Antigen capture ELISA for the heat shock protein (hsp60) of *Chlamydia trachomatis*

P J Horner, M Ali, D Parker, J N Weber, D Taylor-Robinson, M O McClure

Abstract

Aims—To develop an indirect ELISA using the heat shock protein (hsp60) of *Chlamydia trachomatis* as antigen.

Methods—The hsp60 gene was amplified by PCR, expressed in the vector pDEV-107 and transformed into *Escherichia coli*. The recombinant protein, expressed as a β -galactosidase fusion product, was captured onto a solid phase using a monoclonal antibody directed against β -galactosidase. Following incubation with goat anti-human antibody conjugated to peroxidase and colour development on addition of peroxidase substrate, antibody recognition of antigen was quantified by optical density at 492 nm.

Results—A sensitive and relatively specific ELISA to detect hsp60 has been produced, which can be exploited to determine the antibody response to *C trachomatis* hsp60.

Conclusions—This assay will permit the future investigation of the immunopathogenesis of persistent inflammation following *C trachomatis* infection.

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Department of
Genito-urinary
Medicine and
Communicable
Diseases,
Jefferiss Research
Trust Laboratories,
Imperial College
School of Medicine at
St Mary's,
London W2 1NY
P J Horner
M Ali
J N Weber
M O McClure

The MRC Sexually
Transmitted Diseases
Research Group
P J Horner
D Taylor-Robinson

Murex Diagnostics
Ltd., Temple Hill,
Deptford, Kent
DA1 5LR
D Parker

Correspondence to:
Dr P J Horner,
Department of
Genito-urinary Medicine,
Bristol Royal Infirmary,
Bristol BS2 8HW.

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Chlamydia trachomatis is a major sexually transmitted micro-organism, leading to genital and ocular disease in men, women and infants. Two biovars of *C trachomatis* are pathogenic in humans, the trachoma biovar, a pathogen of mucous membranes, and the lymphogranuloma venereum (LGV) biovar. The LGV biovar is much more invasive and causes systemic infections. Serovars A-C cause trachoma; serovars D-K primarily affect the genital tract where they cause urethritis, epididymitis, cervicitis, endometritis, and salpingitis, infection in women often being asymptomatic. Ocular infection with these serovars results in conjunctivitis, particularly in newborn infants of infected mothers.

Infection of fallopian tube mucosa by *C trachomatis* in organ culture has little or no direct deleterious effect.¹ In other words, the changes are insufficient to account for the damage that occurs in vivo. The pathogenesis of the inflammatory damage is, therefore, believed to involve components of the immune system. Much of the understanding of the immunopathogenesis of the *C trachomatis*

trachoma biovar has come from experimental infection of primates and smaller laboratory animals. Studies of *C psittaci* in guinea pigs² and *C trachomatis* in monkeys³ demonstrated that a detergent soluble extract of chlamydial elementary bodies induced conjunctival inflammation in immune, but not in naive animals. This inflammatory response is similar histologically to that produced by whole organisms and that seen in salpingitis in humans. Morrison *et al*^{4,5} showed that the detergent extract of *C psittaci* contained a genus specific protein of 57 kDa which belongs to the family of heat shock proteins (hsp60). This protein, the molecular sequence of which is known, has a considerable degree of homology with the mycobacteria 65 kDa protein, *Escherichia coli* and human hsp60,^{4,7} and is thought to mediate the immunopathology that follows chlamydial infection.^{4,6,7}

Heat shock proteins, popularly referred to as molecular chaperones, have essential roles in the synthesis, transport and folding of proteins. They are among the most conserved proteins in phylogeny with respect to both structure and function. Many hsps are essential for life and their synthesis increases in response to a variety of insults to ensure survival under stressful conditions.⁸ Most hsps have been grouped into families on the basis of their apparent molecular weight. The chlamydial hsp60 can be isolated from the outer membrane of elementary bodies and may participate in the assembly of the chlamydial cell wall.⁷

Wagar *et al*⁹ and Tøye *et al*¹⁰ demonstrated that a serological response to chlamydial hsp60 was associated with the chronic sequelae of pelvic inflammatory disease in patients who had a classic immune response to chlamydial infection. This suggests that the serological response to chlamydial hsp60 may be important in the immunopathogenesis of persistent inflammation of the genital tract in humans. To investigate this possibility further, we have developed an indirect ELISA for the hsp60 of *C trachomatis* serovar E, the development and details of which are presented herein.

