DIAGNOSTIC MICROBIOLOGY

Detection of *Chlamydia trachomatis*-specific antibodies in human sera by recombinant major outer-membrane protein polyantigens

PER MYGIND, GUNNA CHRISTIANSEN, KENNETH PERSSON* and SVEND BIRKELUND

Department of Medical Microbiology and Immunology, University of Aarhus, DK-8000 Aarhus, Denmark and

*Department of Virology, University Hospital, Malmö, Sweden

This study was performed to generate and evaluate recombinant antigens for use in a species-specific *Chlamydia trachomatis* immunoassay. In a molecular genetic approach, fragments of the *C. trachomatis* major outer-membrane protein (MOMP) were produced as fusion proteins to create three different constructs encompassing the variable domains I, II and IV of selected *C. trachomatis* serovars. The recombinant MOMP polyantigens were affinity-purified and used in an enzyme-linked immunosorbent assay. Antibody detection was evaluated with 103 patient sera and the results were compared with titres obtained in the micro-immunofluorescence test. The results showed that the generated MOMP polyantigens detected the presence of *C. trachomatis*-specific human antibodies with little cross-reaction to *C. pneumoniae*-specific antibodies. When compared to the micro-immunofluorescence assay the MOMP polyantigen detected the presence of anti-*C. trachomatis* IgG antibodies with a sensitivity of 80% and a specificity of 91%.

Introduction

The eubacterial genus *Chlamydia* comprises a group of obligately intracellular pathogens that proliferate through a unique biphasic developmental cycle. The organisms alternate between an infective (elementary body, EB) and a replicating form (reticulate body, RB). The genus consists of four species – *C. trachomatis*, *C. pneumoniae*, *C. pecorum* and *C. psittaci*. *C. trachomatis* and *C. pneumoniae* cause a wide variety of infections in man, whereas *C. psittaci* and *C. pecorum* are animal pathogens of particular interest in veterinary medicine. Occasionally, *C. psittaci* causes severe infections of the respiratory tract in man known as psittacosis or ornithosis.

C. trachomatis is divided into serovars, all of which are human pathogens except the mouse pneumonitis serovar (MoPn). The trachoma biovars (serovars A–C) cause conjunctivitis, leading to trachoma, the primary cause of preventable blindness in third world countries. Serovars D–K are all pathogens of the urogenital tract. They are the most common cause of sexually transmitted genital infections, eliciting local

acute epithelial infections, which can lead to pelvic inflammatory disease. Chronic infections by *C. trachomatis* serovars D–K occasionally cause tubal obstruction or ectopic pregnancy. The serovars L1–L3 of *C. trachomatis* infect both macrophages and epithelial cells and are spread systemically through the lymphatic tissue, causing the invasive disease known as lymphogranuloma venereum [1].

C. pneumoniae causes pneumonia, bronchitis and pharyngitis [2]. Recently, this human pathogen has been associated with chronic disease such as asthma, chronic bronchitis, acute myocardial infarction and coronary artery disease [3–5]. Studies show that anti-*C. pneumoniae* antibodies are widespread in the adult population (~50%) [6].

The aim of this study was to develop a serological assay able to measure specific antibodies to *C. trachomatis* genital serovars with little cross-reaction to *C. pneumoniae*-specific antibodies. In cases of upper genital tract infection, it is important to use serology tests that can distinguish antibodies to *C. trachomatis* from those associated with non-genital chlamydiae. This is the main problem of Chlamydia serology today. A large retrospective study showed that antibodies to *C. pneumoniae* account for up to half of all chlamydia IgG-positive patients attending genitourinary medicine clinics [7]. Similar results were obtained when

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Corresponding author: Dr P. Mygind (e-mail: perm@biobase.dk).

analysing sera from anti-chlamydia IgG-positive rheumatological patients [8]. The main reason for this is the low specificity of commonly used chlamydial serology tests.

The chlamydial micro-immunofluorescence test (MIF) is the only differential serodiagnostic tool generally applied [9]. This test is based on the measurement of antibodies to surface-exposed membrane components of purified *Chlamydia* serovars. The outer membrane of *Chlamydia* spp. consists primarily of the major outer-membrane protein (MOMP), outer-membrane protein 2 (Omp2), outer-membrane protein 3 (Omp3) and a loosely associated genus-specific lipopolysaccharide (LPS). MOMP, Omp2 and LPS have been identified as the major immunodominant chlamydial antigens [10, 11].

The only commercially available recombinant chlamydia serology test is based upon LPS (Medac, Germany). LPS is highly immunogenic in human chlamydial infections as it contains a surface-exposed epitope. The epitope is genus-specific and is, therefore, not suitable as an antigen in chlamydial species-specific serology [12, 13].

Omp2 is a general target of the immune system in both *C. trachomatis* and *C. pneumoniae* infections [10, 11, 14]. An analysis of the humoral immune response towards recombinant Omp2 has nevertheless failed to detect any species-specific epitopes [15]. No surface exposure of the protein has been detected [16–18].

MOMP is a primary target of serovar-specific antibodies [19]. It is considered to be a porin, spanning the outer membrane of all *Chlamydia* species. Infections with *C. trachomatis* induce a strong humoral response mainly directed against three variable, surface-exposed segments of this protein (VS-I, VS-II and VS-IV) [20, 21]. These regions are also the basis of the *C. trachomatis* serovar typing. *C. pneumoniae* MOMP does not seem to be surface exposed and it is less immunogenic in infections caused by this pathogen [22, 23]. Therefore, MOMP is an obvious candidate in a *C. trachomatis*-specific serological assay. Unfortunately, MOMP harbours non-resolved immunoglobulin-binding properties, making the mature protein less suitable for chlamydial serology [11].

