INFECTION AND IMMUNITY, Apr. 1991, p. 1579–1582
0019-9567/91/041579-04$02.00/0

Vol. 59, No. 4

Direct Sequence Evaluation of the Major Outer Membrane Protein Gene Variant Regions of *Chlamydia trachomatis* Subtypes D′, I′, and L2′

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Received 27 July 1990/Accepted 7 January 1991

The nucleotide sequences of variable segments (VS) 1, 2, and 4 for the major outer membrane protein gene (*ompl*) of *Chlamydia trachomatis* were determined for serologically defined subtypes D′, I′, and L2′. Asymmetric DNA amplification was used to produce single-stranded DNA for direct sequencing. Amino acid substitutions were detected in VS1, VS2, and VS4 for I′, in VS2 for L2′, and in VS4 for D′. DNA sequencing of *ompl* variant regions may be an important method for evaluating the molecular epidemiology of *Chlamydia* spp.

Chlamydia trachomatis is responsible for significant disease worldwide, including blinding trachoma (4), severe genitourinary pathology (13, 20), and neonatal ophthalmitis and pneumonitis (2, 7). Within this species of *Chlamydia*, 15 serovariants have been identified by the microimmunofluorescence test with polyvalent antisera (17). Recently, this typing method has been improved by using major outer membrane protein (MOMP)-specific monoclonal antibodies (MAbs) (19). The MOMP of *C. trachomatis* is antigenically complex and displays serovar, serogroup, and species specificities (1, 15). MOMP gene (*ompl*) sequence comparisons among serovars has revealed four variable-sequence (VS) regions designated VS1, VS2, VS3, and VS4 (14). MAbs that neutralize infectivity (8, 10, 22) bind serovar-specific epitopes that have been mapped to VS1 and VS2 (22). Alternatively, serogroup- and species-specific determinants have been mapped to VS4 (1, 3, 16). In addition to the importance of MOMP antigens for seroepidemiological evaluations and for the identification of serovariants that are responsible for infection, MOMP antigenic determinants may also be important for eliciting protective immunity (22). Therefore, a molecular understanding of MOMP antigenic determinants is critical for the design of new sero- and molecular epidemiological assays as well as for rational vaccine development.

The entire *ompl* gene has been cloned and sequenced for serovars A (1); B, C, and L2 (14); and L1 (11). Recently, the *ompl* variant regions of serovars A to K and L1 to L3 were sequenced by Yuan et al. (21). On the basis of amino acid sequence comparisons for all four VS, the serovars can be separated into B complex, C complex, and G/F-related groups. Interestingly, prior classification of these serovars by polyvalent antisera or MAbs to MOMP-specific epitopes had grouped them similarly (17, 19). This correlation most likely reflects the antigenic characteristics of the VS regions. The MAb typing method has resulted in the detection of three new chlamydial subtypes: D′, I′, and L2′ (19). Given the identification of new serovariants, the unique antigenic potential of each VS, and the implications for vaccine development, we determined the nucleotide and inferred amino acid sequence variations in VS1, VS2, and VS4 for these new subtypes.

C. trachomatis serovars Ba/AP-2/OT, D/UW-3/Cx, D′/TW-448/OT, I/UW-12/Ur, I′/UW-202/NP, and L2′/TW-396/RT were generously provided by S. P. Wang and C. C. Kuo (University of Washington, Seattle). The passage number in HeLa cells for serovars D′, I′, and L2′ was 10, 14, and 5, respectively. Ten microliters of unpurified elementary bodies for each serovar was incubated in a solution of 10 mM Tris Cl (pH 8.0), 1 mM EDTA, and 20 mM dithiothreitol in a 100-μl volume for 5 min at room temperature and then incubated at 100°C for 10 min. Ten microliters of these preparations was used as template DNA and amplified by polymerase chain reaction (PCR) in a 100-μl volume containing 5 U of *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin-Elmer-Cetus, Norwalk, Conn.)–50 mM KCl–10 mM Tris (pH 8.2)–1.5 mM $MgCl_2$–0.01% gelatin–200 μM (each) dATP, dCTP, dGTP, and dTTP–approximately 200 ng of each oligonucleotide primer pair (Table 1). Each sample was overlaid with 100 μl of mineral oil to prevent evaporation. The samples were placed in a programmable heat block (DNA thermal cycler; Perkin-Elmer-Cetus) for 35 cycles of heating to 94°C for 1 min, cooling to 37°C for 1 min, and heating to 72°C for 1 min. The presence and size of the amplified products were evaluated by agarose gel electrophoresis.

