Characterization of *Chlamydia trachomatis omp1* Genotypes among Sexually Transmitted Disease Patients in Sweden

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A method for detection and genotyping of genital *Chlamydia trachomatis* infections based on *omp1* gene amplification and sequencing was developed. DNA was extracted from urogenital or urine samples using a Chelex-based method, and an approximately 1,100-bp-long fragment from the *omp1* gene was directly amplified and sequenced. Genotyping was performed by BLAST similarity search, and phylogenetic tree analysis was used to illustrate the evolutionary relationships between clinical isolates and reference strains. The method was used to determine the genotypes of *C. trachomatis* in 237 positive urogenital and/or urine specimens collected at a Swedish sexually transmitted disease clinic during 1 year. The most common genotypes corresponded to serotypes E (47%) and F (17%). The *omp1* gene was highly conserved for genotype E (106 of 112 samples without any mutation) and F (41 of 42 samples without any mutation) strains but appear slightly less conserved for genotypes G ($n = 6$) and H ($n = 6$), where the sequences displayed one to four nucleotide substitutions relative to the reference sequence. Genotyping of samples collected at the follow-up visit indicated that two patients had become reinfected, while three other patients suffered treatment failure or reinfection. One woman appeared to have a mixed infection with two different *C. trachomatis* strains. This *omp1* genotyping method had a high reproducibility and could be used for epidemiological characterization of sexually transmitted *Chlamydia* infections.

Sexually transmitted infection with *Chlamydia trachomatis* is a common treatable urogenital infection in young adults worldwide and is associated with a spectrum of clinical diseases, including urethritis and epididymitis among men and cervicitis, salpingitis, and pelvic inflammatory disease among women (4, 10, 21). In Sweden, reporting gonorrhea and syphilis has been mandatory under the Communicable Diseases Act since 1919, and reporting genital infection with *C. trachomatis* has been mandatory since April 1988. The incidence of genital chlamydial infections declined in all Swedish counties until 1996. It has been suggested that this decline was due to contact tracing, screening, and treatment of asymptomatic men and women (10), but data from the Swedish Institute for Infectious Disease Control show that genital infections with *C. trachomatis* are now again increasing. The incidence was 172 cases per 100,000 inhabitants in 1998 and 217 cases per 100,000 inhabitants in 2000 (27).

Characterization of *C. trachomatis* strains can provide valuable information about the variants circulating in the community, and with better knowledge of the epidemiology of *Chlamydia* infection, efforts against spread can probably be more effective. Serotyping with monoclonal antibodies recognizing antigenic determinants located on the major outer membrane protein (MOMP) is the reference method for typing *C. trachomatis* isolates (12, 29, 30). The MOMP is the immunodominant

antigen of *C. trachomatis* and contains four variable domains (VDI to VDIV) that are flanked and interspaced by five constant domains (30). In order to study the epidemiology of *C. trachomatis* infections, new methods—such as PCR, restriction fragment length polymorphism, and sequencing of the *omp1* gene, which encodes the MOMP protein—have recently been described (7, 9, 11, 12, 17, 20, 23, 25).

The aim of the present study was to establish a PCR method for genetic characterization of clinical *C. trachomatis* isolates in a Swedish population by sequence analysis of the *omp1* gene.

MATERIALS AND METHODS

Clinical samples and strains. Urogenital and/or urine samples for diagnosis of *C. trachomatis* were prospectively obtained from all new attendees ($n = 2,195$) of the Outpatient Sexually Transmitted Disease (STD) Clinic, Örebro Medical Centre Hospital, Örebro, Sweden, during 1 year (1 March 1999 to 29 February 2000). The mean age for men ($n = 1,141$) was 28.5 (range, 14 to 68) years, and the mean age for women ($n = 1,054$) was 25.7 (range, 13 to 59) years.

Urethral or endocervical specimens for tissue culture were obtained from males and females, respectively, using sterile Dacron swabs. Swabs were placed into transport medium containing sucrose-phosphate buffer, 5% fetal bovine serum, and antibiotics (2SP medium) and were directly transported to the laboratory and stored at −70°C until processed for tissue culture. At the same examination, first-void urine samples (5 to 10 ml) from both men and women were collected and stored at 2 to 8°C in a sterile screw-cap plastic tube. The first 779 urine samples were tested by the *Chlamydia trachomatis* Amplicor PCR (Roche Diagnostic Systems, Inc., Branchburg, N.J.), and the remaining samples were tested by the COBAS Amplicor *Chlamydia trachomatis* Test (Roche Diagnostic Systems) due to changed diagnostic screening PCR methods during the period of the study. All *C. trachomatis*-positive patients were treated with appropriate antibiotics and requested to come for a checkup visit 4 to 5 weeks after the initial sample was obtained.