The present study selected and purified *C. trachomatis*-specific immunodominant regions of MOMP as recombinant polyantigens (VS-I, VS-II and VS-IV). Fusion proteins that were generated were applied in a *C. trachomatis*-specific serological assay. The study analysed the presence of IgG antibodies against these MOMP polyantigens in a wide panel of human sera with different serological evidence of *C. trachomatis*, *C. pneumoniae* or no chlamydial infection. Results obtained by enzyme-linked immunosorbent assay (ELISA) were compared to the 'gold' standard of

chlamydial serology, the MIF test [9]. Preliminary results of this study were presented at the Ninth International Symposium on Human Chlamydial Infections [24].

Materials and methods

Chlamydial strains

C. trachomatis serovar E was obtained from the American Type Culture Collection (ATCC). Strains used in the MIF protocol have been described previously [25].

Serum samples

Serum samples were collected from genitourinary clinic patients, patients with suspected ornithosis and from apparently healthy, asymptomatic donors.

MIF

The protocol used to obtain MIF results has been described previously [25]. *C. pneumoniae* IOL-207, *C. psittaci* 6BC and *C. trachomatis* serovars D–K were included in the assay. End-point titrations of reactive sera were performed and geometric mean titres were calculated for positive sera. Titres ≥ 16 were considered positive by the assay.

Characterisation of human sera by MIF

Sera were sorted into four groups, according to serum reactivity as measured by MIF with fixed whole cells of *C. trachomatis* and *C. pneumoniae*. Sera of group I (positive for both *C. pneumoniae* and *C. trachomatis* by MIF) were selected from women who were culture-positive for *C. trachomatis* (19 sera). Group II consisted of sera from normal healthy women who had been screened by MIF for anti-chlamydial antibodies and found to be positive for *C. trachomatis* only (20 sera). Sera from patients examined for *C. pneumoniae* infection or ornithosis where only anti-*C. pneumoniae* antibodies had been found constituted group III (19 sera). This group also included 11 sera from apparently healthy donors (asymptomatic patients). Sera without anti-chlamydial antibodies (MIF) at routine screening constituted the fourth group (34 sera).

Cloning of momp gene fragments into expression vector pET9d-6His

Gene fragments encoding a 6-mer histidine tag and selected regions of *C. trachomatis* *momp* genes were introduced into the expression vector pET9d [26, 27]. Four pairs of oligonucleotides (Table 1) were used in the cloning strategy, which is outlined in Fig. 1. The oligonucleotides were synthesised by DNA Technology, Denmark.

Table 1. Oligonucleotides used to generate recombinant MOMP polyantigens

Oligonucleotide set	Oligonucleotide sequences	Encoded protein fragment
H	<p>BamHI BglII</p> <p>5'- <u>GA TCC</u> CAC CAC CAT CAC CAC CAT TAA...-3'</p> <p>3'- ...G GTG GTG GTA GTG GTG GTA <u>ATT CTA G</u> -5'</p> <p style="text-align: center;">S H H H H H H *</p> <p style="text-align: center;">(complementary strands)</p>	-SHHHHHH* (C-term Histag)
1	<p>NcoI</p> <p>5'- <u>CCATGGG</u>GAGACTTTGTTTTCGACCG -3'</p> <p>5'- <u>GGATCCGGGGCC</u>AGCGTATTGGAAAGAAGCCCC -3'</p> <p style="text-align: center;">BamHI ApaI</p>	<i>C. trachomatis</i> serovar E MOMP _{aa66-aa216}
2	<p>ApaI</p> <p>5'-...<u>C</u> GCT CTG GCT GGT TCC GGT AAC ACC ACC</p> <p>3'-<u>CCGGG</u> CGA GAC CGA CCA CGA AGG CCA TTG TGG TGG</p> <p style="text-align: center;">A L A G A S G N T T</p> <p>TCC ACC CTG TCC AAA CTG GTT GAA GCT AAA CCG ACC</p> <p>AGG TGG GAC AGG TTT GAC CAA CTT CGA TTT GGC TGG</p> <p>S T L S K L V E A K P T</p> <p>ACC GAC ACC GGT AAC TCC GCT GCT CCG TCC ACC CTG</p> <p>TGG CTG TGG CCA TTG AGG CGA CGA GGC AGG TGG GAC</p> <p>T D T G N S A A P S T L</p> <p>ACC ACG CGT <u>G</u>... -3'</p> <p>TGG TGC GCA <u>CCT AG</u> -5'</p> <p style="text-align: center;">T T R BamHI</p> <p style="text-align: center;">(complementary strands)</p>	<i>C. trachomatis</i> serovar D-F/G MOMP _{aa86/87-aa102/104}
3	<p>BamHI BglII</p> <p>5'-<u>GA TCC</u> ACA ACC CTT AAC CCA ACT ATT GCA <u>A</u>... 3'</p> <p>3'-...<u>G</u> TGT TGG GAA TTG GGT TGA TAA CGT <u>TCT AG</u>-5'</p> <p style="text-align: center;">S T T L N P T I A</p> <p style="text-align: center;">(complementary strands)</p>	<i>C. trachomatis</i> serovar E MOMP _{aa317-aa324}

A gene fragment encoding a 6-mer histidine tag used in subsequent purification of fusion proteins was initially introduced into the *Bam*HI restriction endonuclease cleavage site of plasmid pET9d (Fig. 1, step 1). The oligonucleotide set H is made up of complementary strands, creating an upstream *Bam*HI sequence overhang and a downstream identical *Bgl*II sequence overhang (Table 1). The pET9d vector was digested with *Bam*HI and re-ligated by using the T4 DNA-ligase in the presence of equimolar amounts of oligonucleotide set H [26] (Boehringer Mannheim GmbH, Germany). Recombinant pET9d plasmids were electrotransformed into *Escherichia coli* XLI-blue competent cells and purified by alkaline lysis of recombinant bacteria [26]. Selected plasmids were DNA sequenced to ensure the correct orientations and nucleotide content of the introduced fragment. This was done bidirectionally with plasmid-specific primers, the ABI Prism dye terminator cycle sequencing ready reaction kit and an ABI Prism 377 DNA sequencer as described by the manufacturer (Perkin-Elmer, CT, USA). Upon ligation of oligonucleotide set H into pET9d, only one *Bam*HI restriction endonuclease site is maintained (upstream of the encoded histidine tag).

