Seroprevalence of antibodies to conserved regions of *Chlamydia trachomatis* heat shock proteins 60 and 10 in women in India

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ABSTRACT

Persistent, untreated chlamydial infection causes chronic stimulation of the host immune system against immunogenic antigens such as chlamydial heat shock proteins (cHSP) 60 and 10. In order to find the seroprevalence of antibodies to cHSPs, enzyme-linked immunosorbent assay (ELISA) is performed using specific peptide sequences to measure antibody response against major outer membrane protein (MOMP), cHSP60 and cHSP10 in patient sera. In this study, 255 patients attending the gynaecology out-patient department (March 2004 to August 2005) of Safdarjung Hospital were enrolled. Of these patients, 107 were diagnosed with cervicitis while 52 had pelvic inflammatory disease (PID)/infertility. *Chlamydia trachomatis* infection in endocervical specimens is diagnosed by a direct fluorescence assay (DFA) and the polymerase chain reaction (PCR). In 75 (29.4%) of the *C. trachomatis*-positive women, 50 (66.7%) were ELISA positive for MOMP, 48 (64.0%) were positive for cHSP60 and 46 (61.3%) were positive for cHSP10. The anti-MOMP index correlated positively with anti-cHSP60 (R=0.522, $P<0.01$) and anti-cHSP10 (R= 0.286, $P<0.05$). Antibody titre for MOMP was significantly higher than that for cHSP60 (1:5; $P<0.01$ and 1:25; $P<0.05$). Moreover, patients with PID/infertility showed significantly higher antibody titres for cHSP60 and cHSP10 when compared to patients with cervicitis at dilutions of 1 in 50, 1 in 250, 1 in 1250 ($P<0.001$) and at 1 in 6250 ($P<0.01$).

KEY WORDS: Chlamydia trachomatis.
Enzyme-linked immunosorbent assay.
Heat-shock proteins.
omp1 protein.

Introduction

Chlamydia trachomatis infection is the most prevalent sexually transmitted bacterial disease worldwide.[1] A high rate of *C. trachomatis* infection (23.3–33%) has been reported in India.[2–5] Up to 80% of women with genital chlamydial infection are asymptomatic, and even upper genital tract chlamydial infection is often silent clinically.[6]

In India, approximately 10 million asymptomatic cases of genital chlamydial infection are reported in the sexually active age groups of the general population,[7] and both symptomatic and asymptomatic women show a similar potential for tubal damage.[8]

Persistent antigen synthesis and an ineffective immune response contribute to chronic inflammation, tissue damage and immunopathology associated with salpingitis/infertility.[9] Protective immunity to *C. trachomatis* infection is limited and repeated episodes of infection are common. Undetected and untreated, chlamydial organisms can ascend the upper genital tract and this may lead to pelvic inflammatory disease (PID), Fallopian tube injury and infertility.[10]

Several case-control studies and cohort analyses have reported a strong epidemiological, histological and serological correlation between chlamydial upper genital tract infection and PID.[11–13]

Numerous seroepidemiological studies have shown a consistent immunopathogenic association between antibody responses to the chlamydial heat shock protein 60 (cHSP60) and the development of PID, ectopic pregnancy and tubal infertility.[14–16] In *C. trachomatis*-induced disease, enhanced antibody and cell-mediated immune (CMI) responses to cHSP60 typically are detected.[17–19] This stress response is believed to interrupt the normal progression of reticulate bodies to infectious elementary bodies, resulting in persistent infection that may serve as an antigenic reservoir for potentially immunopathogenic anti-HSP immune system responses.[20,21]

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In an experimental monkey model, repeated *C. trachomatis* inoculations resulted in extensive tubal damage and occlusion,[22] which suggest the involvement of the host's immune response in tubal pathogenesis. Subsequent studies in monkeys[23] and in guinea pigs[24] demonstrated that exposure to cHSP60 resulted in a delayed hypersensitivity response and marked localised inflammation.