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A total of 240 specimens were found to be *C. trachomatis* positive by tissue culture and/or by the Amplicor PCR or COBAS Amplicor test. Two samples were lost, while 238 were stored at −20°C until used in the study. The first choice for the *omp1* PCR was the urogenital specimen (*n* = 190), and the second choice was the urine sample (*n* = 48), if the urogenital sample was negative or not obtained. One *C. trachomatis*-negative patient sample per every *C. trachomatis*-positive sample was randomly selected each day, and these were used as negative controls in the study. Twenty-four patients who were epidemiologically highly suspected of having a *C. trachomatis* infection, but whose diagnostic tests for *C. trachomatis* were negative, were also included in the study.

The following *C. trachomatis* reference strains were used for optimization of PCR and DNA sequencing: serotypes A/HAR-1/OT, B/TW-5, Ba/AP-2/OT, C/UW-1/OT, D/ICCAL-8/ON, E/DK-20/ON, F/MRC-301/GU, G/IOL-238/R, H/UW-4/GCx, I/UW-12/GU, J/UW-36/GCx, K/UW-31/GCx, L1/440 Bu, L2/434 Bu, and L3/404 Bu, as well as *Chlamydia pneumoniae* strain IOL-207 and *Chlamydia psittaci* strain 6BC. A *C. trachomatis* serotype E strain, used as a control in the diagnostic tissue culture and PCR analyses above (provided from Department of Microbiology, Halmstad Hospital, Halmstad, Sweden), was used as a positive control in each PCR run. All the reference strains were originally from the Institute of Ophthalmology, London, United Kingdom.

Isolation of DNA. DNA was isolated directly from the clinical samples and the reference strains using Chelex 100 resin (catalog no. CA 94547; Bio-Rad Laboratories) (28). A volume of 100 μl from the clinical specimens (in 2SP medium) for tissue culture or a pellet from 1,000 μl of urine was washed in distilled water for 30 min at room temperature and microcentrifuged for 5 min at 18,000 × *g*. The pellet was resuspended to a final volume of 200 μl in distilled water and mixed with 2 μl of 10-mg/ml proteinase K (catalog no. P6556; Sigma) and incubated for 1 h at 37°C. The tubes were then microcentrifuged for 5 min at 18,000 × *g*, and the pellet was resuspended in 200 μl of 5% Chelex 100 resin, thoroughly mixed and incubated at 56°C for 30 min, mixed again, and incubated in boiling water for 8 min. The cell debris was pelleted by centrifugation at 10,000 × *g* for 3 min, and the supernatant containing the DNA was withdrawn and stored at 4°C until used.

omp1 PCR. The optimized *omp1* PCR was carried out as follows. The DNA preparation (10 μl) was added to the PCR mixture (40 μl) containing 1.5 U of *AmpliTaq*Gold DNA polymerase (PE Biosystems, Branchburg, N.J.), 1× PCR buffer (PE Biosystems), 2.0 mM MgCl₂, a 200 μM concentration of each deoxynucleoside triphosphate (PE Biosystems), and a 1.0 μM concentration of each primer (Scandinavian Gene Synthesis AB, Köping, Sweden): primer P1 (5′-ATG AAA AAA CTC TTG AAA TCG G-3′) (7) and primer OMP 2 (5′-ACT GTA ACT GCG TAT TTG TCT G-3′) (designed from *C. trachomatis* strains sequenced in reference 25). The PCR mixture was overlaid with 50 μl of mineral oil and was run in a *GeneAmp* PCR System 9600 (PE Biosystems) at 94°C for 10 min followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min. At the end of the final cycle, an extension at 72°C for 7 min was included before storage at 4°C. A positive control (DNA of *C. trachomatis* serotype E) and a negative reagent control (distilled water), as well as the randomly selected negative sample controls, were included in each PCR run. The amplification product, which consisted of approximately 1,100 bp from the *omp1* gene, was visualized after electrophoresis through a 1.5% agarose gel containing ethidium bromide. The DNA Molecular Weight Marker VI (Boehringer Mannheim, GmbH, Mannheim, Germany) was included in each electrophoresis.