By using the *Nco*I restriction endonuclease site of the pET9d vector located further upstream of the introduced histidine-encoding gene fragment and the generated *Bam*HI restriction endonuclease site, it was possible to include a specific *momp* sequence upstream of the histidine-encoded fragment maintaining the

correct reading frame. *C. trachomatis* serovar E is the most widespread genital strain, causing c. 38–52% of *C. trachomatis* urogenital infections [28–30]. PCR with *C. trachomatis* serovar E genomic DNA and primer set 1 was used to amplify the *momp* gene fragment encoding *C. trachomatis* serovar E MOMP_{aa66-aa216} (Fig. 2, Table 1). The PCR protocol used included 30 amplification cycles (30 s at 94°, 30 s at 50°C and 30 s at 72°C) (Boehringer Mannheim GmbH). The PCR product was ligated into pCRTM-II vector and recombinants were transformed into *E. coli* INVαF' as described (Invitrogen, USA). After plasmid isolation and DNA sequencing, the amplified DNA fragment was moved into the histidine-encoding plasmid by digestion of both the recombinant pCRTM-II vector and the histidine-encoding pET9d with *Nco*I/*Bam*HI restriction endonucleases and subsequent re-ligation with T4 DNA-ligase (Boehringer Mannheim GmbH) (Fig. 1, step 2) [26]. By transformation of recombinant plasmid in *E. coli* XL-I blue and selecting for kanamycin resistance (present in the pET9d vector), clones harbouring the plasmid encoding antigen 1 were obtained (Fig. 2). In addition to the upstream *Nco*I site and a downstream *Bam*HI site, an *Apa*I restriction endonuclease site was introduced downstream of the *momp* encoding sequence, to facilitate subsequent cloning of the fusion construct (Table 1, Fig. 1).

Epidemiological studies have shown that *C. trachomatis* serovars D, E, F and G are responsible for 83–86% of all *C. trachomatis* genital infections [28–30]. To

Fig. 1. Illustration of the cloning strategy applied to create the plasmid constructs encoding antigens 1–3 used in this study. Step 1: digestion of plasmid pET9d with *Bam*HI and re-ligation with phosphorylated, complementary oligonucleotide set H. Step 2: *Nco*I/*Bam*HI digestion of plasmid pET9d and re-ligation with PCR fragment amplified by primer set 1. Step 3: digestion with *Apa*I/*Bam*HI and re-ligation with phosphorylated oligonucleotide set 2. Step 4: *Bam*HI digestion and re-ligation with phosphorylated, complementary oligonucleotide set 3. See *Materials and methods* for details. Colours used in this illustration are as given for Fig. 2.

allow detection of antibodies against heterologous *C. trachomatis* serovars, a gene fragment encoding the MOMP VS-I regions of serovars F/G (identical regions, ⁸⁷ALAGASGNTTSTLSKLVE¹⁰⁴) and serovar D (⁸⁶AKPTTDTGNSAAPSTLT¹⁰²) was introduced (Fig. 2). (Jones *et al.* have reported that this region is

recognised by antibodies in sera from trachoma patients [21].) This was done by synthetically producing a set of oligonucleotides encoding both *momp* fragments (Table 1, oligonucleotide set 2). These complementary oligonucleotides were synthesised to include an upstream *Apa*I and a downstream *Bam*HI sequence overhang. This fragment was introduced into the fusion protein construct encoding antigen 1, by *Apa*I/*Bam*HI digestion of the plasmid and re-ligation in the presence of the oligonucleotide set 2 (Table 1; Fig. 1, step 3). Correct cloning was ensured by DNA sequencing of resulting recombinant plasmids.

MOMP VS-IV is recognised by neutralising murine monoclonal antibodies (MAbs) [20]. Therefore, a gene fragment encoding *C. trachomatis* MOMP_{aa317–aa324} was included in the final fusion protein construct (Fig. 2). This region is conserved in all genital serovars of *C. trachomatis* except for serovar K and is very different from the homologous regions of both *C. pneumoniae* and *C. psittaci* MOMP (Fig. 2). The variable segment IV of *C. psittaci* MOMP has been successfully applied in the serological detection of abortigenic *C. psittaci* infection in ruminants [31]. The coding region was generated by oligonucleotide set 3 and as in the initial cloning of the histidine fragment, upstream *Bam*HI and downstream *Bgl*II restriction endonuclease sequence overhangs were introduced (Table 1). The plasmid construct encoding antigen 2 was digested with *Bam*HI and re-ligated in the presence of oligonucleotide set 3 (Fig. 1, step 4). Sequencing of the resulting construct demonstrated that this final plasmid contained the sequence encoding antigen 3 (Fig. 2).

