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Chlamydia trachomatis Serovar Differentiation by Direct Sequence Analysis of the Variable Segment 4 Region of the Major Outer Membrane Protein Gene

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The polymerase chain reaction method was used to amplify DNA from the fourth variable segment of the gene encoding the major outer membrane protein of *Chlamydia trachomatis*. Direct sequencing of the amplified DNA from prototype strains confirmed previously identified nucleotide sequence differences that were specific for each serovar. This analysis revealed differences in the DNA sequences of prototype strains C/UW-1 and G/IOL-238 from those of prototype strains C/TW-3 and G/UW-57, sequenced previously. This method was also used to determine the serovar types of *C. trachomatis* in 125 urogenital specimens from infected patients. The most common serovars were E (38%), F (17%), and G and D (14% each). Serovar D was found significantly more often in specimens from men than in specimens from women ($P = 0.004$). Conversely, serovar G was found significantly more often in specimens from women than in specimens from men ($P = 0.026$). Only two serovar G isolates gave sequences identical to that of the prototype strain G/IOL-238, suggesting that this strain may be a serovar variant. Three isolates (D^+, G^-, and J′) gave sequences which have not been reported previously. One isolate had the same sequence as the D^- serovar variant. Sequence analysis of amplified DNA reveals subtle differences between *C. trachomatis* strains and provides a very sensitive method for molecular epidemiological analysis.

Chlamydia trachomatis is an important cause of disease worldwide. In developing countries, *C. trachomatis* serovars A, B, Ba, and C are responsible for the blinding disease trachoma, and in developed countries serovars D through K cause oculogenital infections, neonatal conjunctivitis, and pneumonia (11). The more severe genital infection of lymphogranuloma venereum results from infection with serovar L1, L2, or L3.

Recently, monoclonal antibodies have been used to type clinical isolates of *C. trachomatis* (25). These monoclonal antibodies recognize species-, serogroup-, and serovar-specific regions on surface-exposed epitopes of the major outer membrane protein (MOMP) of chlamydiae (2, 23). The MOMP gene (*omp 1*) contains four variable sequence regions (VS1 to VS4) that encode the four variable protein domains (VDI to VDIV) which protrude from the chlamydial membrane (2). VDI and VDII contain serovar-specific epitopes (28), while subspecies-, serogroup-, and species-specific determinants are found in VDIV (2, 23). In addition, evidence suggests either that a serovar-specific epitope is present in VDIV (14, 22, 27) or that VDIV influences antibody binding to VDI and VDII (8, 9). The variable domains are highly immunogenic, and antibodies directed to these domains are thought to have a role in protective immunity (6, 28). MOMP antigens are important not only in serovar epidemiology, but they are also important potential targets for vaccine development.

The extensive variations in the VD sequences of the MOMP are reflected in equally extensive, serovar-specific differences in the VS sequences of *omp 1* genes from different serovars (27). Three serovariants have been identified on the basis of studies with monoclonal antibodies (25), and there is corresponding variation in the VSs of these isolates (9, 14). This has resulted in the designation of three new serovars, Da, Ia, and L2a (26).

We describe here the use of sequence analysis of DNA to differentiate serovars of *C. trachomatis* for epidemiological studies. We have previously used a polymerase chain reaction (PCR) method to amplify VS4 of the *omp 1* gene of *C. trachomatis* from clinical specimens (19, 20). Here we report the use of a technique similar to that of Dean et al. (9) to sequence the amplified DNA. This has allowed us to determine the serovar epidemiology of *C. trachomatis* in our community. We have also identified three new variants of *C. trachomatis*.

MATERIALS AND METHODS

Chlamydiae. *C. trachomatis* serovar strains A/SA-1/OT, B/TW-5/OT, C/UW-1/OT, D/IC-Cal-8, E/DK-20, F/MRC-301/UR, G/IOL-238/R, H/UW-4/CX, I/UW-12/UR, J/UW-36/CX, K/UW-31/CX, L1/440/BU, L2/434/BU, and L3/404/BU were generously provided by T. Forsey (Institute of Opthalmology, London), and serovar strain Ba/AP-2/OT was a kind gift from J. Schachter (University of California, San Francisco). Chlamydiae were concentrated from 100 μl of crude yolk sac extracts by microcentrifugation and then washed twice with phosphate-buffered saline and resuspended in 20 μl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

PCR amplifications. PCR amplifications were carried out essentially as described previously (19, 20). Standard precautions to avoid contamination of specimens and reaction mixtures were taken as described previously (20). Preparations were incubated at 100°C for 5 min, and 10 μl was added to the reaction mixture for DNA amplification by the PCR technique. Amplifications with 0.5 U of DNA polymerase

