Genotyping of *Chlamydia trachomatis* from the endocervical specimens of high-risk women in Hungary

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The distribution of different *Chlamydia trachomatis* serovars in Hungary has not been reported previously. The objective of this study was to determine the distribution and prevalence of *C. trachomatis* serovars in a high-risk population by genotyping. The endocervical specimens of 484 female sex workers (FSWs) were screened for *C. trachomatis* by plasmid PCR. Genotyping was performed in all *C. trachomatis*-positive samples by PCR-based RFLP analysis of the *omp1* gene. A total of 32 specimens (6.6 %) were positive for *C. trachomatis*. Age was an important risk factor for *C. trachomatis* infection in FSWs. The highest prevalence was detected in women under the age of 20 (18.8 %). All positive specimens were successfully genotyped and seven serovars were identified. The most prevalent was serovar D (34.4 %), followed by E (21.9 %), F (18.8 %), G (9.4 %), J (9.4 %), H (3.1 %) and I (3.1 %). A heterogeneous distribution of *C. trachomatis* serovars was observed in the study group, where the most common serovars were D, E and F comprising 75 % of the positive samples. This PCR-based RFLP method could be used in epidemiological studies on the prevalence of *C. trachomatis* infection to provide more information and to compare the serovar distribution among different cohorts.

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INTRODUCTION

Chlamydia trachomatis infection is one of the most prevalent sexually transmitted infections (STIs) worldwide. The estimated global incidence of new cases of *C. trachomatis* infection is about 92 million, in western Europe it is 5 million, and the asymptomatic occurrence is 50 and 70 % in men and women, respectively (WHO, 2001).

The immunodominant surface antigen of *C. trachomatis* is the major outer-membrane protein (MOMP) (Caldwell *et al.*, 1981). The *omp1* gene encoding the MOMP consists of four variable domains (VD1–4), which are flanked and interspaced by five constant domains. The nucleotide sequence polymorphisms in the variable domains account for the different antigenic properties of *C. trachomatis* serovars (Yuan *et al.*, 1989). *C. trachomatis* strains have been classified into at least 15 different serovars based on immuno-epitope analysis of the MOMP with polyclonal and monoclonal antibodies (Caldwell *et al.*, 1981; Wang *et al.*, 1985; Yuan *et al.*, 1989). Additionally, on the basis of amino acid sequence homology, the 15 serovars of *C. trachomatis* have been classified into 3 groups: group B (B,

D, E, L1 and L2), group C (A, C, H, I, J, K, L1 and L3) and an intermediate group (F and G) (Yuan *et al.*, 1989).

Serovars A–C are associated with trachoma, which is hyperendemic in Africa, Asia, and Central and South America, and the second major cause of blindness in the world (Thylefors *et al.*, 1995). Serovars D–K are commonly associated with urogenital infections and perinatal conjunctivitis or pneumonia worldwide (Numazaki, 2004; Paavonen & Eggert-Kruse, 1999). In women, a broad spectrum of clinical manifestations can be present due to urogenital *C. trachomatis* infections including urethritis and cervicitis, and, if left untreated, the ascending infection may cause secondary complications in the upper genital tract, including pelvic inflammatory disease and infertility (Paavonen & Eggert-Kruse, 1999; Paavonen & Lehtinen, 1996). Serovars L1–L3 are associated with lymphogranuloma venereum, a systemic disease most prevalent in tropical and subtropical areas. Nevertheless, several outbreaks among homosexual male populations in western countries have been reported recently, indicating the emerging role of lymphogranuloma venereum (Van de Laar, 2006).

The genotyping methods used recently in many studies for the identification of *C. trachomatis* serovars have mainly involved PCR-based RFLP and sequencing of the *omp1*

Abbreviations: FSW, female sex worker; MOMP, major outer-membrane protein; STI, sexually transmitted infection.