Production and purification of fusion proteins

To express and purify plasmid-encoded fusion proteins, the recombinant pET9d vectors were electrotransformed into *E. coli* BL21-DE3. Cultures were grown in the presence of kanamycin (30 µg/ml). During log-phase growth, the bacterial production of pET9d-encoded fusion proteins was induced with isopropyl β-D-thiogalactoside (IPTG, 0.4 mM) [27]. The C-terminal-encoded histidine tag included in all fusion proteins made it possible to purify the recombinant proteins [32] by affinity chromatography under denaturing conditions with a Ni⁺⁺-NTA resin and a pH gradient (Quiagen, CA, USA) [15]. The eluates were neutralised with a 1 in 10 volume of 1 M Tris-HCl buffer (pH 8) and analysed by SDS-PAGE and Coomassie Blue staining [15].

ELISA

Antigenicity of purified fusion proteins was measured by ELISA with the selected panel of sera. Polysorb microtitation plates (Nunc, Denmark) were coated for 2 h with 50 µl (5 µg/ml) of recombinant protein in 50 mM carbonate buffer (pH 9.6). Excess binding capacity was blocked by adding 200 µl of fetal calf

a

Variable Segment I		
<i>C. trachomatis</i> A	GDFVFDRLVKTVDNKEFQ. M AAPTTRDVAGLEKDPVNVARNPNPAYGKHMQDAEMFTNAAYMALNIWDRFDVFCTLGATTG	
<i>C. trachomatis</i> B	GDFVFDRLVKTVDNKEFQ. MGAKPPTT. TGNAPAP. STLTARENPAAYGRHMQDAEMFTNAACMALNIWDRFDVFCTLGASSG	
<i>C. trachomatis</i> Ba	~~~~~DVNKEFQ. MGAKPATT. TGNATAP. STLTARENPAAYGRHMQ.	
<i>C. trachomatis</i> C	GDFVFDRLVKTVDNKEFQ. MGAAPTTSVAGLQNDPTINVARPNPAYGKHMQDAEMFTNAAYMALNIWDRFDVFCTLGATTG	
<i>C. trachomatis</i> D	~~~~~DVNKEFQ. MGAKPPTT. TGNAPAP. STLTARENPAAYGRHMQ.	
<i>C. trachomatis</i> E	GDFVFDRLVKTVDNKEFQ. MGDKPTST. TGNATAP. TLTARENPAAYGRHMQDAEMFTNAACMALNIWDRFDVFCTLGASSG	
<i>C. trachomatis</i> F	GDFVFDRLVKTVDNKEFE. MGAALAGA. SGNTTSTLSKLVETNPAYGKHMQDAEMFTNAACMTLNIWDRFDVFCTLGATSG	
<i>C. trachomatis</i> G	~~~~~DVNKEFE. MGAALAGA. SGNTTSTLSKLVETNPAYGKHMQ.	
<i>C. trachomatis</i> H	GDFVFDRLVKTVDNKEFQ. MGAAPTNDADLQNDPNTNVARPNPAYGKHMQDAEMFTNAAYMALNIWDRFDVFCTLGATTG	
<i>C. trachomatis</i> I	~~~~~DVNKEFQ. MGAAPTNDADLQNDPNTNVARPNPAYGKHMQ.	
<i>C. trachomatis</i> J	~~~~~DVNKEFQ. MGAAPTSDVAGLQNDPTNVARPNPAYGKHMQ.	
<i>C. trachomatis</i> K	~~~~~DVNKEFQ. MGAAPTSDVAGLQNDPTNVARPNPAYGKHMQ.	
<i>C. trachomatis</i> L1	GDFVFDRLVKTVDNKEFQ. MGAKPAT. TGNAPAP. STLTARENPAAYGRHMQDAEMFTNAAYMALNIWDRFDVFCTLGATSG	
<i>C. trachomatis</i> L2	GDFVFDRLVKTVDNKEFQ. MGAKPPTA. TGNAPAP. STLTARENPAAYGRHMQDAEMFTNAAYMALNIWDRFDVFCTLGATSG	
<i>C. trachomatis</i> L3	GDFVFDRLVKTVDNKEFQ. MGAEPSTSDTAGLNDPTNVARPNPAYGKHMQDAEMFTNAAYMALNIWDRFDVFCTLGATTG	
Amino acid no.	...70.....80.....90.....100.....110.....120.....130.....140....	
<i>C. pneumoniae</i> AR388	GDYVFDRLVKTVDNKEFQ. MGAKP. ... GSAAANYTTAVDRPNPAYNKLHDAEWFTNAGFIALNIWDRFDVFCTLGASNG	
<i>C. psittaci</i> 6BC	GDYNFDRLVKTVDNKEFQ. MGAAPTPTQATGNASNTNQPEANGRNPIAYGRHMQDAEWFSNAAPLALNIWDRFDVFCTLGASNG	
Variable Segment II		
<i>C. trachomatis</i> A	YLKGNASASFNVLGLFGTK. TQSSGFDTANIPNTALNQAVVELYTDTTFAWSVGARAALWECGCATLGASFQYA	TTLNPTIA
<i>C. trachomatis</i> B	YLKGNASASFNVLGLFGNN. ENQTKVSNGAFVPMNSLDQSVVELYTDTTFAWSVGARAALWECGCATLGASFQYA	TTLNPTIA
<i>C. trachomatis</i> Ba	NLVGLFGNN. ENQTKVSNGTFVPMNSLDQSVVELYTDTTFAWSVGARAALWECGCATLGASFQYA	TTLNPTIA
<i>C. trachomatis</i> C	YLKGNASASFNVLGLFGTK. TQSSSFNTAKLIPNTALNEAVVELYINTTFAWSVGARAALWECGCATLGASFQYA	TTLNPTIA
<i>C. trachomatis</i> D	NLVGLFGDN. ENQTKVKAES. VPMNSFDQSVVELYTDTTFAWSVGARAALWECGCATLGASFQYA	TTLNPTIA
<i>C. trachomatis</i> E	YLKGNASASFNVLGLFGDN. ENQSTVTKNS. VPMNSLDQSVVELYTDTTFAWSVGARAALWECGCATLGASFQYA	TTLNPTIA
<i>C. trachomatis</i> F	YLKGNASASFNVLGLFGDG. VNATKPAADS. IPNVQLNQSVVELYTDTTFAWSVGARAALWECGCATLGASFQYA	TTLNPTIA
<i>C. trachomatis</i> G	NLVGLFGDG. ENATQPAATS. IPNVQLNQSVVELYTDTTFAWSVGARAALWECGCATLGASFQYA	TTLNPTIA
<i>C. trachomatis</i> H	YLKGNASASFNVLGLFGTK. TKSSDFNTAKLVPNIALNRVVELYTDTTFAWSVGARAALWECGCATLGASFQYA	TTLNPTIA
<i>C. trachomatis</i> I	NLVGLFGTK. TQSSNFNTAKLVPNIALNRVVELYTDTTFAWSVGARAALWECGCATLGASFQYA	TTLNPTIA
<i>C. trachomatis</i> J	NLVGLFGTK. TQASSFNTAKLVPNIALNRVVELYTDTTFAWSVGARAALWECGCATLGASFQYA	TTLNPTIA
<i>C. trachomatis</i> K	NLVGLFGTK. TQYSKFNTAKLVPNIALNRVVELYTDTTFAWSVGARAALWECGCATLGASFQYA	TTLNPTIA
<i>C. trachomatis</i> L1	YLKGNASASFNVLGLFGDN. ENQSTVKKDA. VPMNSPDQSVVELYTDTTFAWSVGARAALWECGCATLGASFQYA	TTLNPTIA
<i>C. trachomatis</i> L2	YLKGNASASFNVLGLFGDN. ENHATVSDSKLVPMNSLDQSVVELYTDTTFAWSVGARAALWECGCATLGASFQYA	TTLNPTIA
<i>C. trachomatis</i> L3	YLKGNASASFNVLGLFGTK. TQSTNFNTAKLVPNIALNRVVELYTDTTFAWSVGARAALWECGCATLGASFQYA	TTLNPTIA
Amino acid no.	...150.....160.....170.....180.....190.....200.....210..... 317.....324	
<i>C. pneumoniae</i> AR388	YIRGNSTAFNLVGLFGVKGTTVNANE. ... LPNVSLNSGVVELYTDTSFSWSVGARGALWECGCATLGAEFQYA	TAWNPSLL
<i>C. psittaci</i> 6BC	YFKASSAASFNVLGLFGSAASSISTDLPMQLPNVGITQGVVEFYTDTSFSWSVGARGALWECGCATLGAEFQYA	TTWNPSLI

