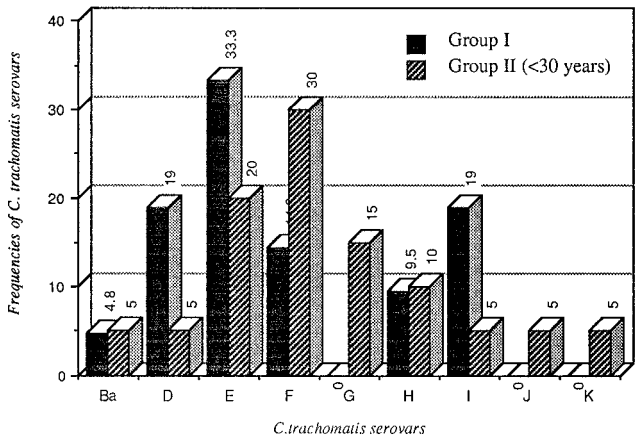

FIG. 1. *C. trachomatis* serovar distributions in younger women (<30 years) in group I (asymptomatic group) and group II (gynecology outpatients).

or biologic factors of *C. trachomatis* were involved. In another study, serovar D was more often detected in the recta of homosexual men than in the cervices of heterosexual women, and an opposite observation was made for serovar E (1).

In conclusion, the present study showed that the prevalence of asymptomatic *C. trachomatis* infections in young women is approximately 9%. Certain serovars (e.g., serovars D and I) might be associated with asymptomatic infection, while others (e.g., serovar G) might be associated with symptomatic infection. This raises the question of whether women infected with possible asymptomatic serovars will develop complications, i.e., PID, tubal infertility, or ectopic pregnancy and, if not, whether these women should be treated. Moreover, it will be interesting to determine whether different *C. trachomatis* serovars differ in their susceptibilities to antibiotic treatment. Studies dealing with these challenging areas are in progress.

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