The major outer-membrane proteins of *Chlamydia trachomatis* serovars A and B: intra-serovar amino acid changes do not alter specificities of serovar- and C subspecies-reactive antibody-binding domains


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The major outer-membrane protein (MOMP) of *Chlamydia trachomatis* is a promising candidate antigen for chlamydial vaccine development. We have sequenced the MOMP genes for a serovar A and a Serovar B isolate and have compared these new sequences with those already reported. Intra-serovar changes in the inferred amino acid sequences of the surface-exposed variable segments known to be responsible for binding of neutralizing antibody were observed. Nevertheless, epitope mapping with solid-phase peptides showed that these intra-serovar changes did not affect the binding of serovar- and subspecies-specific, potentially protective antibodies. Variable segment 1 of *C. trachomatis* serovar A contained two adjacent antibody-binding sites, one of which was C-subspecies specific while the other was serovar A specific. Therefore the subspecies binding site for C-complex organisms is in variable segment 1, whilst that for B-complex organisms is in variable segment 4. This work shows that MOMP sequences are relatively stable within the serovar categorization for isolates taken decades apart from different continents. Within a given serovar, however, limited interchange of functionally related amino acids may occur without impairing the binding of serovar-specific antibody.

Introduction

*Chlamydia trachomatis*, an obligate intracellular bacterium, is a major human pathogen responsible worldwide for both ocular and genital tract infections. *Chlamydiae* have an unusual life-cycle which consists of two morphological forms. The metabolically active reticulate bodies (RBs) undergo replication within inclusions in infected cells, ultimately giving rise to infectious elementary bodies (EBs). Fifteen serovars of *C. trachomatis*, designated A–L3, have been recognized worldwide and may be broadly grouped into two complexes according to which subspecies epitope they carry.

**Abbreviations:** BGMK, buffalo green monkey kidney; EB, elementary body; ELISA, enzyme-linked immunosorbent assay; IB, intermediate body; mAb, monoclonal antibody; MOMP, major outer-membrane protein; PCR, polymerase chain reaction; RB, reticulate body; VS, variable segment.

The nucleotide sequence data reported in this paper have been submitted to the EMBL, GenBank and DDBJ Nucleotide Sequence Databases and assigned the accession numbers M33535 (serovar A) and M33536 (serovar B).

Serovars A, B and C are the aetiological agents of trachoma, one of the world's major causes of preventable blindness. Preliminary trachoma vaccine trials using whole organisms as immunogens gave rise to short-term, serovar-specific protective immunity and, in some cases, long-term genus-specific hypersensitivity reactions which increased disease severity on subsequent reinfec tion (reviewed by Schachter & Dawson, 1978). Attention has therefore focused on the development of a subunit vaccine incorporating protective major outer-membrane protein (MOMP) epitopes from each of the three main serovars but avoiding the damaging hypersensitivity-inducing antigen. Possible intra-serovar antigenic changes in strains circulating in endemic areas therefore assume critical importance for vaccine design.

The most promising candidate antigen for a subunit vaccine is the chlamydial MOMP. This antigen is present on the surfaces of both EBs and RBs (Hatch et al., 1981) and comprises up to 60% of the EB outer envelope (Caldwell et al., 1981). A porin, MOMP plays an essential role in the differentiation of RBs into EBs and is thought to maintain structural rigidity of EBs by disulphide cross-linking (Bavoil et al., 1984). Monoclonal
antibodies (mAbs) have demonstrated that MOMP contains serovar-, subspecies-, species- and genus-specific epitopes (Stephens et al., 1982; Batteiger et al., 1986). The serovar- and subspecies-specific epitopes are surface-exposed, acting as targets for neutralizing antibody, whilst the species- and genus-specific epitopes are inaccessible to antibody and therefore non-neutralizing (Batteiger et al., 1986; Kuo & Chi, 1987; Zhang et al., 1987) although a surface-exposed species-reactive MOMP epitope has recently been described (Collett et al., 1989).

Complete DNA sequences of MOMP genes have been published for serovars L2 (Stephens et al., 1986), B, C (Stephens et al., 1987) and L1 (Pickett et al., 1987) and reported for A and B (Baehr et al., 1988). Comparison of inferred amino acid sequences reveals that MOMP contains four variable segments (VS1, 2, 3 and 4) separated by constant regions. Nucleotide sequences of these variable segments have recently been published for all fifteen serovars of C. trachomatis (Yuan et al., 1989). Antigenic relatedness of serovars reflects the level of DNA homology between MOMP genes, especially within these variable segments.