DNA sequencing. The PCR products were purified using the High Pure PCR product purification kit (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's instructions. Sufficient amounts of purified DNA (1 to 4 μl) were mixed with 2 μl of a 50 μM concentration of one of the primers, S1 (5′-TTG AGT TCT GCT TCC TCC T-3′) or OMP 2 (5′-ACT GTA ACT GCG TAT TTG TCT G-3′) (Scandinavian Gene Synthesis AB), in separate reaction mixtures and sequenced with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems, Warrington, United Kingdom). The DyeEx Spin kit (catalog no. 63106; Qiagen, Hilden, Germany) was used to remove the dye terminators in the cycle sequencing reaction mixtures according to the manufacturer's instructions. The sequence of the *omp1* gene was determined by using an ABI PRISM 310 Genetic Analyser (PE Biosystems). Each PCR product was sequenced twice in each direction, and this gave an overlap of about 200 bp in the middle of the *omp1* gene.

BLAST and phylogenetic analyses. In a first analysis, the individual consensus sequence (about 1,100 nucleotides) of the clinical isolates was determined by comparison to *omp1* nucleotide sequences of known serovars of *C. trachomatis* strains using the BLAST search tool at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Phylogenetic tree analyses were used to illustrate the evolutionary relationships between clinical isolates and the following reference strains of *C. tracho*-

matis obtained from GenBank (accession numbers in parentheses): A/SA1/OT (M58938), Ba/AP-2 (AF063194), B/TW-5 (M33636), C/TW-3/OT (M17343), D/B-120 (X62918), D/B-185 (X62919), D/IC-CAL-8 (X62920), E/DK-20/ON (X52557), F/MRC-301/GU (X52080), G/UW57/Cx (AF063199), H/Wash (X16007), I/UW-12 (AF063200), J/UW36/Cx (AF063202), K/UW31/Cx (AF063204), L1/440 Bu (M36533), L2/434 Bu (M14738), and L3/404 Bu (X55700). MOMP sequences representing *C. psittaci* (AF131889), *C. pneumoniae* (L25436), and a murine variant of *C. trachomatis* (MoPn [M64171]) were used as outgroup sequences to root the tree. The sequences were manually aligned using BioEdit (version 5.0.0) software. Preliminary phylogenetic trees with all sequences were constructed using the DNADIST and NEIGHBOR programs in the PHYLIP (version 3.52c) package (6). A final tree with selected sequences was constructed using a parallelized version of DNAml (5, 6; A. Holmberg et al., unpublished data). Bootstrapping was performed using the SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE programs in the PHYLIP (version 3.52c) package (6). All programs were run under Linux on a custom-built Beowulf cluster, consisting of one master and four slaves (all five were 350-MHz 586 AMD-K6 PCs).

Ethics. The research ethics committee at Örebro County Council, Örebro, Sweden, approved the study.

RESULTS

omp1 PCR. All tested prototype isolates of serovars A to L3 of *C. trachomatis* were successfully amplified in the *omp1* PCR, whereas the *C. pneumoniae* and *C. psittaci* strains were PCR negative. The *omp1* PCR showed high concordance with the diagnostic tests for *C. trachomatis*, and out of 238 *C. trachomatis*-positive clinical samples analyzed with the diagnostic tests, 235 were found to be positive in the *omp1* PCR. Consequently, three clinical samples were found to be negative in the *omp1* PCR and cell culture but positive in the Amplicor PCR. Retesting with the COBAS Amplicor test showed that two of the three samples were negative, and thus these were considered false positives in the Amplicor PCR. The remaining COBAS Amplicor-positive sample was repeatedly negative in the *omp1* PCR.

Samples from 2 of the 24 *C. trachomatis*-exposed but -negative partners were found to be positive in the *omp1* PCR. When these two samples were retested with the COBAS Amplicor test, one was positive and the other was negative.

All clinical samples included in the study as negative controls (one per positive sample) were negative in the *omp1* PCR.

Sequence and phylogenetic analyses. Optimization of the DNA sequence analysis was performed using reference isolates for *C. trachomatis* serovars A, B, Ba, C, D, E, F, G, H, I, J, K, L1, L2, and L3. All isolates were successfully amplified and sequenced.

Sequence analysis of the *omp1* gene from amplified DNA from the 237 clinical strains revealed that the most prevalent genotypes corresponded to *C. trachomatis* serovar E (47.3%), followed by F, K, D/B-120, D, J, Ia, D/B-185, G, H, and 1 strain of Ba. All nucleotide sequences were easy to read and interpret when compared by BLAST similarity search. The results are summarized in Table 1, and the evolutionary relationships between clinical isolates and reference strains are shown in Fig. 1.