Further immune response to cHSP10 is associated with the pathogenic sequelae of chronic chlamydial infection[25] and tubal occlusion.[26] In addition, co-expression of cHSP10 with cHSP60 has been reported;[27] however; detection of anti-cHSP10 antibodies does not parallel that of anti-cHSP60 antibodies,[25,28] and the latter are reported to be an independent marker.

In order to assess the significance of the possible association of the response to cHSPs with disease progression, this study aims to evaluate the seroprevalence of *C. trachomatis* infection and the severity of disease by measuring antibody levels against cHSP60 and cHSP10 with respect to anti-MOMP. This will help to provide a clinically useful antibody screening test with which to predicting or confirming PID and infertility in infected women.

Materials and methods

Study population

A total of 255 women (aged 16 to 45 years) attending the out-patient department of Safdarjang Hospital, New Delhi, India, for gynecological complaints (cervical discharge, lower abdominal pain, pelvic pain, ectopy, erosion, PID and infertility) were enrolled in the study. Of these, 107 patients were diagnosed with cervicitis (presented with mucopus in endocervical exudate) while 52 had PID. The study was approved by the hospital ethics committee and informed consent was obtained from each patient.

On recruitment, a detailed history was taken. Routine haematological tests (erythrocyte sedimentation rate [ESR], white blood count [WBC] and haemoglobin) were requested and chest X-ray and endometrial biopsy were performed in the PID/infertility group to rule out infection with *Mycobacterium tuberculosis*.

Collection of samples

The vulva and cervix were examined for evidence of lesions and vaginal/cervical discharge. After cleaning the endocervix with a cotton swab, two cervical specimens were collected on separate cotton swabs and placed in sterile vials containing phosphate-buffered saline (PBS).[4,5] Cells were extracted by vortex mixing and then direct fluorescence assay (DFA) and polymerase chain reaction (PCR) methods were used to detect endocervical *C. trachomatis* infection.[3,29]

Diagnosis of other sexually transmitted disease (STD) pathogens was achieved by culture (*Neisseria gonorrhoeae*, *Mycoplasma hominis*, *Ureaplasma urealyticum*) and microscopy of Gram-stained smears (*Candida* spp., bacterial vaginosis, *Trichomonas vaginalis*).[30]

Non-heparinised venous blood was drawn, the serum was separated and then stored at –70°C for the detection of antibodies against chlamydial MOMP, HSP60 and HSP10.

Detection of antibodies against cMOMP

Enzyme-linked immunosorbent assay was used to detect IgG antibodies to *C. trachomatis* using MOMP-specific antigen ([288]SATAIFDTTLNPTIAGAGDVKTGAEGQLG[317]) (Cisbio, USA).[31] Briefly, 2.5 µg antigen was coated on microtitre plates and incubated overnight at 4°C. After washing with PBS-Tween 20 (PBS-T), the non-specific sites were blocked with 3% PBS-BSA. Serum samples (1 in 500 dilution in PBS-T) were added to the wells and incubated for 2 h at 37°C. After washing, a dilution (1 in 10,000) of horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG antibody (Bangalore Genei, India) was used to achieve colorimetric detection. Positive samples were defined as those yielding an absorbance (*A*) value at least

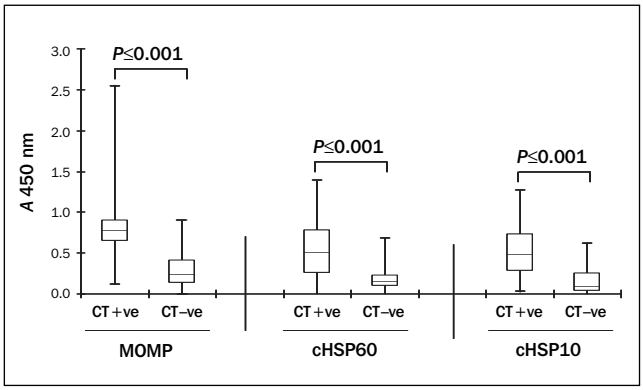

Fig. 1. Quantitation of chlamydial antigen-specific antibodies in human sera by ELISA using peptide-specific sequence of antigens. Median values of anti-MOMP, anti-cHSP60 and anti-cHSP10.