Two sets of oligonucleotide primer pairs were constructed to flank the 5′ and 3′ ends of the *ompl* sequence encompassing both VS1 and VS2 (VS1-VS2). The first set of primers was designed to amplify VS1-VS2 from serovars Ba, D, D′, and L2′ (B complex-related serovars). The second pair was designed to amplify VS1-VS2 from serovars I and I′ (C complex-related serovars). A third set of primers to flank VS4 on conserved regions of the *ompl* sequence was synthesized for amplification of Ba, D, D′, I, I′, and L2′. Oligonucleotide primers were synthesized on a 380B DNA synthesizer (ABI, Foster City, Calif.) by the solid-phase triester method. Synthetic products were deprotected, evaporated to dryness, and purified by polyacrylamide gel electrophoresis.

To ensure sufficient quantities of double-stranded DNA for asymmetric PCR amplification, even from cultures containing few organisms, an initial symmetric amplification was

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TABLE 1. Oligonucleotides used in primer extension of double-stranded and single-stranded DNA from *C. trachomatis ompl* VS1, VS2, and VS4

Primer	Sequence	VS and serovars sequenced
DF-1[a]	5′-AAAGGATCCATGGGTGCCAAGCCT-3′	VS1 and VS2 for D, D′, and L2′
DB-2[b]	5′-AGCGAATTCAGCAAAAGTAGTATCTGT-3′	
DF-3[c]	5′-ACTGATGTGAATAAAGAATTTCAG-3′	VS1 and VS2 for I and I′
DB-2	5′-AGCGAATTCAGCAAAAGTAGTATCTGT-3′	
DF-4[a]	5′-TCCTTACATTGGAGTTAAATGGTCTC-3′	VS4 for D, D′, I, I′, and L2′
DB-4[b]	5′-CTAGATTTCATCTTGTTCAATTGC-3′	

[a] 5′-Upstream oligonucleotide used for amplification of designated VS and serovars; the primer sequence was based on a B complex-conserved region of the MOMP gene.
[b] 3′-Downstream oligonucleotide used for amplification of designated VS and serovars; the primer sequence was based on a B complex-conserved region of the MOMP gene.
[c] 5′-Upstream oligonucleotide used for amplification of designated VS and serovars; the primer sequence was based on a C complex-conserved region of the MOMP gene.

performed with unpurified chlamydial DNA. The double-stranded DNA product of this reaction was purified with a Centricon 30 microconcentrator (Amicon, Danvers, Mass.) to remove excess primers and nucleotides. The purified double-stranded DNA was then used as a substrate for asymmetric amplification (6) by using a 1:100 ratio of the 5′-flanking primer to the 3′ primer to produce single-stranded DNA for direct sequencing. The temperatures and times for the asymmetric PCR were the same as for the symmetric reactions. DNA sequencing was performed by the dideoxynucleotide chain-termination method of Sanger et al. (12). The 5′ oligonucleotide primer was employed for each sequencing reaction, and sequencing was performed by using α-^{35}S-dATP and Sequenase as described by the manufacturer (Sequenase [70700] DNA sequencing kit; U.S. Biochemical Corp., Cleveland, Ohio). DNA sequences were determined following separation on 5% polyacrylamide gels and autoradiography. A second amplification and sequencing reaction was performed on the original unpurified chlamydial DNA for each serovar to verify the respective sequences.