The phylogenetic analysis of the *omp1* gene showed that the serovars of *C. trachomatis* are segregated into three main clusters. Two clusters were characterized by small genetic distances within each cluster; one consisted of genotypes F and G, and the other consisted of A, I, J, H, C, K, and L3. The third cluster consisted of genotypes B, Ba, D, D/B-120, D/B-185, E, L1, and L2 and displayed larger genetic distances (Fig. 1).

TABLE 1. *omp1* genotype distribution of 237 urogenital
C. trachomatis strains isolated in Örebro, Sweden, from
March 1999 to February 2000

Genotype	No. of persons infected with genotype			
	Men (*n* = 138)	Women (*n* = 99)	Total (*n* = 237)	%^{a}
Ba	1	0	1	0.4
D	9	3	12	5.1
D/B-120	8	6	14	5.9
D/B-185	3	3	6	2.5
E	71	41	112	47.3
F	24	17	41	17.3
G	2	4	6	2.5
H	3	3	6	2.5
Ia	4	3	7	3.0
J	6	4	10	4.2
K	7	14	21	8.9
Mixed (E + F)	0	1	1	0.4

^{a} Values are percentages of total numbers of *C. trachomatis*-positive specimens (*n* = 237).

Detailed analysis revealed that there were limited sequence differences within genotypes (Table 2 and Fig. 1). However, some differences from the reference sequences were observed, and these are listed in Table 2. Some of these sequence variants were observed in a single sample, whereas others were observed in several samples. Notably, all of the sequences within genotypes G and H differed from the respective reference sequence by one or more nucleotides (Table 2). Some of these nucleotide substitutions resulted in amino acid replacements and could thus potentially alter the function and antigenicity of the MOMP. For genotypes D, D/B-120, D/B-185, E, F, J, and K a majority of the *omp1* gene sequences from the clinical samples had sequences that were identical to those reported for the respective reference isolates. However, in genotypes E, J, and K several clinical isolates displayed identical nucleotide differences relative to the reference sequence. Thus, 4 of 113 genotype E strains displayed an A→G substitution at position 420, and 3 strains displayed a G→A substitution at position 997 (compared to E/DK-20/ON [19]). The latter substitution resulted in an alanine-to-threonine amino acid replacement, whereas the former was silent. Similarly, 4 of 10 genotype J sequences displayed a silent C→T substitution at position 369 (compared to J/UW36), and 3 of 21 genotype K sequences displayed a silent C→T substitution at position 132 (compared to K/UW 31/Cx).

As shown in Table 3, three women and two men were found to be *C. trachomatis* positive at follow-up visits 1 to 5 months after treatment of their initial infections. The *omp1* sequence of isolates obtained from the initial sample was compared to those obtained from the follow-up samples. The sequence analysis suggested that a reinfection had occurred in two of these five individuals. Thus, one woman was found to have *C. trachomatis* genotype K sequence in the initial endocervical sample and a genotype D/B-185 sequence 1 month later. Similarly, in the initial urethral specimen from one man and also in the second sample (received 2 months later) a genotype E sequence was found, while the third urethral sample, 5 months after the initial sample, contained genotype D/B 120. The
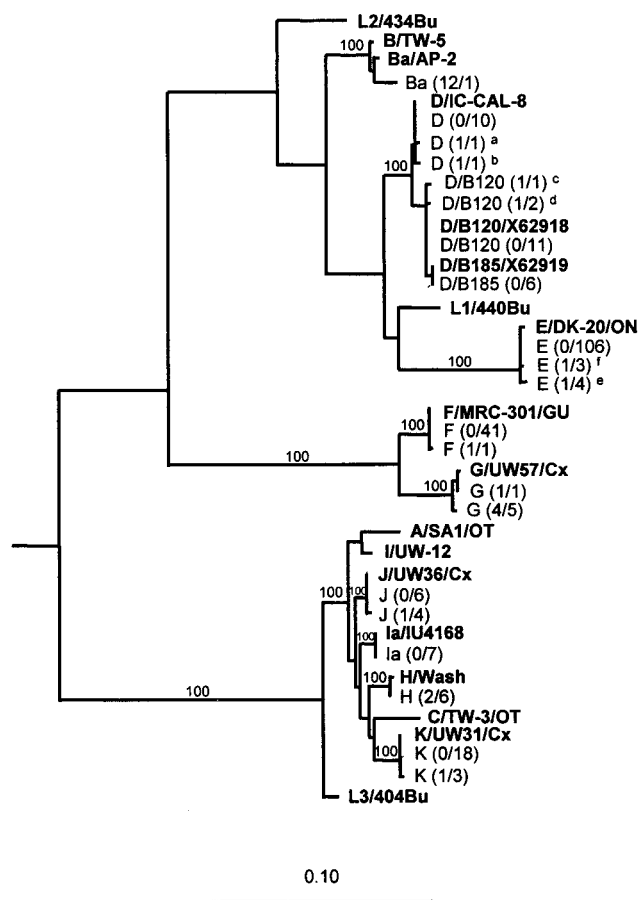