Binding of mAbs to fragments of MOMP genes expressed in λgt11, and to synthetic peptides, mapped the serovar-specific epitopes of serovars B, C, L1 and L2 to sequences within VS2 (Baehr et al., 1988; Conlan et al., 1988; Stephens et al., 1988) whilst a serovar-specific region of serovar A MOMP was localized to VS1 (Baehr et al., 1988). Subspecies- and species-specific regions for organisms in the B complex were located in VS4 (Baehr et al., 1988; Conlan et al., 1988; Stephens et al., 1988).

To evaluate the antigenic stability of MOMP epitopes we sequenced MOMP genes from a different isolate of serovar A to that previously sequenced and from a recent field isolate of serovar B. We observed intra-serovar amino acid changes between the MOMP sequences of these strains and those previously reported for the same serovars. Nested synthetic peptides encoding portions of these MOMPs were probed with mAbs to locate the serovar-specific epitopes. The observed intra-serovar amino acid changes did not inhibit binding of serovar-specific mAbs and we envisage that ultimately such epitopes will be combined to form a multi-component vaccine.

Methods

Bacterial strains and vectors. Chlamydia trachomatis A/SAl/OT was originally isolated in Saudi Arabia and was obtained from Dr J. Trehanne (Institute of Ophthalmology, London, UK). Serovar B strain B/Jali-20/OT was collected from a trachoma case by Dr D. Mabey (London School of Hygiene and Tropical Medicine, UK) in 1985 in the village of Jali, The Gambia. Escherichia coli strains used were JM101 [Δ(lac-pro) thi supE F' traD36 proAB lacI^Z ΔM15; Messing et al., 1981] and Q358 (r^C m^T su^ φ80); Karn et al., 1980). ΔEMBL3 DNA was obtained from Stratagene. DNA from JL47.1 was originally obtained from Professor W. Brammar, University of Leicester. M13mp18 and M13mp19 (Yanisch-Perron et al., 1985) were obtained from Boehringer Mannheim.

Enzymes. Restriction endonucleases, T4 DNA ligase and calf intestinal phosphatase were obtained from Boehringer Mannheim and used as recommended by the manufacturer. DNA fragments generated by restriction endonuclease digestion were usually separated using 1% (w/v) submarine agarose gels in Tris/acetate/EDTA buffer.

Oligonucleotide synthesis. Oligonucleotides were synthesized using β-cyanethyl phosphoramidite chemistry on an automated DNA synthesizer (Applied Biosystems).

Chlamydial DNA. C. trachomatis strains A/SAl/OT and B/Jali-20/OT were grown in cycloheximide-treated BGMK cells and EBs were purified as previously described (Salari & Ward, 1981). DNA was extracted from EBs by proteinase K/Sarkosyl treatment (Wenman & Lovett, 1982) followed by phenol/chloroform extraction and ethanol precipitation. Chlamydial DNA was digested to completion with relevant restriction endonucleases and 150 ng of these fragments was ligated into 1 μg of BamHI-cut ΔEMBL3 (A/SAl/OT) or JL47.1 (B/Jali-20/OT). Ligated DNAs were packaged in vitro using Gigapack Gold packaging extract (Stratagene). Bacteriophages were plated out and amplified in E. coli Q358. Approximately 3000 plaques from each library were transferred to Hybond-N (Amersham) and prepared for blotting as described by the manufacturer.

Southern blotting and plaque hybridization. Chlamydial DNA was digested to completion with a range of restriction enzymes. After separation by electrophoresis, the fragments were transferred to Hybond-N by vacuum blotting.