Methods

AMPLIFICATION OF CHLAMYDIAL HSP60 DNA BY PCR

A conserved region of the DNA sequence encoding the hsp60 gene for *C trachomatis* serovar A was used to design primers for PCR for the amplification of the hsp60 gene from *C trachomatis* serovar E. The primer sequences were: (a) 5'> TCC CCC GGG ATA TGG

TCG CTA AAA ACA TT <3' and (b) 5'> GAG CCC GGG ATT AAT AGT CCA TTC CTG CGC C <3'.

C. trachomatis serovar E, grown in McCoy cells, was purified on a sucrose gradient and DNA extracted using standard methods.¹¹ The PCR reaction was carried out in a final volume of 50 µl containing 5 µl 10× buffer (500 mM KCl, 100 mM Tris-HCl (pH 8.0), 1 mg/ml gelatin, and 1% Triton X-100), 4 µl of dNTPs (1 mg/ml), 1.5 mM MgCl₂, 1 µl of Taq polymerase, 1 µl (100 ng) of oligonucleotide primers (a) and (b), and 200 ng of genomic chlamydial DNA. The DNA was annealed at 60°C for 120 seconds, extended at 72°C for 150 seconds and denatured at 95°C for 150 seconds for 35 cycles using a thermal cycler (Perkin Elmer, UK), with an extension time of seven minutes in the final cycle.

SUBCLONING OF PCR AMPLIFIED PRODUCT

The PCR amplified DNA was purified on a 1% agarose gel and a band of the appropriate size was excised and isolated using the GeneClean (BIO101, UK) protocol. The DNA recovered was digested with the restriction enzyme Sma I and further purified using the GeneClean method. To construct a recombinant clone that contained the hsp60 protein, the prokaryotic expression vector pDEV-107 (Murex Diagnostics, Deptford, Kent, UK) (fig 1), which produces a β-galactosidase carboxyl terminal fusion protein, was digested with Sma I and purified on a 1% agarose gel, as described previously. The PCR product (10 ng) and expression vector pDEV-107 (5 ng) were blunt-end ligated and transformed with DH5a (Gibco BRL, Paisley, UK) competent *E. coli* cells using standard protocols.¹¹ The transformed cells were plated onto 1.6% agar containing X-gal (800 µg/ml) and ampicillin (100 µg/ml) and recombinant clones isolated and characterised by restriction digestion of mini DNA preparations.¹¹ The above procedure was also carried out with pDEV-107 alone.

Figure 1 Restriction map of the pDEV-107 expression vector.

SOUTHERN BLOT ANALYSIS

After restriction enzyme digestion, the DNA was analysed by agarose gel electrophoresis and DNA from clones successfully transformed by pDEV-107 and containing the chlamydial PCR product was transferred to a Genescreen (Dupont, UK) membrane. This was probed with an oligonucleotide, the sequence of which (5' ATT TCT GCT AAT AAT GAT GCA GAA ATC 3') was located between the primer pairs and corresponded to a conserved region of the hsp60 gene in both *C. trachomatis* serovar A and *C. psittaci*. When recombinant clones were identified, glycerol stocks were prepared: one containing chlamydial PCR product insert and one with pDEV-107 alone.