b

Fig. 2. (a) Multiple alignment of chlamydial MOMP amino acid sequences. Only regions of the protein used in this study are illustrated (variable segments I, II and IV). (b) The compositions of the generated fusion proteins (antigen 1–3) are shown. The colour coding refers to the multiple alignment above. Light grey depicts the protein fragment of *C. trachomatis* (E) VS I–II (aa66–aa216). Dark grey depicts *C. trachomatis* D, F/G VS–I (aa86/87–aa102/104). Areas depicted in black show the *C. trachomatis*-specific VS–IV fragment (aa317–aa324). Amino-acid fragments generated by restriction endonuclease sites in the cloning strategy are depicted by single letter code between fusion partners.

serum 20% in phosphate-buffered saline (pH 7.4) including Tween-20 0.05% (PBS-T) (Sigma). Sera were diluted 1 in 100 in the blocking agent to absorb any cross-reacting antibodies directed against fetal calf serum. Antigen-coated microtitration plates and sera dilutions were stored overnight at 4°C. Diluted sera were added to the plates and incubated for 2 h. Bound antibody was detected by incubation for 1 h with peroxidase-labelled goat anti-human IgG (BioRad, CA, USA) diluted 1 in 3000 in PBS-T. Tetramethylbenzidine was the chromogen used in this assay, as described by the manufacturer (Sigma). Incubation was at room temperature and all intermediate steps in the assay were followed by washing the microtitration wells four times in PBS-T. Colour development was terminated

after 20 min by addition of an equal amount of 1 M HCl. Optical density at 450 nm (OD₄₅₀) was measured on a Bio-Kinetics Reader with the KC3 software program (Bio-Tek Instruments, Winooski, VT, USA). The results are average values of two independent measurements, with SDs of <10%. In all data presented in this paper, the background OD₄₅₀ value measured for each serum upon binding to plates without antigen was subtracted from the OD₄₅₀ measured when antigen was used in the ELISA. This background was in the order of OD₄₅₀ 0.0–0.3. The OD₄₅₀ cut-off values for each antigen, chosen empirically so as to obtain the highest possible sensitivity and specificity of the ELISA compared to MIF, were 1.5 (antigen 1), 0.8 (antigen 2) and 0.8 (antigen 3).

DNA sequences

Chlamydial *momp* sequences were obtained from the EMBL/GenBank/DDBJ database (accession nos. M58938, M17342, M17343, X52557, X52080, X16007, M36533, M14738, X55700) and from the paper by Yuan *et al.* [33].