An N-terminal probe for serovar A MOMP of 461 bp was prepared by polymerase chain reaction (PCR; Saiki et al., 1985) using a thermal DNA cycler (Cetus), Taq DNA polymerase (Perkin-Elmer) and serovar A genomic DNA as template. Oligonucleotides used to prime the PCR contained a sequence 50 bp upstream of the MOMP gene, 5' CCGCCGAAAAAGATGAC 3' and a sequence spanning nucleotides 394–410 within the gene, 5' ACATCAGAAAGATCCCA 3'. The PCR product was simultaneously labelled by incorporation of digoxigenin-dUTP (Boehringer Mannheim). Southern blots and EMBL3 plaque lifts containing A/SAl/OT DNA were prehybridized for 4 h and hybridized overnight with 50 ng of denatured probe per filter, all at 68 °C. Filters were washed in 2× SSC for 15 min at 65 °C then in 2× SSC, 0.1% SDS for 30 min at 65 °C (1× SSC is 0.15 M-NaCl/0.015 M-trisodium citrate, pH 7.0). Hybridizations and immunological detections utilized a non-radioactive labelling and detection kit (Boehringer Mannheim), according to the manufacturer's instructions.

Southern blots and JL47.1 plaque lifts containing B/Jali-20/OT DNA were probed with the purified C. trachomatis L1 MOMP gene originally cloned in JL47.1 (Pickett et al., 1987). This probe was labelled with [α-32P]dCTP (Amersham) using a nick-translation kit (Amersham) and was denatured by boiling before use. The JL47.1 plaque lifts were prehybridized and hybridized at 65 °C under conditions described by the manufacturer. Filters were washed at 65 °C, twice in 2× SSC then once in 2× SSC with 1% (w/v) SDS. After drying, the filters were autoradiographed overnight using Kodak X-Omat AR film. Single plaques from both libraries showing homology with the appropriate probes were picked, plated at low density and re-probed. Bacteriophage DNA was prepared from single positive plaques amplified to high titre and subjected to restriction mapping.

DNA sequencing. Appropriate restriction endonuclease fragments were subcloned into bacteriophages M13mp18 and M13mp19 using E. coli and Quick Change II Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's instructions.
coli JM101 as host. A template was prepared and DNA sequencing was performed by the dideoxy chain-termination method using modified T7 polymerase (Sequenase; Cambridge Biocience) and [35S]dATP (410 Ci mmol⁻¹; 15:2 TBq mmol⁻¹; Amersham). A set of 17-mer oligonucleotides used as primers for PCR and DNA sequencing of the A/SA1/OT MOMP gene was based on conserved regions of all published MOMP gene sequences.

Monoclonal antibodies. Mice were immunized intraperitoneally with BGMK cells infected with C. trachomatis A/SA1/OT and boosted prior to fusion with NS-1 cells. Hybridomas were screened by ELISA with C. trachomatis A EBs as antigen. The B/Ba-serovars-specific mAb, B3/B9, was a kind gift of Dr K. Persson, Malmö, Sweden. Specificities of mAbs were determined by dot-blot assay against formalin-killed whole EB standards of all serovars of C. trachomatis (Washington Research Foundation, Seattle), by Western blotting and by immunogold labelling against purified EB of serovars A and C. Tissue culture supernatant (serovar A specific mAbs, A21, A30 and A36) or ascites fluid (mAb B3/B9) was used without further purification.

Peptide synthesis and immunoassay. Peptides were synthesized by the method of Geyser et al. (1984) on solid polyethylene support pins (Cambridge Research Biochemicals) and assayed by ELISA in accordance with the manufacturer's instructions and as previously described (Conlan et al., 1988). The peptides chosen for synthesis are shown in Fig. 3, as are hexamers covering 139TKTQSSGFDTANIVPNT515 from serovar A V52.

Immunogold labelling. Immunogold labelling was performed as previously described (Conlan et al., 1989), using tissue culture supernatants and protein G–gold conjugate (15 nm; Biocell) at a final dilution of 1 in 10.

Results

Screening genomic libraries of C. trachomatis serovars A and B

Southern blotting of genomic DNA from C. trachomatis serovar B showed that the entire MOMP gene from this strain was carried on a 11-5 kb BamHI restriction fragment (data not shown). However, unlike other MOMP sequences, the serovar A MOMP gene contained a previously unreported internal BamHI site. Further Southern blotting experiments indicated that the A/SA1/OT MOMP gene was located on a 5-5 kb BglII fragment. Different λ vectors were chosen for the cloning of serovar A and B MOMP genes to ensure that no confusion arose during the genetic manipulations. The λEMBL3 recombinant selected for further study contained the complete A MOMP gene within a 5-5 kb BglII fragment of A/SA1/OT DNA plus an extra 7 kb BglII genomic fragment which was cloned concurrently because λEMBL3 only accepts DNA inserts in the size range 9–23 kb. The entire MOMP gene of B/Jali-20/OT was cloned in λL47.1 on a unique 11-5 kb BamHI fragment.