EXPRESSION OF CHLAMYDIAL HSP60 β-GALACTOSIDASE CARBOXY-TERMINAL FUSION PROTEIN AND β-GALACTOSIDASE

A 10 µl volume of glycerol stock was added to 15 ml LB broth containing 100 µg/ml ampicillin and incubated overnight at 37°C. This seeding culture was added to 300 ml LB broth containing 100 µg/ml ampicillin, incubated at 37°C for one hour and induced with 50 µg/ml IPTG for a further three and a half hours. After centrifugation at 10 000 × g for 20 minutes, the pellet was washed twice in TEN buffer (25 mM Tris HCl (pH 8.0), 1 mM EDTA and 100 mM NaCl) and resuspended in 3.0 ml TEN buffer per g *E. coli* in the presence of 2 µg/ml leupeptin, 2 µg/ml pepstatin, 2 µg/ml E64, and 300 µg/ml phenyl methyl sulphonyl fluoride (PMSF). To this was added 20 mg/ml lysozyme and the mixture kept on ice for 90 minutes. Deoxycholic acid (3 mg per g *E. coli*) was then added with gentle agitation for 20 minutes before addition of MgSO₄ (final concentration 10 mM) and incubated with DNase I (final concentration 100 µg/ml) at 4°C overnight. This lysate was stored at 20°C in 500 µl aliquots.

SEMI-PURIFICATION OF HSP60 PROTEIN

An aliquot of the lysate was centrifuged at 13 000 × g and the pellet resuspended on ice for 10 minutes in 1 ml TEN buffer containing protease inhibitors (leupeptin, pepstatin, E64, and PMSF) and 0.5% Triton X-100 (lysis buffer), then centrifuged at 13 000 × g for three minutes at 4°C. The supernatant was discarded and the procedure repeated twice. The pellet was then washed three times with ice-cold TEN buffer containing protease inhibitors, but without detergent, washed in lysis buffer and then resuspended on ice for five minutes in lysis buffer without the protease inhibitors, but containing 4 M urea. This preparation was centrifuged at 13 000 × g for one minute at 4°C and the supernatant discarded. This step was repeated and the pellet resuspended in 1 ml lysis buffer containing 7 M urea and kept on ice for 10 minutes. Following centrifugation at 13 000 × g for 10 minutes at 4°C, the supernatant was diluted 1 in 1 in glycerol to a final dilution of 1 in 4. This could be stored at -70°C.

For all absorption experiments, using *E coli* containing β -galactosidase, the pellet mentioned earlier was washed three times and resuspended in phosphate buffered saline (PBS) following the washes in TEN buffer containing protease inhibitors, but no detergent.

CHARACTERISATION OF EXPRESSED RECOMBINANT PROTEIN

Samples were solubilised in sample buffer (62.5 mM Tris-HCl (pH 6.8) containing 10% SDS, 5% β -mercaptoethanol, 10 mM EDTA, 10% glycerol, and 0.5% bromophenol blue) and subjected to 7.5% polyacrylamide gel electrophoresis (SDS-PAGE).^{12,13} Two identical gels were prepared, one of which was stained for protein visualisation with Coomassie blue and the other transferred to a nitrocellulose membrane (VDF-Immobilon/Millipore, UK). After transfer, the membrane was blocked with blocking solution (PBS containing 10% lamb serum (LS; Gibco) and 5% non-fat milk powder; PBSB) for two hours and washed three times with PBS containing 0.1% Tween 20 (PBST). A mycobacterial hsp60 monoclonal antibody, ML-30, which recognises the chlamydial, but not the *E coli* hsp60 antigen, was diluted 1 in 400 in blocking solution (0.1% Tween 20 and 5% *E coli* lysate containing pDEV-107) and incubated with the membrane for one hour at 37°C. The membrane was then washed three times with PBST and incubated with the second antibody (goat anti-mouse IgG conjugated to peroxidase; Sigma, Poole, Dorset, UK) at a dilution of 1 in 500, and incubated with the membrane which was then washed, as described. Immunostaining was visualised following addition of DAB (3'3-diaminobenzidinetetrahydrochloride) substrate (6 mg/ml in 50 mM Tris-HCl (pH 7.5) and 10 μ l 30% hydrogen peroxide).