gene (Dean *et al.*, 1995; Gao *et al.*, 2007; Lan *et al.*, 1993, 1995; Lima *et al.*, 2007; Lysén *et al.*, 2004; Morré *et al.*, 2000a; Ngandjio *et al.*, 2004; Sturm-Ramirez *et al.*, 2000; Van Duynhoven *et al.*, 1998). Serovar determination of *C. trachomatis* by different typing methods is a challenging area of chlamydial research. The geographical distribution and prevalence of different serovars give insights into the molecular epidemiology of *C. trachomatis* and typing may reveal the circulation of *C. trachomatis* serovars and variants in a certain community (Lysén *et al.*, 2004; Sturm-Ramirez *et al.*, 2000). The possible relationship between urogenital serovars and clinical manifestations has also been studied, but the results are controversial. Some studies have found associations between clinical symptoms and certain serovars (Dean *et al.*, 1995; Morré *et al.*, 2000a; Van Duynhoven *et al.*, 1998), whilst others have concluded that urogenital infections are not strongly influenced by serovars (Geisler *et al.*, 2003; Lysén *et al.*, 2004). Newer molecular approaches, in particular amplified fragment length polymorphism analysis, a fingerprinting technique (Morré *et al.*, 2000b), and different multi-locus sequence typing methods, have also been developed to study the genomic differences in *C. trachomatis* serovars and to gain more information on their association with clinical disease (Klint *et al.*, 2007; Pannekoek *et al.*, 2008).

In Hungary, limited information is available on *C. trachomatis* prevalence in certain populations and there are no data about the circulating serovar distribution (Deák *et al.*, 1997; Ujházy *et al.*, 2007). The aims of the present study were to determine and characterize the prevalence of *C. trachomatis* serovars in a high-risk population by the genotyping method of *omp1* PCR-based RFLP analysis.

METHODS

C. trachomatis strains. Extracted DNA samples from *C. trachomatis* strains of different serovars were kindly provided by Dr H. Jalal (Addenbrooke's Hospital, Cambridge, UK) for use as reference controls as described in a published study (Jalal *et al.*, 2006).

Clinical specimens. Endocervical samples were obtained from 484 street-based female sex workers (FSWs) attending a mobile clinic during an anonymous screening in Budapest and the suburban area from January 2006 to July 2006. The specimens were collected with Dacron-tipped swabs and transported to the laboratory. The swabs were placed into sterile Eppendorf tubes containing 400 µl transport medium (Bartels FlexTrans; Trinity Biotech). After incubation at 4 °C for 24 h, the swabs were removed following vortexing and the samples were stored at -20 °C until further analysis.

DNA extraction. The endocervical samples were centrifuged at 10 000 g for 10 min, the pellets were resuspended in PBS and the DNA was extracted using a High Pure DNA template preparation kit (Roche Diagnostics) according to the manufacturer's instructions. The elution step with 75 µl elution buffer was repeated once and the extracted DNA was stored at -20 °C.

PCR detection of *C. trachomatis*. The samples were first screened by a PCR specific for the human β -globin gene to act as a control for

the efficacy of DNA extraction and the presence of PCR inhibitors using primers PC04 (GenBank accession no. A26623) and GH20 (GenBank accession no. A26624). For the detection of *C. trachomatis* DNA in the samples, a diagnostic PCR was used to amplify a 495 bp fragment of the cryptic plasmid gene of *C. trachomatis* with slightly modified primers of CTpl1 and CTpl2 described by Ossewaarde *et al.* (1992) (Table 1). The PCR was performed in an Eppendorf thermocycler, in a volume of 20 µl containing 10 × ReddyMix buffer (ABgene), 200 µM dNTPs, 0.5 µM each primer and 0.5 U ThermoPrime *Taq* DNA polymerase (ABgene). The PCR conditions consisted of a denaturation step at 95 °C for 4 min, followed by 40 cycles of amplification at 95 °C for 30 s, 64 °C for 30 s and 72 °C for 45 s, with an elongation step at 72 °C for 4 min. The PCR products were visualized on a 1 % agarose gel stained with ethidium bromide.

Genotyping. *C. trachomatis*-positive samples were selected for *omp1* PCR-based RFLP genotyping. The primers used for the *omp1* PCR have been described by Lan *et al.* (1993, 1995) (Table 1). Briefly, a semi-nested PCR was performed with the outer primer set consisting of NLO and NRO, with 3 µl extracted DNA sample added to the reaction mixture in a final volume of 20 µl. Following amplification, 5 µl of the primary PCR product was used for the nested PCR in a final volume of 50 µl. The primers used for the second PCR run were NLO and SERO2A, generating a fragment of the *omp1* gene of approximately 1070 bp. Both amplification conditions consisted of a denaturation step at 95 °C for 4 min, 45 cycles of 95 °C for 30 s, 45 °C for 50 s and 72 °C for 60 s, and a final elongation step at 72 °C for 6 min. The *omp1* nested PCR products were visualized by 1 % agarose gel electrophoresis.