Results

Production of purified recombinant antigens 1–3

Three recombinant plasmids encoding MOMP polyantigens were generated by a combined cloning strategy. Antigen 1 contained the variable segment I–II of *C. trachomatis* serovar E (MOMP_{aa66–aa216}) (Fig. 2). The gene fragment encoding VS-I of serovar F/G and serovar D was introduced in antigen 2 (Fig. 2). The final construct (antigen 3) included the gene fragment encoding the species-specific region of *C. trachomatis* MOMP VS-IV (Fig. 2). The intervening sequences encoded by nucleotides of restriction enzyme palindromes between fusion partners are depicted in Fig. 2. All three antigens were purified by nickel-affinity chromatography and analysed by SDS-PAGE (not shown). The antigen preparations were found to be >95% pure, as judged by a Coomassie Blue-stained polyacrylamide gel.

Reactivity of sera with recombinant antigens 1–3

To evaluate the efficacy of the purified MOMP polyantigens in detecting species-specific *C. trachomatis* antibodies, a panel of 103 human sera was tested in duplicate by ELISA. The sera were sorted into four groups according to the presence or absence of *C. trachomatis* or *C. pneumoniae* antibodies, or both, in the samples as measured by MIF. First, the antigen containing *C. trachomatis* serovar E MOMP (VS I–II) was used in the ELISA (antigen 1, Fig. 2). It was found that >56% (22 of 39) of *C. trachomatis* MIF-positive sera were positive by ELISA (serum groups I and II, Fig. 3). In contrast, 80% (51 of 64) of MIF-negative sera were negative in the ELISA assay (serum groups III and IV, Fig. 3). The species specificity of this antigen is evident, because there was no increase in the positive rate of samples reported positive by *C. pneumoniae* MIF (serum groups I and III) as compared to serum groups II and IV (negative by *C. pneumoniae* MIF) (Fig. 3).

To increase the sensitivity and specificity of the assay, antigen 2 containing, additionally, VS-I from *C. trachomatis* serovars D and F/G was tested (Fig. 2). When this recombinant MOMP polyantigen was used in ELISA, 12 of 19 sera (63%) that were positive by *C. trachomatis* MIF and culture isolation were positively identified (group I, Fig. 3). In group II (not confirmed

Fig. 3. ELISA results obtained with recombinant MOMP polyantigens 1, 2 and 3. The results are compared with MIF. The percentages of sera positive by ELISA are shown in black, whereas the white colouring shows the percentage considered negative by ELISA. The cut-off OD₄₅₀ values were 1.5, 0.8 and 0.8 respectively.

by culture isolation, but *C. trachomatis* positive and *C. pneumoniae* negative by MIF), this antigen gave positive results with 12 of 20 (60%) of the sera (Fig. 3). In the group of *C. trachomatis*/*C. pneumoniae* MIF-negative sera (serum group IV, Fig. 3) there was a decrease in the false-positive detection of sera. In all, antigen 2 was superior to antigen 1 in the detection of *C. trachomatis*-specific antibodies, as both sensitivity and specificity were enhanced.

Finally, to generalise the species-specificity of the assay, antigen 3 was tested (Fig. 2). By including the species-specific MOMP fragment VS-IV in the poly-antigen, the ELISA detection of the *C. trachomatis* MIF-positive sera was clearly increased (groups I and II, Fig. 3).

Comparison of results obtained by MIF and ELISA with antigen 3

The qualitative correlation between results obtained by ELISA with antigen 3 and the results of *C. trachomatis*

MIF was analysed. The sensitivity of this antigen was close to 80%, as 31 of 39 *C. trachomatis* MIF positive sera were positively identified in this third assay. The specificity of this assay was 91%, as 58 of 64 sera were negative by both *C. trachomatis* MIF and the ELISA with antigen 3. The quantitative ELISA results with antigen 3 were correlated with *C. trachomatis* MIF titres of all sera, and the results are presented in Fig. 4. There was no statistical correlation between MIF titres and antibodies measured in this assay ($p > 0.05$), but a trend was evident (Fig. 4). Sera with higher MIF titres gave higher OD₄₅₀ values in the ELISA.

Discussion

Serology has provided important information regarding the wide range of disease associated with *C. trachomatis* infection, including infant pneumonia, acute reactive arthritis and chronic genital infections. Whole-cell chlamydia organisms and particularly the MIF assay have generally been applied in the species-

Fig. 4. The quantitative relationship between titres obtained with *C. trachomatis* antigen in MIF and ELISA readings with recombinant antigen 3. Sera were diluted 1 in 100 in the ELISA measurements. Each circle represents one serum. The mean OD₄₅₀ values of sera with identical MIF titres are indicated by a solid line, whereas the cut-off point of the ELISA is marked by a dotted line.

specific serological detection of chlamydial infections [9]. However, the MIF test is difficult to standardise, and is technically demanding. As a result, the use of this assay has been restricted to a relatively small number of qualified research laboratories [34]. The results presented in this paper suggest that a more widely applicable enzyme immunoassay, based upon recombinant MOMP polyantigens, may be helpful in the serodiagnosis of *C. trachomatis* infections. A similar approach has been applied successfully in serological detection of cytomegalovirus-specific antibodies in human sera [35]

To evaluate the ELISA with MOMP polyantigens (Fig. 2), 103 sera from different patient groups were selected and grouped according to MIF results (Fig. 3). Almost 80% (79.5%) of *C. trachomatis* MIF-positive sera (31 of 39), were identified in the assay with the third MOMP polyantigen (antigen 3, Fig. 3), and <10% of *C. trachomatis* MIF-negative sera (6 of 64) were identified as positive with this antigen. The divergent results need to be investigated. However, the detection of a few *C. trachomatis* MIF-negative sera as positive by ELISA is not due to the presence of cross-reacting *C. pneumoniae* antibodies. The detection of positive sera by the ELISA was similar in group III (*C. pneumoniae*-positive by MIF) and group IV (*C. pneumoniae*-negative by MIF) sera (Fig. 3).