ENZYME LINKED IMMUNOSORBENT ASSAY

The solid phase (flat bottomed microtitre plates; Nunc, Denmark) was pre-coated with 100 μ l of a monoclonal antibody directed against β -galactosidase, BG-79 (Murex Diagnostics), at 7.5 μ g/ml in PBS, 0.1% NaN₃ and incubated overnight at room temperature. The plates were washed four times in PBST and blocked with 200 μ l PBSB and 0.1% NaN₃ for two hours at room temperature, then washed three times in PBST. The antigens, chlamydial hsp60 β -galactosidase carboxyl terminal fusion protein and β -galactosidase, were captured onto the solid phase at a dilution of 1 in 160 in lysis buffer with 0.4 M urea, and incubated overnight at room temperature. The solid phase was washed four times with PBST and blocked, as before, with PBSB and then washed three more times in the same buffer. Serum samples were diluted 1 in 100 in serum diluent, namely PBSB plus 2.5% *E coli* lysate containing pDEV-107 to remove antibodies directed against *E coli* and β -galactosidase, and 100 μ l was added to each of the captured antigens on the solid phase. This was incubated for one hour at 37°C. After six washes in PBST, 100 μ l goat anti-human peroxidase conjugate,

at a dilution of 1 in 3000 in PBSB, was added to each well and incubated and washed, as above. To each well, 100 μ l OPD (1,2-phenylenediamine; Dako, High Wycombe, UK) hydrogen peroxide substrate was added. The reaction was developed in the dark for 20 minutes and stopped with 100 μ l of 1 M H₂SO₄. Samples were read spectrophotometrically at 492 nm.

SERUM SAMPLES USED AND CALCULATION OF THE ELISA CUT-OFF VALUE

The ELISA cut-off value, below which any serum sample assayed was considered to be hsp60 antibody negative, was calculated from assaying 148 serum samples taken from 93 subjects known to be antibody negative to *C trachomatis* or *C pneumoniae* by micro-immunofluorescence (MIF).¹⁴ The cut-off value for the assay was calculated from these samples as the mean (corrected) optical density (OD) + 3 SD. In addition, serum samples from patients with acute chlamydial urethritis, confirmed by direct fluorescent antibody testing, who had antibody directed against *C trachomatis* measured by MIF were used as positive controls.

Results

MOLECULAR AND BIOCHEMICAL

CHARACTERISATION OF RECOMBINANT PROTEIN
Subcloning of the chlamydial hps60 gene into *E coli* was confirmed by Southern blotting (fig 2). Both the chlamydial hsp60 PCR product and the fragment subcloned into the pDEV-107 expression vector and transformed into *E coli* hybridised readily with the molecular probe.

SDS-PAGE of the recombinant fusion protein indicated the expected molecular weight of approximately 175 kD and western blot analysis with the ML-30 monoclonal antibody indicated specific recognition (fig 3).

EVALUATION OF THE ELISA

Recombinant antigen captured on ELISA plates by the anti- β -galactosidase monoclonal antibody, BG-79, constituted the basis of the *C trachomatis* ELISA. Absorbance values (OD

Figure 2 Southern blot using a conserved sequence common to hsp60 of *C trachomatis* and *C psittaci* as a probe (see text), showing hybridisation of the probe with hsp60 PCR amplified DNA. Lane 1, PCR amplified DNA; lane 2, DNA amplified from uninfected McCoy cells; lane 3, water control; lane 4, PCR product in pDEV-107 expression vector. Non-equivalent amounts were loaded onto the gel.

readings) due to any reaction of the 148 chlamydia antibody negative serum samples with β -galactosidase were in each case subtracted from the OD obtained when serum was

Figure 3 Western blot of recombinant hsp60 protein using ML-30. Lane 1, sucrose gradient purified *C trachomatis* fractionated from McCoy cells; lane 2, *E coli* expressing β galactosidase; lane 3, *E coli* lysate containing hsp60 β -galactosidase fusion protein (and breakdown products); lane 4, purified hsp60 β -galactosidase fusion protein extracted in 7 M urea as described in the text.