For RFLP analysis, 10 µl of the PCR products was digested with *AluI* (Promega). The restriction patterns of the products were analysed by 1.5 % agarose gel electrophoresis. Serovars belonging to group C were further digested with *HinfI*, *EcoRI* and *DdeI* (Promega). The variants of serovar D were differentiated by restriction with *CfoI* (Promega).

In silico RFLP analysis. The sequences of known reference *C. trachomatis* strains, determined by searching GenBank, were analysed by using NEBcutter version 2.0 software (<http://tools.neb.com/NEBcutter2/index.php>). The following strain sequences were used: D/IC-Cal8 (GenBank accession no. X62920), E/Bour (X52557), F/IC-Cal3 (X52080), G/UW57 (AF063199), H/UW4 (X16007), I/UW12 (AF063200), J/UW36 (AF202457) and K/UW31 (AF063204). These reference sequences, appropriate for the *omp1* PCR product, were pasted into NEBcutter version 2.0 software and the *in silico*-obtained length of the restriction bands were compared with the gel-based genotyping results.

Table 1. Primer sequences used for the *C. trachomatis* plasmid PCR and the *omp1* PCR

Primer	Sequence (5'→3')	Position (nt)
CTpl1*	GGCTATTTCTCTTGACCACAGC	329–350
CTpl2*	TACTCTCCCATTTCTCCACA	804–824
NLO†	ATGAAAAAACTCTTGAAATCG	1–21
NRO†	CTCAACTGTAACCTGCGTATTT	1108–1128
SERO2A†	TTTCTAGATTTTCATCTTGTT	1045–1064

*According to the sequence of the *C. trachomatis* cryptic plasmid gene (GenBank accession no. X06707).

†According to the *omp1* sequence of *C. trachomatis* serovar E (GenBank accession no. X52557).

Table 2. Positive results for *C. trachomatis* in relation to age ($n=275$)

Age group (years)*	Observation of positive test		Prediction of positive test†	
	<i>n</i>	Percentage	Percentage	95 %CI
<20	3/16	18.8	31.0	24.5–37.4
20–29	17/180	9.4	10.0	7.3–12.7
39–30	3/62	4.8	2.6	1.8–3.4
49–40	0/13	0	0.6	0.4–0.8
>50	0/4	0	0.2	0.1–0.2
Total	23/275	8.4	8.9	5.3–12.4

*No age data were available for 209 women.

†Age effect: $P<0.001$.

CI, Confidence interval.

Statistical analysis. Logistical regression analysis was used to assess the effect of age on the probability of *C. trachomatis* infection. A comparison between predicted and observed probabilities was carried out to evaluate the validity of the fitted model. Data analysis was performed with SAS version 9.1 software.

RESULTS AND DISCUSSION

The efficiency of the PCR-based RFLP method for the genotyping of different *C. trachomatis* serovars in clinical specimens has been proven by many studies (Gao *et al.*, 2007; Lan *et al.*, 1993, 1995; Morr *et al.*, 2000a; Ngandjio *et al.*, 2004; Van Duynhoven *et al.*, 1998). To the best of our knowledge, this is the first study to determine the serovar distribution of *C. trachomatis* in a particular population in Hungary.

Prevalence of *C. trachomatis*

A total of 484 endocervical specimens from FSWs was analysed in the study. All samples were successfully screened by β -globin PCR and no inhibition was detected. Of the 484 samples, 32 (6.6%) were found to be positive by *C. trachomatis* cryptic plasmid PCR. FSWs are regarded as a high-risk group for infection and transmission of *C. trachomatis* and other STIs. In a review article by Cwikel *et al.* (2008), it was pointed out that the prevalence of *C. trachomatis* in FSWs varies between 0.61 and 46.2% worldwide. According to these data and other studies (Folch *et al.*, 2008; Mak *et al.*, 2005; Papadogeorgaki *et al.*, 2006; Rabenau *et al.*, 2000; Resl *et al.*, 2003; Ward *et al.*, 2004), this rate usually appears to be less than 10% in Europe (Belgium 7.3%, Czech Republic 5.5%, Germany 9.6%, Greece 5.9%, Spain 5.9%, UK 4.8%) as detected by nucleic acid amplification tests, and these findings correspond with our results.