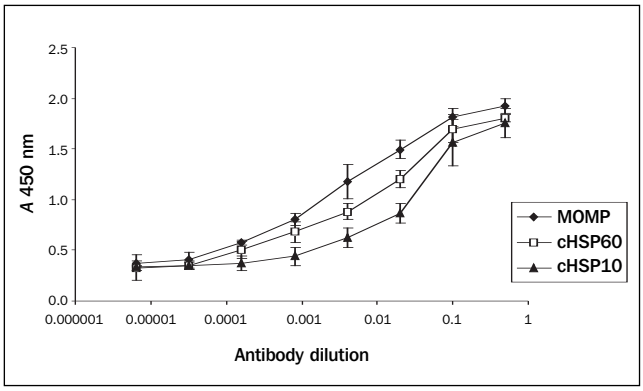

Fig. 2. Positive patients' sera (n=75) were diluted and ELISAs of MOMP, cHSP60 and cHSP10 were expressed as mean (A values) ± standard deviation. Statistically significant differences in A values between anti-MOMP and anti-cHSP60 were P<0.01 at 1:50 and P<0.05 at 1:250. Antibody titres of anti-cHSP60 were significant at dilutions of 1:50 (P<0.01) and at 1:250 and 1:1250 (P<0.05).

Table 1. Clinical details of the patients included in the study.

	C. trachomatis +ve n=75 (29.4%)		*C. trachomatis* –ve n=180 (70.6%)	*P*
	cHSP60 +ve n=48 (64%)	cHSP10 +ve n=46 (61.3%)		
Mean age (y)	30 (16–45)	29 (15–43)	32 (20–45)	
PID/infertility	13 (27.0%)	10 (21.7%)	29 (16.1%)	>0.76
Cervicitis	25 (52.1%)	27 (58.6%)	55 (30.6%)	>0.67

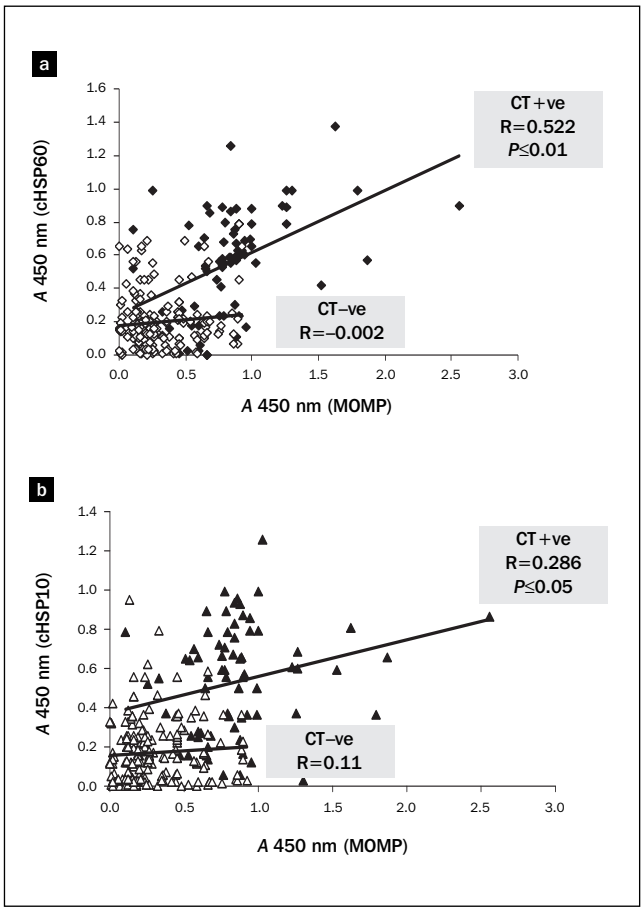

Fig. 3. Scatter plot showing the correlation of the serological response to immunogenic chlamydial antigens: a) anti-MOMP versus anti-cHSP60; b) anti-MOMP versus anti-cHSP10.