Figure 4 Histogram showing (corrected) OD at 492 nm for *C trachomatis* and *C pneumoniae* antibody negative control serum samples.

incubated with the chlamydial hsp60 fusion protein. The results are shown in fig 4.

The ELISA cut-off value was calculated from these serum samples. Using this, three positive control serum samples were identified from those patients with acute chlamydial urethritis, having OD values higher than the cut-off value.

The specificity of the assay was confirmed by western blot analysis using lysates of *C trachomatis* and uninfected McCoy cells as sources of antigen. Lanes 1 and 13 in fig 5 confirm a band of 60 kD when monoclonal antibody ML-30 is used. This identified the chlamydial hsp60 as the upper band of a protein doublet. The antibody status of the three positive control serum samples was positive by western blotting (fig 5, lanes 10–12). Of the 148 antibody negative serum samples, 15 (including the group of five outliers with the highest OD values in fig 4) were also subjected to western blotting. The five samples lying outside the main group distribution, with the highest OD values, turned out to be seropositive by western blotting. (Four of these are shown in fig 5, lanes 6–9.) The remaining 10, four of which are shown in fig 5, were confirmed as being seronegative (lanes 2–5). The cut-off value, therefore, was amended by excluding the five samples that were positive for hsp60, by western blotting, from the 148 chlamydia antibody negative serum samples, as described previously.

Discussion

We have subcloned the hsp60 gene from *C trachomatis* serovar E into *E coli* and expressed it as a β -galactosidase carboxyl terminal fusion protein. This was used to develop a sensitive and relatively specific ELISA for the detection of antibodies directed against chlamydial hsp60.

The hsp60 genome is highly conserved. From the nucleotide sequences of *C psittaci* and *C trachomatis* hsp60 more than 80% homology has been demonstrated.⁵ We assumed, therefore, that the homology between *C trachomatis* serovars would be such that PCR primers designed for *C trachomatis* serovar A would subclone the hsp60 gene from *C trachomatis* serovar E. This was, indeed, the case. In addition, a Southern blot probe was derived from a DNA sequence homologous to *C psittaci* and *C trachomatis*. The same principle was used to determine whether the subcloned chlamydial hsp60 gene was inserted into pDEV-107 in the correct orientation following blunt-end ligation. The hsp60 gene of *C trachomatis* serovar A has one Bam H1 restriction site at position 133. The pDEV-107 expression vector has a Bam H1 restriction site at the 3' end of the cloning site. Thus, the correct orientation was confirmed by restriction enzyme digestion with Bam H1. The probe used for the Southern blot analysis recognised both the PCR product and the insert subcloned into pDEV-107 from the successfully transformed *E coli*. Two PCR products hybridised with the molecular probe. The lower molecular weight product of approximately

Figure 5 Lanes 1 and 13, western blot of sucrose gradient purified *C trachomatis* lysate using ML-30; lanes 10–12, patient serum samples known to be antibody positive for *C trachomatis* by MIF, which had OD values higher than the ELISA cut-off values; lanes 2–5, patient serum samples known to be antibody negative by MIF; and lanes 6–9, antibody negative serum samples which gave the highest OD values in the ELISA (see text).