Complete DNA sequences of MOMP genes from C. trachomatis serovars A and B

The complete C. trachomatis A and B MOMP coding sequences were determined for both strands by a series of overlapping MOMP18 and MOMP19 subclones. Inferred amino acid sequences of MOMP from A/SA1/OT and B/Jali-20/OT compared with those of other strains of serovars A and B are shown in Fig. 1. Nucleotide differences are summarized in Table 1.

Comparison of the MOMP gene sequence of serovar A/SA1/OT with the serovar A/Har-13 DNA sequence kindly provided by W. Baehr revealed six nucleotide differences distributed throughout the genes; most of these were transversions in the third base position of codons. Five of the changes gave rise to alterations in the inferred amino acid sequence. VS2, 3 and 4 (as defined by Yuan et al., 1989) were identical in the two isolates but two amino acid changes occurred in VS1. One of these changes in VS1 was an alanine to valine substitution at residue number 80 within the serovar-A-specific epitope defined by Baehr et al. (1988): 139DVAGLEKDPVA80. The other change was a serine to arginine substitution at residue 69, just in front of the epitope. The effects of the amino acid changes observed in VS1 on binding of three serovar-specific mAbs were investigated by solid-phase peptide synthesis. VS2 of serovar A, identical in both A/SA1/OT and A/Har-13, was also synthesized as overlapping peptides and probed with the same mAbs. The remaining three amino acid changes in serovar A occurred in regions which are normally constant and which have not been implicated in immunogenicity.

Comparison of the B/Jali-20/OT MOMP gene sequence with the B/TWS/OT MOMP gene sequences published by Stephens et al. (1987) and reported by Baehr et al. (1988) revealed twelve nucleotide changes, which gave rise to five amino acid changes. In VS2, an alanine to threonine substitution at residue 150 occurred within the serovar-B-specific epitope defined by Stephens et al. (1988) as 139NNEQTKVSNGAFV152. The effect of this change on binding of a serovar-specific mAb was investigated by solid-phase peptide synthesis. The equivalent sequences from VS2 of B/TWS/OT and the related serovar Ba/AP-2 (Yuan et al., 1989) were also synthesized as overlapping peptides and probed with the B/Ba-serovar-bispecific mAb, B3/B9.

At amino acid 74 in VS1, an alanine in B/Jali-20/OT was replaced by valine in the two published B/TWS/OT sequences. However, neither serovar- nor subspecies-specificities have been allocated to VS1 of serovar B. VS3 and VS4 were identical for the three serovar B sequences compared.

Monoclonal antibody specificities

mAb A21 was specific for serovars A and J by dot-blot and with serovars A and C by ELISA. mAb A30 was specific for serovars A and C by dot-blot and for serovars A, C, L1, L3 and B by ELISA. Therefore mAbs A21 and
A30 showed limited subspecies specificity, depending upon the assay system. mAb A36 was specific for C. trachomatis serovar A by dot-blot and ELISA. mAbs A21, A30 and A36 all labelled intact EBs of serovar A, but not serovar C, with colloidal gold (Fig. 2). mAb B3/B9 was specific for C. trachomatis serovars B and Ba by micro-immunofluorescence, and polyclonal antipeptide sera with serovar specificity covering the binding site of mAb B3/B9 have been shown to recognize the surface of EBs (Conlan et al., 1989). Western blotting demonstrated that each of the four mAbs used was specific for MOMP. mAb specificities are summarized in Table 2.

**Localization of subspecies- and serovar-specific epitopes on serovar A MOMP**

mAbs A21, A30 and A36 were assayed against overlapping hexapeptides spanning VS1 and VS2 of C. trachomatis A/SA1/OT. No reactivities were observed with VS2 peptides for any of the mAbs assayed. The
Table 1. (a) **Amino acid and corresponding nucleotide changes between isolates of C. trachomatis serovar A**

The *C. trachomatis* A/SA1/OT sequence is from this study; the A/Har-13 sequence was supplied by W. Baehr (Rocky Mountain Laboratories, Hamilton, Mont., USA).