two standard deviations (SDs) above the mean value obtained from the panel of samples taken from the negative subjects (i.e., A_{450} >0.72 [mean +2SD of the negative samples]).[32,33]

Detection of antibodies against cHSP10 and cHSP60 IgG
Enzyme-linked immunosorbent assay was used to detect cHSP60 and cHSP10 antibodies in patient sera against synthetic cHSP60 ([151]SANNDAEIGNLI[162]) and cHSP10 ([79]SGQELTVEG[87]) peptides (Techno Concept, India).[34,35] The technique employed was as used above, and ELISA-positive samples were those that produced *A* at 450 nm >0.522 (cHSP60) and >0.49 (cHSP10).[32,33]

Statistical analysis
Spearman's rank method was used to find any correlation between anti-chlamydial antigens. The level of significance among groups was compared using the $\chi 2$ test. Significance of antibody titres was calculated by independent *t*-test.

Results

Diagnosis of STD pathogens in the cervix
Cervical *C. trachomatis* infection was diagnosed in 75 (29.4%) patients. None were found to be infected with other STD pathogens. Among the *C. trachomatis*-negative patients,

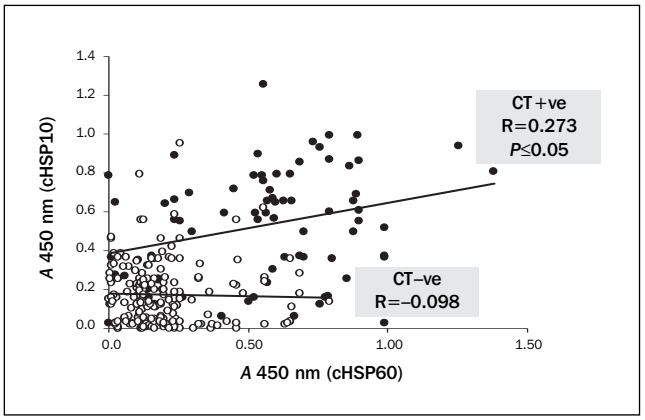

Fig. 4. Scatter plot showing the correlation between the chlamydial heat shock proteins. A significant positive correlation was seen between anti-cHSP60 and anti-cHSP10 in infected patients, and a negative correlation between the two in uninfected patients.

11 (6.1%) were infected with *Candida* spp., 17 (9.4%) showed bacterial vaginosis, 13 (7.2%) were infected with *M. hominis*, and 48 (26.7%) with *U. urealyticum*. No cases of *N. gonorrhoeae* or *T. vaginalis* were detected (data not shown).

Detection of antibodies against MOMP, cHSP60 and cHSP10
Enzyme-linked immunosorbent assay results for cMOMP, cHSP60 and cHSP10 showed higher *A* values in *C. trachomatis*-positive patients than in *C. trachomatis*-negative patients (median values: 0.794 *vs* 0.212, 0.584 *vs* 0.152 and 0.564 *vs* 0.13, respectively; Fig. 1). Positive samples were defined as those with an *A* value greater than the mean +2SD of the negative samples. Out of the 75 positive patients, 50 (66.7%) showed ELISA positivity for anti-MOMP, 48 (64.0%) for anti-cHSP60 and 46 (61.3%) for anti-cHSP10.

Antibody titres for anti-MOMP, anti-cHSP60 and anti-cHSP10
Five-fold serially diluted (1:2, 1:10, 1:50, 1:250, 1:1250, 1:6250, 1:31,250 and 1:156,250) positive sera were used. A significant increase in anti-MOMP titre was found at 1:50 ($P<0.01$) and 1:250 ($P<0.05$) compared to that of anti-cHSP60. The anti-cHSP60 titre was higher than that of anti-cHSP10 at 1:50 ($P<0.01$) and at 1:250 and 1:1250 ($P<0.05$).