The inclusion of additional *C. trachomatis*-specific epitopes in the antigen 1 construct (antigen 2 and antigen 3) clearly increased both sensitivity of specificity of the ELISA assay (Figs 2 and 3). Indeed, the OD mean value of *C. trachomatis* MIF-negative sera is low with antigen 3 (OD ~0.43, Fig. 4). A closer look at the three sera of group I not detected by antigen 3 revealed that two had low *C. trachomatis* MIF titres (16 and 64) with high MIF titres against *C. pneumoniae* (both 512) (data not shown). Thus, a certain amount of anti-*C. pneumoniae* cross-reacting antibodies in the *C. trachomatis* MIF cannot be ruled out because of the presence of the genus-specific LPS epitope in the assay.

When the anti-*C. trachomatis* IgG antibody levels obtained by ELISA were correlated with the results of the MIF, no strong quantitative correlation was found. This indicates that different antibodies are measured in the two assays. Whereas this ELISA is based upon linear MOMP epitopes, the chlamydial MIF includes detection of conformational MOMP epitopes and epitopes on other *Chlamydia* species or *C. trachomatis* serovar specific antigens, or both. The specific antigens involved in the *C. pneumoniae* MIF assay have not been identified.

Even though MIF has been considered the 'gold' standard in chlamydial serology for many years, a recent investigation has shown that the inter-laboratory variation of MIF results is of the order 10–40% [34].

Therefore, a discrepancy when comparing other tests to the MIF assay is inevitable.

This paper describes the first evaluation of the use of recombinant antigens in the differential serodiagnosis of *C. trachomatis* urogenital infections. The results indicate that recombinant MOMP polyantigens could become a valuable tool in the development of a standardised serological assay for the detection of *C. trachomatis* infections. However, no correlation of ELISA results and disease stages was attempted. This is clearly an important issue, especially in respect to chronic chlamydial infections. To perform such a study, larger, clinically well characterised patient groups need to be tested. Serology may prove to be of importance in the diagnosis of subclinical and persistent *C. trachomatis* infections. The test described in this paper offers practical advantages over MIF including simplicity, low cost and objectivity. However, improvements in the sensitivity and specificity of such a serological test are imperative. Further investigation including detection of serum IgA and IgM is warranted. Perhaps the use of carefully selected peptides in an enzyme immunoassay may prove to be more sensitive in the serodiagnosis of human *C. trachomatis* infections.

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References

1. Moulder JW. Interaction of chlamydiae and host cells in vitro. *Microbiol Rev* 1991; **55**: 143–190.
2. Grayston JT, Kuo C-C, Wang S-P, Altman J. A new *Chlamydia psittaci* strain, TWAR, isolated in acute respiratory tract infections. *N Engl J Med* 1986; **315**: 161–168.
3. Hahn DL, Dodge RW, Golubjatnikov R. Association of *Chlamydia pneumoniae* (strain TWAR) infection with wheezing, asthmatic bronchitis, and adult-onset asthma. *JAMA* 1991; **266**: 225–230.
4. Kuo CC, Jackson LA, Campbell LA, Grayston JT. *Chlamydia pneumoniae* (TWAR). *Clin Microbiol Rev* 1995; **8**: 451–461.
5. Saikku P, Leinonen M, Mattila K *et al*. Serological evidence of an association of novel *Chlamydia*, TWAR, with chronic coronary heart disease and acute myocardial infarction. *Lancet* 1988; **2**: 983–986.
6. Li D-K, Daling JR, Wang S-P, Grayston JT. Evidence that *Chlamydia pneumoniae*, strain TWAR, is not sexually transmitted. *J Infect Dis* 1989; **160**: 328–331.
7. Moss TR, Darougar S, Woodland RM, Nathan M, Dines RJ, Cathrine V. Antibodies to *Chlamydia* species in patients attending a genitourinary clinic and the impact of antibodies to *C. pneumoniae* and *C. psittaci* on the sensitivity and specificity of *C. trachomatis* serology tests. *Sex Transm Dis* 1993; **20**: 61–65.
8. Friedank HM, Terreri MT, Peter HH, Bredt W. Comparison of serological tests for the detection of antibodies against *Chlamydia trachomatis* and *Chlamydia pneumoniae* in rheumatological patients. *Int J Med Microbiol Virol Parasitol Infect Dis* 1993; **279**: 518–525.
9. Wang SP, Grayston JT. Immunologic relationship between genital TRIC, lymphogranuloma venereum, and related organ-