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G/UW-57     TTG GCA AAA CCT GTT GTA GAT ATT ACA ACC CTT AAC CCA ACT ATT GCA GGA TGC GGC AGT GTA GTC GCA GCT AAC TCG GAA GGA CAG ATA TCT
                                                                                                                     Ser
G/IOL-238                                                                                                            GCG
                                                                                                                     Ala

C/TW-3      TTG GCT GAA GCA ATC TTG GAT GTC ATC ACT CTG AAC CCG ACT ATC GCT GGT AAA GGA AGT GTG GTC TCT GCC GGA ACC GAT AAC GAA CTG GCT
                                                Val         Thr                                     Ala
C/UW-1                                          CTA         ACC                                     TCC
                                                                                                    Ser

K/UW-31     TTG GCT GAA GCA ATC TTG GAT GTC ACT ACT CTA AAC CCG ACT ATC ACT GGT AAA GGA GCT GTC GTC TCT TCC GGA AGC GAT AAC GAA CTG GCT
                                                                            Thr
K/UW-31                                                                     GCT
(This study)                                                                Ala
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FIG. 1. Nucleotide sequences of VS4s from the *C. trachomatis omp 1* genes from prototype strains of serovars G, C, and K. Nucleotide and amino acid changes are noted below the prototype sequences determined by Yuan et al. (27). The species-specific conserved sequences are underlined.

(AmpliTaq; Perkin-Elmer Cetus, Norwalk, Conn.) took place in a 40-μl final volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.5 mM $MgCl_2$, 0.001% gelatin, 200 μM each deoxynucleoside triphosphate, and 1 μM each primer. To prevent evaporation, 100 μl of paraffin oil was overlaid onto each reaction mixture. Thirty cycles of 25 sec at 94°C, 1 min at 54°C, and 2 min at 72°C in a thermocycler (Thermal Reactor; Hybaid, Middlesex, England) were carried out, with a further 0.5 U of polymerase added at the tenth and twentieth cycles. The length of time of the initial denaturation step at 94°C was increased to 75 sec to ensure template denaturation. Following the last cycle, all reaction mixtures were incubated for a further 7 min at 72°C to ensure that the extension step was complete. Amplified products (277 or 280 bp) of each reaction were visualized after electrophoresis through 2% agarose gels containing ethidium bromide. The PCR primers were based on published sequences of the *omp 1* gene of serovar L2 (22). The primer sequences flanked VS4 of the *omp 1* gene and corresponded to regions which are conserved in all serovars for which the DNA sequences were known, thus allowing amplification of all serovars (19, 20). The two oligonucleotide primer sequences selected were 5′-GGCAAGCAAGTTTAGCTCTCTCT-3′ and 5′-GTTCCT ACTGCAATACCGCAAGA-3′.

DNA sequencing. The amplified product of each reaction was precipitated by adding 1 volume of 4 M ammonium acetate and 2 volumes of cold 95% ethanol (−20°C), washed twice with cold 70% ethanol, evaporated to dryness in a Speed Vac Concentrator (Savant Instruments Inc., Farmingdale, N.Y.), and then dissolved in 6 μl of TE buffer in preparation for direct double-stranded sequencing. Three μl of each template was used in the sequencing reactions. Sequencing was carried out with 50 ng of the 5′ oligonucleotide primer with ^{35}S-dATP (1,000 Ci/mmol; Amersham International, Amersham, England) and Sequenase (70770 Sequenase version 2.0 DNA sequencing kit; U.S. Biochemicals, Cleveland, Ohio) by using a modification of the method described by Casanova et al. (7). The double-stranded DNA template was denatured at 90°C for 5 min in the presence of primer and reaction buffer and then transferred immediately into a dry ice-ethanol bath for 10 sec to anneal primer to template. Labelling mix was added to the reaction mixture, which was rapidly thawed, vortexed, and briefly microcentrifuged before aliquots were added to the dideoxynucleotide termination reaction mixtures. Termination reactions were stopped after the mixtures had been incubated at 37°C for 2 min. The reaction mixtures were analyzed on a 6% denaturing polyacrylamide gel in which a salt gradient was established by incorporating 4% potassium acetate in the lower buffer. This allowed the determination of the DNA sequence of each VS4 on a single gel. Sequences were determined after autoradiography for 4 to 5 days with Kodak X-Omat-AR film (Eastman Kodak Co., Rochester, N.Y.).