Correlation between anti-MOMP with anti-cHSP60 and anti-cHSP10
A highly significant ($P<0.01$) positive correlation (R= 0.522) was seen between anti-MOMP and anti-cHSP60 in the *C. trachomatis*-positive patients. In the *C. trachomatis*-negative patients an insignificant correlation (R= –0.002) was observed between anti-MOMP and anti-cHSP60 (Fig. 3a). A positive correlation (R= 0.286; $P<0.05$) was seen between anti-MOMP and anti-cHSP10 in *C. trachomatis*-positive patients, but an insignificant correlation (R= 0.11) was seen in *C. trachomatis*-negative patients (Fig. 3b).

Correlation between anti-cHSP60 with anti-cHSP10
A significant positive correlation between anti-cHSP10 and anti-cHSP60 (R= 0.273; $P<0.05$) was seen in *C. trachomatis*-positive patients, but a negative correlation (R= –0.098) was found in *C. trachomatis*-negative patients (Fig. 4).

Antibody titres for anti-cHSP60 and anti-cHSP10 in PID and cervicitis

In the *C. trachomatis*-infected, cHSP60 ELISA-positive patients ($n=48$), 13 (27.0%) had PID/infertility and 25 (52.1%) had cervicitis. In the cHSP10 ELISA-positive patients ($n=46$), 10 (21.7%) had PID/infertility and 27 (58.6%) had cervicitis.

Sera from *C. trachomatis*-infected women positive for cHSP60 and cHSP10 were five-fold serially diluted (1:2, 1:10, 1:50, 1:250, 1:1250, 1:6250, 1:31,250 and 1:156,250) and antibody titres were compared between the PID/infertility and cervicitis groups.

cHSP60 ELISA positivity produced significantly higher titres at 1:50, 1:250, 1:1250 ($P<0.001$) and at 1:6250 ($P<0.01$) in PID/infertility patients compared to those with cervicitis (Fig. 5a). Similar observations were made with cHSP10; however, titres were not as well demarcated (Fig. 5b).

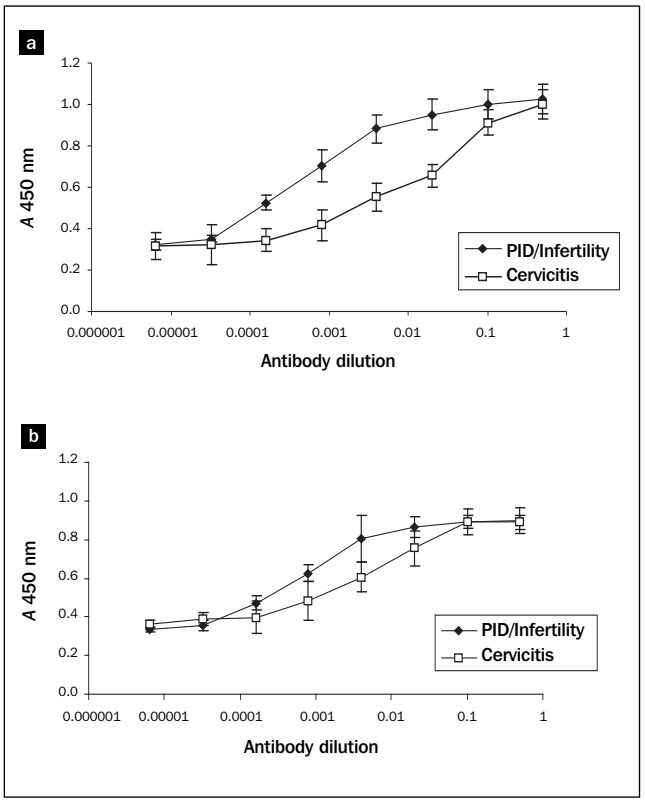

Fig. 5. Sera ($n=75$) positive for cHSP60 and cHSP10 were diluted and antibody titres were compared between PID/infertility and cervicitis cases: a) *A* values for anti-cHSP60 showed statistically significant differences at 1:50, 1:250 and 1:1250 ($P<0.001$) and at 1:6250 ($P<0.01$); b) *A* values for anti-cHSP10 showed significant differences at 1:250 and 1:1250 ($P<0.05$).