We utilized the PCR technique to obtain chlamydial DNA from unpurified elementary bodies and asymmetrically amplified this DNA to produce single-stranded DNA for direct sequencing of *C. trachomatis ompl* VS1, VS2, and VS4. *C. trachomatis ompl* VS1, VS2, and VS4 for serovars Ba, D, D′, I, I′, and L2′ were amplified by using oligonucleotides constructed to the respective *ompl* regions (Table 1). The respective 5′ primer was then used for the desired sequencing reaction. The nucleotide and inferred amino acid sequences of VS1, VS2, and VS4 for serovars D′, I′, and L2′ are shown in Fig. 1 in comparison with their respective prototype sequences. Amino acid substitutions were detected in VS4 for serovar D′; in VS1, VS2, and VS4 for serovar I′; and in VS2 for L2′. We have confirmed the reliability of this approach by comparing the nucleotide sequences of serovars Ba, D, and I with previously published sequences (21). This method is technically straightforward, rapid, and requires few organisms. These are distinct advantages over standard cloning and sequencing procedures.

The serovariants D′, I′, and L2′ were identified by immunotyping techniques in which a two-step microimmunofluorescence test with MOMP-specific MAbs is used (19). Recent epitope mapping studies by Baehr et al. (1) found that serovar-specific epitopes mapped to MOMP VS1 and VS2 for serovars A and L2, respectively. In addition, subspecies-, serogroup-, and species-specific epitopes were found to map to VS4. Stephens et al. (16) showed that the serovar-specific determinants of B, C, and L2 mapped to VS2 and that species-specific epitopes mapped to VS4. Although these MAbs used for typing (19) have not been mapped, the sequencing results for I′ and L2′ are probably consistent with the immunotyping and epitope mapping data for C complex- and B complex-related serovars, respectively. It has previously been proposed on the basis of presumed secondary structures that VS2 and VS4 are likely to contain serovar-specific antigens (14). In addition, Yuan et al. (21) have sequenced A to K and L1 to L3 and found considerable amino acid sequence heterogeneity in VS4 among serovars in the B complex- and C complex-related serogroups. These findings lend support to the proposition that VS4 may contain a serovariant-specific determinant or may modulate antibody binding to VS1 and VS2. However, it is also possible that sequence diversity in other regions of the MOMP may account for these findings and influence a nonlinear, conformational epitope. Additional epitope mapping studies are required to accurately define the complexity of antigenic determinants within VS4 for each *C. trachomatis* serovar and subtype.

On analysis of the nucleotide sequences for D′ and L2′, there are a few base pair substitutions in conserved regions of the MOMP gene (data not shown), which suggests that the emergence of these subtypes is not recent. Therefore, it is reasonable to assume that *ompl* accumulates point mutations that may be good markers of evolutionary and immune-selected changes within this gene. Genetic analysis of multiple samples for each serovar from different geographic regions of the world may allow for an understanding of the mechanisms of gene mutation and antigenic variation among *C. trachomatis* serovariants. The DNA extraction and sequencing method we describe will be useful for evaluating rapidly evolving base pair substitutions in *ompl* and allow for the identification of additional chlamydial subtypes. This technique provides a molecular signature of chlamydiae which should offer a more precise classification of *C. trachomatis* than current immunotyping procedures. In addition, this method may be of particular value for evaluating the molecular epidemiology of chlamydiae in trachoma-endemic areas. Usually, one or two serovars have been found to predominate in a certain geographic region. However, these serovars may represent only select types that can be recognized by current serological assays. The sequence determination of *ompl* variant regions of chlamydial isolates