Data on age were obtained from 275 individuals. The FSWs were aged from 18 to 59 years and the mean age was 27.5

years. A significant association was observed between FSW age and the prevalence of *C. trachomatis* infection (Table 2). Younger women had a higher prevalence rate, indicating that age is an important risk factor for chlamydial infection. These results are comparable to other studies showing the same age effect in relation to infection in different cohorts such as FSWs, pregnant women, patients of STI clinics and asymptotically infected women (Dek *et al.*, 1997; Lan *et al.*, 1995; Lima *et al.*, 2007; Papadogeorgaki *et al.*, 2006).

Genotyping of *C. trachomatis*

For genotyping purposes, the *C. trachomatis*-positive samples were analysed further by an *omp1* PCR. All samples positive for the plasmid PCR were successfully amplified by the semi-nested PCR method and were suitable for further genotyping analysis. The DNA controls for reference *C. trachomatis* strains were also successfully amplified and genotyped.

Among the positive samples, seven different serovars of *C. trachomatis* were identified by RFLP genotyping, showing the same restriction patterns as the control strains. Serovars D, E, F and G were differentiated by *AluI* digestion. The group C serovars with similar *AluI* restriction patterns were further distinguished by *EcoRI* digestion, which differentiates serovars H, I and J from serovar L3. Restriction digestion with *DdeI* and *HinFI* differentiated serovars H, I and J. The *CfoI* digestion of samples identified as serovar D showed a distinct restriction pattern in one sample, which referred to the D⁻ variant identified by Lan *et al.* (1993).

In our study population, a heterogeneous distribution of *C. trachomatis* serovars was observed. All serovars belonging to the D–K urogenital group could be identified except for serovar K, which was not detected. Serovar D was the most prevalent (11/32, 34.4%), followed by E (7/32, 21.9%), F (6/32, 18.8%), G (3/32, 9.4%), J (3/32, 9.4%), H (1/32, 3.1%) and I (1/32, 3.1%). The *in silico* RFLP analyses of the GenBank reference sequences of *C. trachomatis* strains correlated well with the gel-based restriction patterns of the control strains as well as those of the samples. The actual trend in serovar distribution was similar to previous findings. According to these results, the three most frequently observed serovars varied among D, E, and the intermediate group serovars (F and G), according to different typing methods worldwide (Gao *et al.*, 2007; Lan *et al.*, 1993, 1995; Lima *et al.*, 2007; Lysn *et al.*, 2004; Morr *et al.*, 2000a; Ngandjio *et al.*, 2004; Sturm-Ramirez *et al.*, 2000). In our study, 75% of the positive specimens comprised the serovars D, E and F in agreement with other findings, indicating that most genital infections with *C. trachomatis* are caused by a small number of serovars.

The present study had some limitations that should be acknowledged. The occurrence of false-positive results during the plasmid PCR should have been overcome, as all positive samples were confirmed with the *omp1* PCR

performed for genotyping. Nevertheless, the true prevalence of *C. trachomatis* in the study group may have been rather underestimated. Although the possibility of the presence of inhibitors in the samples was well controlled with the β -globin PCR, the plasmid PCR was not confirmed using other methods, e.g. Southern blot hybridization, and so could have resulted in false-negative results. However, the obtained prevalence rate in this study was similar to those observed in FSW populations in other European countries. Furthermore, our findings on serovar distribution correspond to other studies from different geographical regions worldwide. The prevalence of different *C. trachomatis* serovars has been examined in different cohorts comparing it to risk groups (FSWs and non-FSWs), gender and clinical symptoms, and the most prevalent serovars were also D, E and F/G (Gao *et al.*, 2007; Geisler *et al.*, 2003; Morré *et al.*, 2000a; Van Duynhoven *et al.*, 1998). In addition, the *in silico* analysis of RFLP patterns of the reference strains showed that this method could be a good alternative tool for characterizing *C. trachomatis* serovars if control strains are not available in the laboratory.

In conclusion, to the best of our knowledge, the present study is the first description of *C. trachomatis* serovar distribution in a high-risk group in Hungary. The predominating serovars were D, E and F, as has been reported from other countries. A higher prevalence of *C. trachomatis* infection was found among younger women. This PCR-based RFLP analysis is a valuable molecular method for investigating the prevalence of *C. trachomatis* serovars in epidemiological surveys. Further studies are required to compare the distribution of *C. trachomatis* serovars in different cohorts as well as in the general population, and to investigate the correlations between serovars and clinical manifestations in Hungary.

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