Clinical specimens. A total of 125 urogenital specimens containing *C. trachomatis* were examined. The specimens had been collected from 119 patients (74 men and 45 women) who attended the Sexually Transmissible Diseases Clinic at Dunedin Hospital between 1985 and 1990. The specimens had been shown by cell culture isolation, immunofluorescence, or enzyme immunoassay to contain *C. trachomatis*. Endourethral swabs from men or endocervical swabs from women were collected and placed in chlamydia transport medium (cell culture isolation), rolled onto a slide prior to placing in transport medium (immunofluorescence), or collected into a dry specimen container prior to the addition of the manufacturer's buffer (Pharmacia Chlamydia EIA; Pharmacia, Uppsala, Sweden) (enzyme immunoassay). Specimens were stored at −70°C after the initial investigation. For PCR amplification and sequence analysis, the specimens were thawed and the entire volumes (200 to 1,200 μl) were prepared for PCR amplification as described above.

Statistical analysis. The significance of differences in the proportions of serovars between men and women was determined by the χ^2 test.

RESULTS

Sequencing of prototype serovars. We first determined the sequences of VS4 from prototype serovars of *C. trachomatis* and compared the results to those obtained by Yuan et al. (27). Our results were identical to those of Yuan et al. (27), except for single nucleotide base differences for serovars G and K and three nucleotide base changes for serovar C (Fig. 1). Differences from the published sequences were verified by sequencing the amplified VS4 complementary strand with the 3′ oligonucleotide primer. The differences between our sequences and those of Yuan et al. (27) for serovars C and G presumably reflect that we used different prototype strains and that immunotyping had not differentiated between them. We used a strain of serovar K which was the same as that of Yuan et al. (27) but which had been obtained from a different source, and the single nucleotide base change that we found may indicate that point mutations occurred during repeated passages in cell or yolk sac cultures.

Sequence analyses of clinical specimens. We then determined the serovars of *C. trachomatis* for 125 clinical speci-

TABLE 1. Distribution of 125 *C. trachomatis* serovars from the urogenital specimens of 119 patients

Serovar	No. of infections		Total (n = 125)	%[a]
	Men (n = 78)	Women (n = 47)		
B/Ba[b]		1	1	0.8
D	16	1	17	13.6
E	30	18	48	38.4
F	12	9	21	16.8
G	7	11	18	14.4
H	4	3	7	5.6
J	1	1	2	1.6
K	1	2	3	2.4
Mixed				
G/J		1	1	1.6
E/K	1		1	
Variant				
D^-	1		1	4.8
D^+	1		1	
G'	2		2	
G^-	1		1	
J'	1		1	

[a] Values are percentages of total number of specimens (125).
[b] Not distinguished.

mens. We were able to determine serovar types from specimens which gave very little PCR-amplified product and also to differentiate serovar types for mixed infections. The results are summarized in Table 1. Serovar E was the serovar most prevalent in both men and women. The second and third most prevalent serovars were serovars G and F, respectively, for women and serovars D and F, respectively, for men. Together these four serovars accounted for 83% of infections. We identified significantly more serovar G infections for women than for men (P = 0.026). In contrast, significantly more specimens from men than from women contained serovar D (P = 0.004). The reasons for these differences are not apparent. Few serovars in the C-complex group (C, A, H through K, L3) were identified, and serovars A, C, Da, I, Ia, L1, L2, L2a, and L3 were not found.

Table 2 compares the results of our study with those of studies at other centers. The distribution of serovars is similar to those found in the other studies, except that we found an unusually high proportion of serovar G for our specimens.

Mixed infections. Two (1.6%) specimens gave superimposed sequences which were different at some positions and were concluded to have arisen from mixed infections. One endourethral specimen contained serovars E and K, and one endocervical specimen contained serovars G and J. In a larger study, Barnes et al. (4) detected mixed infections in 2% of the positive specimens.

Reinfections. Two women and four men in our study had second chlamydial infections 4 to 21 months after their initial infections. Both endocervical specimens and one of the endourethral specimens contained serovar G on the subsequent occasion, after initially having contained serovar E (two specimens) or serovar F. The other three males had infections with the same serovar (D, E, or F) 12 to 15 months after their initial infections. Two of these men probably had reinfections, as *C. trachomatis* was not found in endourethral specimens obtained between episodes. The other male had not been investigated for chlamydiae during the 15 months between determination of positive specimens, but he had received tetracycline therapy following the initial diagnosis. This suggests that a reinfection had occurred subsequent to the first infection rather than that a reactivation had occurred after failure to eradicate *C. trachomatis* when it was first diagnosed.

Identification of serovar variants. Six serovar variants were identified following analysis of DNA from the clinical specimens. All six variant sequences were verified by repeating both the PCR amplification and the sequencing reactions. Figure 2 shows the nucleotide and inferred amino acid sequences of VS4 of the serovar variants, compared with their respective prototype sequences. Three of these variants (D^+, G^-, and J') have not been described previously.