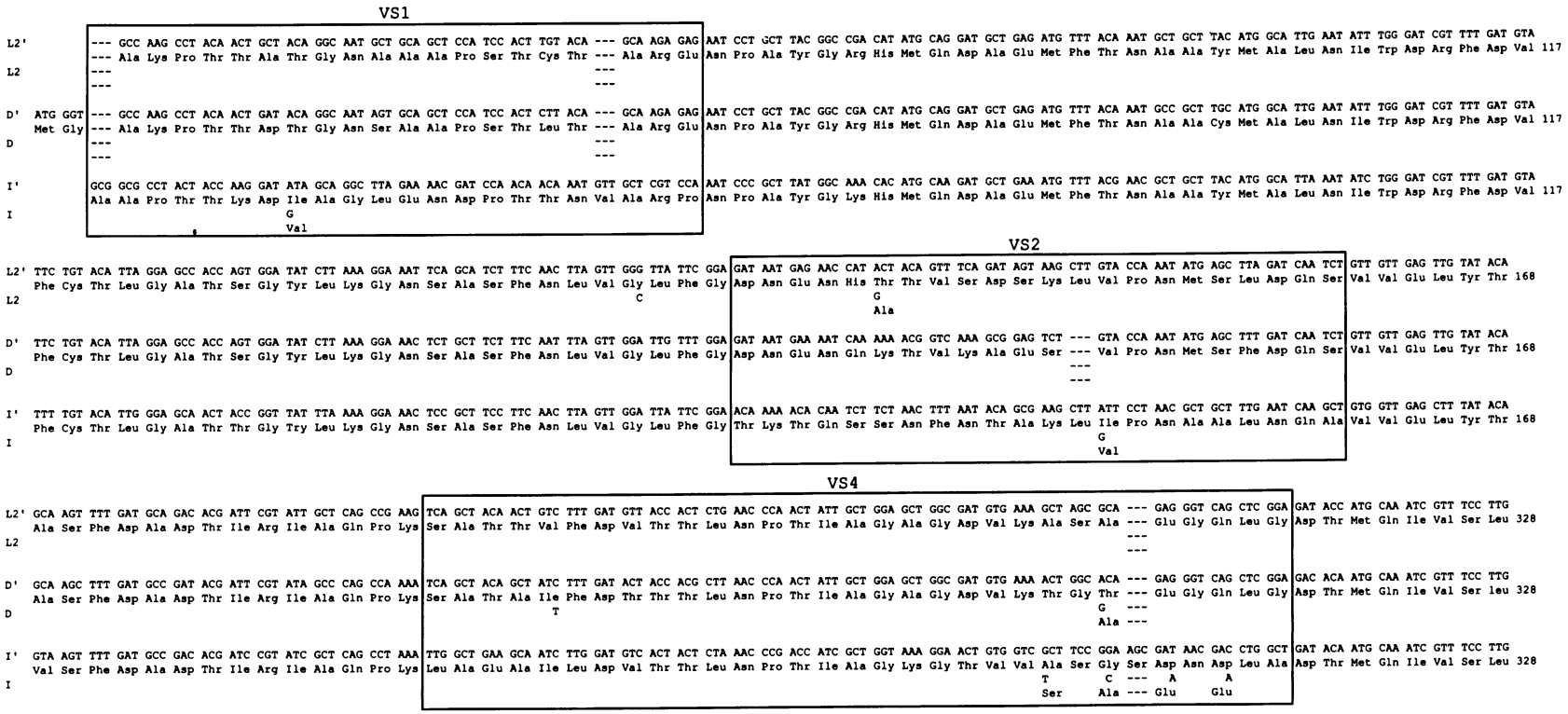

FIG. 1. Nucleotide and amino acid sequences of *C. trachomatis omp1* VS1, VS2, and VS4 for subtypes D′, I′, and L2′. Each subtype is shown in relation to prototype serovars D, I, and L2, respectively. Nucleotide and amino acid changes in the prototype serovar sequence are noted below the respective position in the subtype. The areas with three hyphens represent an absence of nucleotide sequence for the respective serovar. The boxed areas define the sequences for VS1, VS2, and VS4.

may identify new, additional subtypes that would help to evaluate *C. trachomatis* transmission patterns within households and communities in trachoma-endemic areas.

Molecular signature analysis of chlamydiae offers a precise means for detecting genetic drift within the MOMP gene. Evaluation of genetic drift would be important because the emergence of certain mutations under selective immune pressure could give rise to different antigenic determinants that may elicit unpredictable host immune responses. Previous vaccine trials have documented serovar-specific protective immune responses among humans (5) and primates (9, 18) challenged with homologous and/or heterologous serovars of chlamydiae. The emergence of new serovariants based on newly defined molecular variations in MOMP would have significant implications for vaccine development.

This research was supported by funds from the Edna McConnell Clark Foundation and by Public Health Service grants EY07757, AI21912, and EY07058 from the National Institutes of Health, and equipment funds were provided by the University of California-San Francisco Academic Senate. R.S.S. is a recipient of a research award from Research to Prevent Blindness. D.D. is the recipient of a postdoctoral fellowship from the Giannini Foundation.

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