Discussion

Upper female genital tract infection due to *C. trachomatis* is often asymptomatic. In such cases a strong association between tubal factor infertility (TFI) and circulating chlamydial antibodies has been reported.[36,37] Immunity to *C. trachomatis* HSP60 and HSP10 is associated more typically with chronic upper genital tract infection and Fallopian tube damage than it is with acute infection of the lower genital tract.[15,25,26]

Although the presence of cHSP antibodies has been reported previously, correlation with disease and seroprevalance in patients with cervicitis/infertility in India is largely unexplored.

The results of the present study agreed with previous data which show a 23–30% incidence of chlamydial infection in the lower genital tract in Indian women.[2–5] Various serological studies suggest that the host's immune response is directed predominantly against *C. trachomatis* immunodominant proteins such as MOMP and cHSP60.[38,39]

In persistent chlamydial infection the ratio between chlamydial HSP60 and MOMP increases dramatically *in vitro*; however, the production of cHSP60 antibodies may predominate over the production of MOMP antibodies, which play a major role in protective immunity against the initial stages of infection.[40,41]

The results of the present study suggest that MOMP may be more immunogenic than cHSP60 in *C. trachomatis*-positive individuals. Similar observations have been reported in another study comparing MOMP and cHSP60 antibody titres.[42]

Many reports suggest that seropositivity for HSP60 antibodies is associated with the increased prevalence of histological salpingitis, pelvic adhesions and a past history of PID.[15–18,43] Furthermore, it has been reported that the ratio between cHSP60 and MOMP increases dramatically in subclinical chlamydial infection.[21]

Co-expression of cHSP60 and cHSP10 is a recognised feature and a positive correlation between the two HSPs in *C. trachomatis*-positive samples, and a negative correlation in *C. trachomatis*-negative patients, has been reported.[27] This suggests that cHSP60 and cHSP10 may express simultaneously during chlamydial infection, which confirms previous findings.[29]

Immune sensitisation of the host to specific regions of the chlamydial HSP60 might be responsible for initiating a delayed hypersensitivity reaction within the Fallopian tubes, resulting in tubal distortion or peritubal pelvic adhesions. *C. trachomatis*-induced diseases, enhanced antibody and other responses to cHSP60 have been detected in patients.[17–19]

Human HSP60 is a highly conserved protein that shares 48% amino acid sequence identity with cHSP60,[44] and specific antibodies have been found to cross-react with human HSP60, which suggests that these antibodies may play a role in disease pathogenesis.[38]

During rapid chlamydial cell growth or differentiation, or following environmental stress such as inflammation, host HSP60 synthesis is induced. Prolonged or repeated exposure to cHSP60 could result in the breakdown of immunological tolerance, leading to self-HSP60-directed immunity through cross-reactive T-cell and B-cell epitopes.[33] In general, the activation of a self-HSP60-specific immune response is considered to be detrimental to the well-being of the host and is associated with a shift from a protective immune response to a pathological response.[17–19]

The present study found high cHSP60 and cHSP10 antibody titres in PID patients. Similar results were obtained in a study of 306 patients, which showed a significant correlation between cHSP60 antibody, PID and occluded Fallopian tubes.[45] In addition, anti-cHSP60 titres were higher than anti-cHSP10 titres in PID patients. Furthermore, the

association between cHSP10 seropositivity and TFI was greater in *C. trachomatis*-positive patients than in *C. trachomatis*-negative patients.[25,26] This implies that seropositivity for both cHSP60 and cHSP10 may be a useful marker for disease severity. □

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