The transition in the D^+ variant resulted in a glycine-to-serine amino acid substitution. All serovars in the B-complex group have a serine residue at this position, except for L1, D (prototype strain), and D^- (serovar variant). Sequence analysis of the rest of the *omp 1* gene may clarify the

TABLE 2. Distribution of *C. trachomatis* serovars according to different studies

Country (date)[a]	Reference	No. of isolates	% of no. of isolates for the following serovars:																			
			B complex									Intermediate				C complex						
			B	Ba	D	E	B/Ba	B/D/E	D/E	B/E	Total	F	G	F/G	Total	C	H	I	Ia	J	K	Total
United States (1983)	13	493	4		14	10		2	20		50	17	4	4	25		6	7		3	6	22
Canada (1991)	12	40		13	23	33					69	13			13				3	8	8	19
Finland (1987)	21	51	2		5	26			26		59	10	4		14		6	6		8	4	24
Sweden (1989)	18	38			11	37					48	21	3		24		13			8	5	26
Sweden (1990)	17	1,009	1		13	40					54	25	4		29	1	2	1		5	8	17
England (1972)	10	23			17	39					56	13	26		39							
Holland (1988)	24	190			18	24					42	21	2		23		11	2	1	6	15	35
France (1990)	15	53		6	6	62					74	9	2		11		4			6	2	12
Germany	16	56			29	36					65	27	4		31						5	5
Australia (1990)	1	31	48			19				16	83	3			3						12	12
New Zealand (1992)	This study	125			14	38	1				53	17	14		31		6			2	2	10

[a] A study in Ghana (5) typed three isolates which were all serovar G. Antibodies in the populations studied were most commonly directed against serovars D, E, F, and G.

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D/UW-3      TCA GCT ACA GCT ATT TTT GAT ACT ACC ACG CTT AAC CCA ACT ATT GCT GGA GCT GGC GAT GTG AAA ACT GGC GCA --- GAG GGT CAG CTC GGA
                                                                                Ala                     Gly
D-                                                                              ACT
                                                                                Thr
D+                                                                                                      AGC
                                                                                                        Ser

G/UW-57     TTG GCA AAA CCT GTT GTA GAT ATT ACA ACC CTT AAC CCA ACT ATT GCA GGA TGC GGC AGT GTA GTC GCA GCT AAC TCG GAA GGA CAG ATA TCT
                                                                                                                Ser
G'                                                                                                              GCG
                                                                                                                Ala
G-                                                                                                              TTG
                                                                                                                Leu

J/UW-36     TTG GCT GAA GCA ATC TTG GAT GTC ACT ACT CTA AAC CCG ACC ATC GCT GGT AAA GGA ACT GTG GTC GCT TCC GGA AGC GAA AAC GAC CTG GCT
                                                                                                Val Ala                     Asp
J'                                                                                              ATC GCC                     GAA
                                                                                                Ile                         Glu
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FIG. 2. Nucleotide sequences of *C. trachomatis omp 1* VS4s from the serovar variants. One isolate of each variant was obtained, except for G′ (two isolates). Variant sequences are shown below the serovar prototype VS4 sequences. Codon and amino acid changes are shown below the respective positions in the prototype serovars. The underlined nucleotides indicate the conserved species-specific regions of VS4.

relationship between the D^+ variant and other serovars in the B-complex group. One serovar D variant had a transition identical to that of the D^- variant described by Lampe et al. (14). This transition results in an amino acid substitution of threonine for alanine and causes a loss of reactivity with the subgroup-specific monoclonal antibody, BB-11 (14).

One serovar G variant (G^-) in which a base transition resulted in a serine-to-leucine substitution was identified. Two other variants (G′) had a different mutation in the same codon and had the same sequence as strain G/IOL-238/R, the prototype strain we sequenced. It is likely that the G′ strains which we identified and the prototype G/IOL-238/R strain represent a serovar subtype or variant. The remaining serovar G strains had VS4 nucleotide sequences identical to that of the serovar G prototype strain G/UW-57/CX determined by Yuan et al. (27). Sequence analyses of other variant G strains may indicate whether the amino acid which is altered in the G′ and G^+ variants is at a critical site for selective pressure from the immune system.

We found one C-complex variant whose sequence was different from those of all known strains. We assigned this variant to serovar J (J′), although it has three nucleotide differences from the sequence of the prototype strain, one of which is silent. It would be most interesting to investigate how this variant reacts with monoclonal antibodies specific for C-complex-related serovars and in particular with those specific for serovar J.