FIG. 1. Phylogenetic tree showing the evolutionary relationship between the *C. trachomatis* nucleotide sequences of the *omp1* gene from one representative of each genetic variant in the clinical material and 18 serovars of *C. trachomatis* available from GenBank. Sequences representing *C. psittaci*, *C. pneumoniae*, and a murine variant of *C. trachomatis* (MoPn) were used as outgroups to root the tree. The tree was calculated by the maximum-likelihood method (transition/transversion ratio, 1.4; global rearrangement option on) from an alignment of 1,032 aligned nucleotides. Relevant bootstrap values (as a percentage of 1,000 replicates) are also given. Reference strains are shown in boldface text. The clinical strains (*n* = 237) are illustrated with genotype and number of mutations per number of samples (within parentheses). Footnote letters: a, genotype D with one mutation at position 331 compared to D/IC-CAL-8; b, genotype D with one mutation at position 1092 compared to D/IC-CAL-8; c, genotype D/B120 with one mutation at position 975 compared to the strain X62918 (GenBank); d, genotype D/B120 with one mutation at position 1045 compared to the strain X62918 (GenBank); e, genotype E with one mutation at position 420 compared to E/DK20/ON; f, genotype E with one mutation at position 997 compared to E/DK20/ON.

remaining three individuals showed no change in genotype, which suggests either treatment failure or reinfection with the same genotype. Further on, one woman showed evidence of double infection, since she was found to have *C. trachomatis* genotype E in an endocervical sample and genotype F in the urine sample (Table 1).

The epidemiological information suggested that eight patients had been infected abroad. One woman and one man, both infected with genotype E, were likely to have acquired their infections in Bulgaria. Like most other genotype E se-

TABLE 2. Mutations found in 237 clinical specimens compared to strains in GenBank

Genotype	No.[a]	No. of mutations	Mutation		Amino acid change	GenBank accession no.
			Nucleotide	Position		
D	1 (12)	1	G→A	331	R→H	X62920
D	1	1	G→A	1092	A→T	X62920
D/B-120	1 (14)	1	A→T	975	Silent	X62918
D/B-120	2	1	T→C	1045	Silent	X62918
E	4 (113)	1	A→G	420	Silent	X52557
E	3	1	G→A	997	A→T	X52557
F	1 (42)	1	A→T	1409	Silent	X52080
J	4 (10)	1	C→T	369	Silent	AF063202
K	3 (21)	1	C→T	132	Silent	AF063204
H	6 (6)	2	A→G	440	D→S	X16007
			C→T	1018	Silent	X16007
G	1 (6)	1	T→G	1003	S→A	AF063199
G	5	4	T→A	228	Silent	AF063199
			G→A	487	G→S	AF063199
			G→C	700	E→Q	AF063199
			T→A	1003	S→T	AF063199

[a] Number of clinical strains with mutations. The values in parentheses are the total numbers of strains of the same genotype.

quences these two *omp1* gene sequences were identical to that of the reference strain. The other five patients infected abroad included one genotype Ia infection from the United States, one genotype F infection from Greece, one genotype D/B-120 infection from Austria, one genotype D infection from Switzerland, one genotype D infection from Norway, and one genotype Ba infection from Thailand. Interestingly, this genotype Ba infection was the only genotype Ba sample in the entire study.

