Charactetization 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 been reinfected, while three other patients suffered treatment failure or reinfecion. 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.
A total of 240 specimens were found to be C. trachomatis positive by tissue culture amplification PCR for COBAS AmpliCARE test. Two samples were lost, while 238 were stored at −20°C until use. The first choice for the omp1 PCR was the urethral specimen (n = 190), and the second choice was the urine sample (n = 48), if the urethral specimen 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/JICAL-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 (X57000). MOMP sequences representing C. psittaci (AF313889), C. pneumoniae (L29546), and a murine variant of C. trachomatis (MoPu [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 AmpliCARE test showed that two of the three samples were negative, and thus these were considered false positives in the Amplicor PCR. The remaining COBAS AmpliCARE-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 AmpliCARE 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).
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 sequence variations resulted in amino acid replacements. Thus, 4 of 113 genotype E strains displayed an A substitution at position 331 compared to D/IC-CAL-8; f, genotype E with one mutation at position 1045 compared to the genotype D/B120 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 with one mutation 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 gene sequences of the isolate 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 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-
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 Thailand, one genotype D infection from Norway, and one genotype Ba infection from Austria. 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 238 of 237 samples were positive by the diagnostic Chlamydia tests and positive in the omp1 PCR. One reason for this false-negative result could be that the omp1 gene is 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, 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 theomp1 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

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of mutations</th>
<th>Mutation</th>
<th>Amino acid change</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>1 (12)</td>
<td>G→A</td>
<td>R→H</td>
<td>X62920</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>G→A</td>
<td>A→T</td>
<td>X62920</td>
</tr>
<tr>
<td>D/B-120</td>
<td>1 (14)</td>
<td>A→T</td>
<td>975</td>
<td>X62918</td>
</tr>
<tr>
<td>D/B-120</td>
<td>2</td>
<td>T→C</td>
<td>1045</td>
<td>X62918</td>
</tr>
<tr>
<td>E</td>
<td>4 (113)</td>
<td>A→G</td>
<td>420</td>
<td>X52557</td>
</tr>
<tr>
<td>E</td>
<td>3</td>
<td>G→A</td>
<td>997</td>
<td>X52557</td>
</tr>
<tr>
<td>F</td>
<td>1 (42)</td>
<td>A→T</td>
<td>1409</td>
<td>X52080</td>
</tr>
<tr>
<td>J</td>
<td>4 (10)</td>
<td>C→T</td>
<td>369</td>
<td>AF063202</td>
</tr>
<tr>
<td>K</td>
<td>3 (21)</td>
<td>C→T</td>
<td>132</td>
<td>AF063204</td>
</tr>
<tr>
<td>H</td>
<td>6 (6)</td>
<td>A→G</td>
<td>440</td>
<td>X16007</td>
</tr>
<tr>
<td>G</td>
<td>1 (6)</td>
<td>T→G</td>
<td>1003</td>
<td>AF063199</td>
</tr>
<tr>
<td>G</td>
<td>5</td>
<td>T→A</td>
<td>228</td>
<td>AF063199</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Patient sex</th>
<th>Genotype in initial sample</th>
<th>Genotype after:</th>
<th>1 mo</th>
<th>2 mo</th>
<th>5 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>E</td>
<td>D/B-120</td>
<td>E</td>
<td>D/B-120</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>K</td>
<td>D/B-185</td>
<td>Negative</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>E</td>
<td>K</td>
<td>E</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>K</td>
<td>D/B-120</td>
<td>Negative</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>D/B-120</td>
<td>D/B-120</td>
<td>Negative</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

* ND, not done (diagnostic samples not obtained).
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 associated with trachoma, and genotype L3, which is associated with genital infections, were closely related to each other. However, in a recent Finnish serological study it was stated that serovar G could be associated with subsequent development of cervical squamous cell carcinoma.

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...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. This 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.

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REFERENCES