DISCUSSION

We have studied the serovars of *C. trachomatis* for 125 clinical specimens by sequencing the DNA of VS4 of the *omp 1* gene after PCR amplification. Very little amplified DNA is required, and this method is able to distinguish mixed infections. The distribution of serovars (Table 1) was broadly similar to the distributions found in other centers (Table 2) by using polyclonal or monoclonal antibodies or restriction fragment length polymorphism to differentiate serovars. In this and other studies, serovars E, D, and F were the serovars most commonly found in urogenital infections (Table 2). Serovar G was found more frequently in our specimens (14.4%) than in specimens from other centers, except for the study of Dwyer et al. (10), in which only a small number of specimens was studied. Serovar G was found more frequently in women. The reason for this is not apparent. By contrast, serovar D was found significantly more often in men than in women in the present study. This finding is similar to that of another study comparing *C. trachomatis* serovars from women and homosexual men, in which serovar D was found significantly more often in men (3). We were unable to determine whether the men with serovar D in our study belonged to any particular subgroup of the male population.

In addition, three of the prototype strains which we analyzed gave sequences different from those obtained previously (27) (Fig. 1), reflecting the fact that different prototype strains were used. In one case, a change had apparently occurred during passage of the strain in culture. Of the 125 clinical specimens in the present study, 6 contained *C. trachomatis* variants (Fig. 2). This apparently high rate of serovar variation may reflect the power of DNA sequencing to detect subtle differences between isolates, differences which may not be detected by antibody-based methods or restriction fragment length polymorphism analysis. In particular, DNA sequencing is able to detect differences which do not correspond to changes in the amino acid sequence of the MOMP, and three such changes were detected in this study (Fig. 1 and 2). This rate of variation may also reflect the genetic diversity of *C. trachomatis* in geographically distant regions. VS4, the segment we sequenced, is the largest of the variable regions within the *omp 1* gene and with VS1 encodes the most immunoreactive domains of the MOMP (29). A greater frequency of amino acid substitutions would be expected to occur within these domains than elsewhere in the protein. In addition, more than one VD can be spanned by a single monoclonal antibody (8). Amino acid substitutions inducing a conformational change in VDIV could alter antibody binding to the other VDs. Sequence analysis of the other VSs, particularly VS1, may identify other serovar subtypes and variants.

Zhong and Brunham (29) found that in general the surface-exposed epitope of VDIV was toward the N-terminal end of the domain in B-complex serovars and toward the C-terminal end in C-complex serovars. These exposed antigenic epitopes would be expected to be subject to the most intense selection pressure for antigenic change, and most amino acid substitutions would be expected to be in these regions. In the variants found in this study and prototype serovars G/IOL-238/R and C/UW-1/OT, all the amino acid substitutions were toward the C-terminal end of VDIV, including substitutions in the two B-complex D variants. Our results are therefore consistent with the epitope observations of Zhong and

Brunham for the C-complex variant (J′) but not for the B-complex variants (D^- and D^+). However, Baehr et al. (2) mapped the B-complex serogroup- and subspecies-specific epitopes to the C-terminal end of VDIV, and our results are consistent with their findings. The amino acid substitutions in the intermediate group variants (G′, G^+, and G/IOL-238/R) were all toward the C-terminal end of VDIV. Nucleotide analyses of other variants will indicate which regions of VDIV are most prone to change.

The nonapeptide species-specific epitope (TTLNPTIAG) is present in VS4 of the MOMP (2), and the nucleotide sequence encoding this part of the protein is shown in Fig. 1 and 2. Although this sequence is conserved in all serovars (27), recent amino acid substitution experiments (29) demonstrate that this region has some tolerance for residue substitutions. We found no nucleotide sequence changes in this region for the variants we identified among our clinical specimens. Two nucleotide transitions in the prototype strain C/UW-1/OT in this region were identified, but these did not result in amino acid substitutions (Fig. 1). The nucleotide difference from the sequence published by Yuan et al. (27) which we identified in the nonapeptide coding region of prototype strain K/UW-31/CX results in an alanine residue at that site, consistent with all other serovars of the C-complex group. Given the amino acid change observed by Yuan et al. (27) for this strain, it is likely that substitutions at this site are tolerated.

In conclusion, the method described here provides a simple and very powerful means for differentiating serovars of *C. trachomatis*. PCR amplification of VS4 of the *omp 1* gene followed by sequencing of DNA can be applied to clinical specimens to determine serovar epidemiology and to identify serovar variants. The ability to precisely identify variants may contribute important information to the design of peptide sequences for recombinant vaccine studies.

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