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(b) **Amino acid and corresponding nucleotide changes between isolates of C. trachomatis serovar B**

The *C. trachomatis* B/Jali-20/OT sequence is from this study; the B/TWS/OT (Stephens) sequence is from Stephens *et al.* (1987). The B/TWS/OT (Baehr) sequence was supplied by W. Baehr.

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Table 2. **Immunological specificity of mAbs determined by dot-blot, ELISA and immunogold labelling**

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* Specificity of B3/B9 determined by micro-immunofluorescence.

Reactivities of VS1 peptides with representative mAbs A30 and A36 are summarized in Fig. 3(a, b).

mAb A30 reacted with three peptides in VS1, spanning the sequence <sup>69</sup>RDVAGLEK<sup>76</sup>. The minimum epitope included in all three peptides was <sup>71</sup>VAGL<sup>74</sup>. This four-amino-acid sequence is common to both serovar A strains and it also occurs in VS1 of C-complex serovars C, I and J (Yuan *et al.*, 1989). Reactivity of mAb A21 with synthetic peptides was identical to that of A30.

mAb A36 reacted with four peptides in VS1, spanning the sequence <sup>77</sup>AGLEKDPV<sup>80</sup>. The minimum epitope required for binding was <sup>77</sup>EKD<sup>77</sup>, which is unique to serovar A, occurring in both A/SA1/OT and A/Har-13 (Yuan *et al.*, 1989). Reactivity of the mAb with hexapeptide GLEKDP, in which the binding site is centrally located, was half of that with peptides AGLEKD, in which the binding site is located at the C terminus, and LEKDPV. Presumably this reflects the influence of flanking amino acid sequences on both sides of the critical binding site, which promote mAb recognition but are not essential.

Taken together, these data localize two distinct epitopes to serovar A VS1, one a subspecies-specific epitope (VAGL) and the other a serovar-specific epitope (EKD). Neither of the amino acid changes that we observed in VS1 of serovar A are included within these epitopes; hence the substitutions do not affect binding of serovar- and C-complex specific, potentially neutralizing antibodies.
Fig. 2. Immunogold labelling of viable C. trachomatis EBs. (a) Serovar A, mAb A21 (tissue culture supernatant); (b) serovar A, mAb A30 (tissue culture supernatant); (c) serovar A, mAb A36 (ascites); (d) serovar C, mAb A21 (tissue culture supernatant). The gold particles are 15 nm in diameter. The bar represents 0.1 μm.

No evidence was found for serovar- or subspecies-specific epitopes in VS2 of serovar A; here serovar A MOMP clearly differs from serovars B, C, L1 and L2.

Localization of serovar-B-specific epitopes

mAb B3/B9 was assayed against overlapping synthetic hexapeptides spanning VS2 of C. trachomatis B/TW5/OT (Stephens et al., 1987), B/Jali-20/OT and Ba/AP-2 (Yuan et al., 1989). The results are summarized in Fig. 3(c–e). For B/TW5/OT, mAb B3/B9 recognized two peptides which spanned the sequence 149GAFVPM155 and the minimum epitope required for binding was 150AFVPM153 (Fig. 3c). For B/Jali-20/OT, mAb B3/B9 recognized two peptides spanning the sequence 149GTFVPM155 with a minimum binding site of 150TFVPM154 (Fig. 3d). For Ba/AP-2, mAb B3/B9 recognized two peptides spanning the sequence 149GTFVPM155 with a minimum binding site of 150TFVPM153 (Fig. 3e). These data demonstrate that although the amino acid substitution within serovar B occurred at the critical binding site of mAB B3/B9, the substitution of threonine for alanine did not alter binding and serovar specificity.