1000 kD corresponds to sequence 997–1028 on the *C trachomatis* genome, which has 14 base pair homology with the reverse primer used.⁵

The chlamydial hsp60 β -galactosidase carboxy terminal fusion protein was insoluble when expressed in *E coli*, with 7 M urea being required for solubilisation. However, this protein when stored at -70°C lost immunogenicity over time, as shown by using positive control serum in the ELISA. SDS-PAGE indicated that this was not a result of proteolytic degradation of the stored protein (data not shown). The solubilised protein may be stored at -70°C in 50% glycerol.

Previous studies have indicated that some subjects have a serological reactivity to *E coli* derived β -galactosidase. It was important, therefore, to block this response in order to avoid false positive results. This was done by preincubating the serum samples with a lysate to *E coli* expressing pDEV-107 and β -galactosidase. However, *E coli* also contains endogenous hsp60 which has 60% homology with the chlamydial hsp60. Thus, the absorbance could potentially interfere with the chlamydial hsp60 serological response. The *E coli* hsp60 was, therefore, removed by differential solubilisation in Triton X-100, the chlamydial hsp60 fusion protein remaining insoluble in detergent. This was confirmed on a western blot of Triton X-100 *E coli* using a monoclonal antibody directed against *E coli* hsp60.

The results of initial experiments using the mouse anti- β -galactosidase monoclonal antibody indicated that many of the serum samples produced a high background OD value. When we used this background OD value to calculate the threshold OD value for the ELISA, the cut-off value was such that the assay was insufficiently sensitive to detect two of the three positive controls. A subtraction assay, therefore, was used to remove the high background. As can be seen from fig 4, this gave a surprisingly unambiguous normal distribution for the negative results. Previous work from Toye *et al*¹⁰ indicated that samples that were

chlamydia negative on the basis of MIF serology were also almost invariably negative when tested for chlamydial hsp60 antibody by ELISA. We therefore used this criterion to select our negative controls for calculation of the cut-off value. To confirm the validity of this approach, we used western blots with chlamydial lysate to test the "negative" control serum samples. The result of the western blot indicated that those serum samples with the highest OD on ELISA (distinct outliers on the histogram, fig 4), were chlamydial hsp60 antibody positive. The remainder were negative as judged by testing 10 randomly selected serum samples. Interestingly, the band density on western blot of the three positive serum samples, as defined by the ELISA cut-off value, corresponded to the relative OD on ELISA.

It is not clear why our results should differ from those of Toye *et al*,¹⁰ who found that women with chlamydial hsp60 antibodies by ELISA were almost always chlamydia positive by MIF. Antibodies directed against hsp60 are not uncommon in humans. As this protein is highly conserved, one would expect to find subjects who have never been infected with chlamydiae to have cross-reactive hsp60 antibodies, as has been observed with mycobacterial hsp65.¹⁵ Toye *et al*¹⁰ only detected chlamydial hsp60 antibody in 1 of 160 chlamydia antibody negative patients. A possible explanation could be that as hsp60 is expressed on the outer membrane of chlamydial elementary bodies, patients who have cross-reactive hsp60 antibodies have serum that reacts positively in MIF, even though they have no anti-chlamydial antibodies. However, using ML-30 we were unable to identify whole *C trachomatis* elementary bodies by direct immunofluorescence (B Thomas, personal communication). This could indicate that hsp60 is not immunologically recognisable on the elementary bodies or it may associate only with the ML-30 epitope. Our data suggest that the chlamydial hsp60 is not recognised by anti-hsp60 antibodies on chlamydial elementary bodies. Another explanation for the difference in findings between ourselves and Toye *et al*¹⁰ is that the glucuronidase hsp60 fusion protein has a different immunogenic profile from the β -galactosidase hsp60 fusion protein we used in the ELISA. In support of this is our observation that the immunogenicity of our fusion protein varied widely depending on how it was solubilised and stored. An alternative explanation may be that Toye *et al*¹⁰ routinely used serum samples diluted 1 in 500 and it may be that at this dilution their assay was insufficiently sensitive to detect cross-reactive anti-chlamydial hsp60 antibodies.

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