- isms in an new microtitre indirect immunofluorescence test. *Am J Ophthalmol* 1970; **70**: 367–374.
10. Freidank HM, Herr AS, Jacobs E. Identification of *Chlamydia pneumoniae*-specific protein antigens in immunoblots. *Eur J Clin Microbiol Infect Dis* 1993; **12**: 947–951.
 11. Newhall WJ, Batteiger B, Jones RB. Analysis of the human serological response to proteins of *Chlamydia trachomatis*. *Infect Immun* 1982; **38**: 1181–1189.
 12. Brade H, Brade L, Nano FE. Chemical and serological investigations on the genus-specific lipopolysaccharide epitope of *Chlamydia*. *Proc Natl Acad Sci USA* 1987; **84**: 2508–2512.
 13. Chernesky M, Luinstra K, Sellors J *et al.* Can serology diagnose upper genital tract *Chlamydia trachomatis* infections? Studies on women with pelvic pain, with or without chlamydial plasmid DNA in endometrial biopsy issue. *Sex Transm Dis* 1998; **25**: 14–19.
 14. Hanuka N, Glasner M, Sarov I. Detection of IgG and IgA antibodies to *Chlamydia trachomatis* in sera of patients with chlamydial infections: use of immunoblotting and immunoperoxidase assays. *Sex Transm Dis* 1988; **15**: 93–99.
 15. Mygind P, Christiansen G, Persson K, Birkelund S. Analysis of the humoral immune response to outer membrane protein 2. *Clin Diagn Lab Immunol* 1998; **5**: 313–318.
 16. Evereet KD, Hatch TP. Architecture of the cell envelope of *Chlamydia psittaci* 6BC. *J Bacteriol* 1995; **177**: 877–882.
 17. Mygind P, Christiansen G, Birkelund S. Topological analysis of *Chlamydia trachomatis* outer membrane protein 2. *J Bacteriol* 1998; **180**: 5784–5787.
 18. Watson MW, Lambden PR, Everson JS, Clarke IN. Immunoreactivity of the 60 kDa cysteine-rich proteins of *Chlamydia trachomatis*, *Chlamydia psittaci* and *Chlamydia pneumoniae* expressed in *Escherichia coli*. *Microbiology* 1994; **140**: 2003–2011.
 19. Su H, Caldwell HD. In vitro neutralization of *Chlamydia trachomatis* by monovalent Fab antibody specific to the major outer membrane protein. *Infect Immun* 1991; **59**: 2843–2845.
 20. Batteiger BE, Lin P-M, Jones RB, Van der Pol BJ. Species-, serogroup-, and serovar-specific epitopes are juxtaposed in variable sequence region 4 of the major outer membrane proteins of some *Chlamydia trachomatis* serovars. *Infect Immun* 1996; **64**: 2839–2841.
 21. Jones HM, Schachter J, Stephens RS. Evaluation of the humoral immune response in trachoma to *Chlamydia trachomatis* major outer membrane proteins by sequence-defined immunoassay. *J Infect Dis* 1992; **166**: 915–919.
 22. Campbell LA, Kuo C-C, Wang S-P, Grayston JT. Serological response to *Chlamydia pneumoniae* infection. *J Clin Microbiol* 1990; **28**: 1261–1264.
 23. Christensen G, Østergaard L, Birkelund B. Analysis of the *Chlamydia pneumoniae* surface. In: Orfila J *et al.* (eds) *Chlamydia infections: Proceedings of the Eighth International Symposium on Human Chlamydial Infections*. Italy, Esculapio. 1994: 173–176.
 24. Mygind R, Hünich BS, Christiansen G, Persson K, Birkelund S. Detection of *Chlamydia trachomatis* infections by the use of recombinant MOMP polyantigens. In: Stephens RS *et al.* (eds) *Chlamydial infections: Proceedings of the Ninth International Symposium on Human Chlamydial Infection*. International *Chlamydia* symposium. San Francisco, 1998: 615–618.
 25. Persson K, Treharne J. Diagnosis of infection caused by *Chlamydia pneumoniae* (strain TWAR) in patients with 'ornithosis' in southern Sweden 1981–1987. *Scand J Infect Dis* 1989; **21**: 675–679.
 26. Sambrook JE, Fritsch EF, Maniatis T (eds). *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press. 1989.
 27. Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW. Use of T7 RNA polymerase to direct expression of cloned genes. In: Goeddel DV (ed) *Gene expression technology*. (*Methods Enzymol* 185) San Diego, Academic Press. 1990: 60–89.
 28. Morré SA, Moes RM, Van Valkengoed IGM, Boeke JAP, Meijer CJLM, van den Brule AJC. *Chlamydia trachomatis* genotyping in urine specimens will facilitate large epidemiological studies. In: Stephens RS *et al.* (eds) *Chlamydial infections: Proceedings of the Ninth International Symposium on Human Chlamydial Infection*. International *Chlamydia* symposium. San Francisco, 1998: 63–66.
 29. Poole E, Lamont I. *Chlamydia trachomatis* serovar differentiation by direct sequence analysis of the variable segment 4 region of the major outer membrane protein gene. *Infect Immun* 1992; **60**: 1089–1094.
 30. Rodriguez P, de Barbeyrac B, Persson K, Dutilh B, Bebear C. Evaluation of molecular typing for epidemiological study of *Chlamydia trachomatis* genital infections. *J Clin Microbiol* 1993; **31**: 2238–2240.
 31. Kaltenboeck B, Heard D, DeGraves FJ, Schmeer N. Use of synthetic antigens improves detection by enzyme-linked immunosorbent assay of antibodies against abortigenic *Chlamydia psittaci* in ruminants. *J Clin Microbiol* 1997; **35**: 2293–2298.
 32. Hochuli E, Bannwarth W, Döbeli H, Gentz R, Stüber D. Genetic approach to facilitate purification of recombinant protein with a novel metal chelate adsorbent. *Biotechnology NY* 1988; **6**: 1321–1325.
 33. Yuan Y, Zhang Y-X, Watkins NG, Caldwell HD. Nucleotide and deduced amino acid sequences for the four variable domains of the major outer membrane proteins of the 15 *Chlamydia trachomatis* serovars. *Infect Immun* 1989; **57**: 1040–1049.
 34. Peeling RW, Wang SP, Grayston JT *et al.* *Chlamydia* serology: inter-laboratory variation in microimmunofluorescence results. In: Stephens RS *et al.* (eds) *Chlamydial infections: Proceedings of the Ninth International Symposium on Human Chlamydial Infection*. International *Chlamydia* symposium. San Francisco, 1998: 159–162.
 35. Landini MP, Lazzarotto T, Maine GT, Ripalti A, Flanders R. Recombinant mono- and polyantigens to detect cytomegalovirus-specific immunoglobulin M in human sera by enzyme immunoassay. *J Clin Microbiol* 1995; **33**: 2535–2542.