Discussion

Epitope mapping of VS1 and VS2 from C. trachomatis serovar A using solid-phase peptides revealed adjacent subspecies- and serovar-specific epitopes in VS1, with no evidence for serovar-specific epitopes in VS2. Baehr et al. (1988) identified the proposed A-specific epitope 70DVAGLEKDPV80 in VS1 of A/Har-13 by binding of an A serovar-specific mAb to a minimal sequence shared by overlapping Agt11 subclones of the serovar A MOMP gene. We now report that this sequence contains two epitopes, 71VAGL74 (subspecies-specific) and 75EKD77 (serovar-specific), which are adjacent and non-overlapping. By contrast, in serovar B, serovar-specificity is located in VS2 and two adjacent B-subspecies specific epitopes are found in VS4 (Baehr et al., 1988; Conlan et al., 1989). The C-complex-specific mAb A30 recognized 71VAGL74 in VS1 of serovars A and C by dot-blot and ELISA but surface-labelled whole EBs of serovar A only. This suggests that this C-subspecies specific epitope is surface-exposed in serovar B but not in serovar C. mAb A30 also reacted in ELISA with EBs of serovars L1, L3 and B. Serovar L3 MOMP contains the related sequence, 71TAGL74, where the threonine at position 71 may be an acceptable substitution for valine within the binding site of mAb A30. Serovars L1 and B do not contain VAGL or any closely related peptide within the primary sequence of MOMP. It is possible that mAb A30 reacts in ELISA with other antigens of these serovars which contain an appropriate binding site, or that it has some additional reactivity of which we are not aware.

Comparison of inferred amino acid sequences of MOMPs of two serovar A strains revealed five amino acid changes, none of which affected binding of the C-complex or serovar-specific mAbs. Of these amino acid changes, two occurred in VS1. Firstly, at residue 69, a neutral serine in A/Har-13 was replaced in A/SA1/OT by a basic arginine. This replacement did not occur within the critical binding sites of mAbs A21, A30 and A36 and so did not affect reactivity of these mAbs. Secondly, at position 80, alanine in A/Har-13 was replaced by a valine in A/SA1/OT. Both of these amino acids are neutral and hydrophobic. Although this substitution occurs within the A-specific epitope defined by Baehr et al. (1988), it did not affect binding of mAbs A21, A30 and A36. At position 259, the hydrophobic phenylalanine in A/Har-13 is replaced with hydrophilic serine in A/SA1/OT. This change lies outside the four variable segments and is not thought to be located within a serovar- or subspecies-specific epitope. The other two amino acid changes between the serovar A strains involve exchanges between neutral, hydrophobic amino acids in constant regions of the molecule which have not been associated with immunogenicity.

The C. trachomatis MOMP gene sequences published to date have all been from laboratory-adapted strains. We were concerned that such strains might differ from...
MOMP genes of Chlamydia trachomatis

Disease-causing strains circulating in endemic areas. C. trachomatis B/Jali-20/OT was one of a series of recent trachoma-causing serovar B isolates from our field study centre in The Gambia. The amino acid sequence of MOMP was remarkably conserved between this strain and the laboratory-adapted B/TWS/OT, with only five amino acid differences. One of these amino acid changes occurred in VS2 within the B-serovar specific region defined by Stephens et al. (1988), where a hydrophobic alanine in B/TWS/OT is replaced in B/Jali-20/OT by a hydrophilic threonine at position 150 of the mature protein. Despite the critical position of this substitution and the differing polarity of the exchanged amino acids, binding of the serovar-specific mAb B3/B9 was not affected, both minimal recognition sites SoAFVP153 and SoTFVP153 being acceptable. In VS1, an alanine in B/Jali-20/OT is replaced by a valine in B/TWS/OT. These are both neutral, hydrophobic residues and it is unlikely that this substitution affects a protective epitope since VS1 of serovar B has not been associated with serovar- or subspecies-specificity. The three other amino acid changes between the compared serovar B strains occurred outside variable segments.

Conlan et al. (1989) have shown that a synthetic peptide encompassing the critical binding site of mAb B3/B9 (150AFVP153) is immunogenic in rabbits. Antisera to this peptide reacted with the surfaces of native EBs and intermediate bodies (IBs), indicating that this epitope is surface-exposed in situ. Serovar-specific, surface-exposed epitopes have potential to induce neutralizing, protective antibodies and remain accessible to them. We have now defined subspecies-specific epitopes for B-complex (Conlan et al., 1989) and C-complex trachoma-causing serovars. We have demonstrated that serovar-specific epitopes of serovars A and B retain the ability to bind subspecies- and serovar-specific antibodies, despite amino acid substitutions in or around the critical antibody-binding sites. Such antigenic stability
of the protective subspecies- and serovar-specific epitopes within the serovars is encouraging for future chlamydial vaccine design. Whether these epitopes also show immunogenic stability is the subject of our current research.

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References