DISCUSSION

In this study we have established a method for PCR amplification and sequence analysis of the *omp1* gene of *C. trachomatis* from clinical specimens. Our *omp1* PCR worked successfully with both urogenital samples and urine samples and showed no cross-reactions with *C. pneumoniae* or *C. psittaci* or other false-positive reactions. All but 1 of 238 samples that were positive by the diagnostic *Chlamydia* tests were positive in the *omp1* PCR. One reason for this false-negative result could be that the *omp1* gene is only present in one copy per organism, whereas the plasmid that is targeted by the diagnostic tests is present in approximately 10 copies per organism (13, 18, 24). However, the *omp1* PCR was also positive in 2 of 24 diagnostic test negative samples from individuals epidemiologically highly suspected of having a *C. trachomatis* infection. One of these two samples was positive after retesting by the diagnostic test,

TABLE 3. Patients still *Chlamydia* positive at follow-up visit after initial antibiotic treatment

Patient sex	Genotype in initial sample	Genotype after:		
		1 mo	2 mo	5 mo
Male	E	ND[a]	E	D/B-120
Female	K	D/B-185	Negative	ND
Male	E	E	ND	ND
Female	K	K	Negative	ND
Female	D/B-120	D/B-120	Negative	ND

[a] ND, not done (diagnostic samples not obtained).

perhaps due to the freeze-thawing, as recently described (3). The remaining sample, which was found positive in the *omp1* PCR only, could contain a plasmid-free variant of *C. trachomatis* (1).

C. trachomatis in these Swedish clinical isolates was most frequently genotyped as E (47%) or F (17%). Also relatively prevalent were *omp1* genotypes D, D/B-120, J, and K (5 to 10%), whereas the remaining genotypes (Ba, D/B-185, G, H, and Ia) were more infrequently encountered. The genotype distribution in our 237 clinical specimens was broadly similar to the serovar distributions reported previously (15, 20). There was a high level of conservation of the *omp1* gene in infections caused by genotype E, of which 106 out of 112 had 100% similarity to the strain E/DK-20/ON. These findings are in agreement with those of Rodriguez et al. (22), who reported a high level of conservation of E strains from different geographic origins. However, we found minor sequence variations within the different genotypes in our clinical material. Some of these genetic variants were detected only in a single sample, which makes it difficult to exclude the possibility that they represent sequencing artifacts, but most of them were independently detected in two or more samples, which strongly indicates that they were accurate. Thus, 4 of 10 clinical genotype J strains were found to have one identical substitution, but at a different position than that described by Morré et al. (14). Similarly, five of six of genotype G sequences had identical substitutions that were different from the substitutions reported previously (14, 16), and all six genotype H sequences harbored two identical mutations. It has been speculated that such *omp1* genovariants occur as a result of point mutations and recombination events selected by immune pressure (8). However, several of the nucleotide substitutions that we detected were synonymous (i.e., silent), which suggests that they were evolutionarily neutral.

The phylogenetic tree analysis of all the genotype sequences found indicates that there is no simple correlation between the disease manifestation and *omp1* gene phylogeny. The *omp1* sequences of genotype H, I, J, and K, which were represented in our material from patients with symptomatic or subclinical

genital infections, were closely related to each other. However, they were also closely related to genotypes A and C, which are associated with trachoma, and genotype L3, which is associated with lymphogranuloma venerum. These observations are broadly the same as those reported by Stothard et al. when they compared *omp1* sequences from clinical strains from the United States to sequences registered in GenBank (26). Few studies have addressed the possible correlation between specific serovars or genotypes and disease manifestations and severity of disease. However, in a recent Finnish serological study it was stated that serovar G could be associated with subsequent development of cervical squamous cell carcinoma (2). This finding is interesting and requires further study, especially due to the fact that some genotype G sequences in our study differ from the prototype sequence by three amino acids (Table 2).

Genotyping was also useful in the follow-up of the *Chlamydia*-infected patients, and five patients were still *C. trachomatis* positive at the follow-up visit. In two of these patients, the initial sample and follow-up sample showed different *omp1* genotypes, which indicates that new sexual partners had reinfected these patients. In the other three patients the genotypes were identical in the initial and follow-up samples, suggesting treatment failure or reinfection with the same genotype. Furthermore, one woman was found to have a mixed infection with two genotypes of *C. trachomatis*, and she had had two sexual partners, each carrying one of these different genotypes.

In conclusion, we have established a sensitive and relatively simple method for the genotyping of *C. trachomatis* strains in clinical samples based on sequencing of the *omp1* gene. Genotypes E and F dominated in our Swedish material, and the individual sequences were stable and showed limited variation. This *omp1* genotyping method provided interesting results concerning double infections and reinfections and could be useful for epidemiological characterization of circulating *C. trachomatis* strains in the community.

ACKNOWLEDGMENTS

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