Chlamydial Heat Shock Proteins (cHSP) 60 and 10 in Immunopathogenesis of Female Genital *Chlamydia trachomatis* Infection

THESIS

Submitted in partial fulfillment
of the requirements for the degree of
DOCTOR OF PHILOSOPHY

BY

RAJNEESH JHA

UNDER THE SUPERVISION OF

DR. ARUNA SINGH

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE
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BIRLA INSTITUTE OF TECHNOLOGY & SCIENCE
PILANI RAJASTHAN

CERTIFICATE

This is to certify that the thesis entitled "**Chlamydial Heat Shock Proteins (cHSP) 60 and 10 in Immunopathogenesis of Female Genital *Chlamydia trachomatis* Infection**" which is submitted by **MR. RAJNEESH JHA,** (ID No. 2006PHXF023P) for award of Ph.D. Degree of the Institute, embodies original work done by him under my supervision.

Signature in full of the Supervisor — A. Singh

Name in capital block letters — (DR. ARUNA SINGH)

Designation — SCIENTIST 'F'

Institute of Pathology

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Place: New Delhi

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Rajneesh Jha

(Rajneesh Jha)

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Abstract

Chlamydia trachomatis is an important etiologic agent of Sexually Transmitted Infections (STIs) worldwide. Female genital *C. trachomatis* infections are often asymptomatic or show mild symptoms and thus often remain untreated. *C. trachomatis* female urogenital tract infections, includes cervicitis, salpingitis, Pelvic Inflammatory Disease (PID), endometritis, tubal factor infertility (TFI). Persistent, untreated *C. trachomatis* infection causes chronic stimulation of the host immune response against immunogenic antigens such as chlamydial heat shock proteins (cHSP) 60 and 10. There is a need to study the seroprevalence of cHSP60 and cHSP10 in symptomatic *C. trachomatis* infected women and also elucidate their role in the pathogenesis of female genital tract infection. IgG antibodies to conserved region of Chlamydial Major Outer Membrane Protein (cMOMP), cHSP60 and cHSP10 were detected by Enzyme-linked immunosorbent assay (ELISA) in *C. trachomatis* infected symptomatic patient's sera attending the gynecology out-patient department of Safdarjung Hospital, New Delhi. IgG antibody titers detected for anti-cMOMP were significantly higher than that for anti-cHSP60 (1:5; $P < 0.01$ and 1:25; $P < 0.05$). Moreover, patients with PID/infertility showed significantly higher antibody titers for anti-cHSP60 and anti-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 ($P < 0.01$). Further, considering IgG anti-MOMP ELISA as test standard, anti-cHSP60 antibodies showed higher sensitivity (90.91%) and specificity (89.47%) than anti-cHSP10 ELISA (75.6% and 73.87%) in the secondary infertile women. Anti-cHSP60 antibodies detection had a sensitivity of 67.33% and a specificity of 90.67% in secondary infertile women when compared with Direct Fluorescence Assay (DFA) and Polymerase Chain Reaction (PCR). This study will help in providing a

clinically useful antibody screening test for predicting or confirming PID and infertility in infected women.

Further, relative transcript levels and intracellular expression of cHSP60 and cHSP10 in cervical cells had different pattern of expression in *C. trachomatis*-infected fertile women compared to infertile women. The difference in expression pattern may reflect an abnormal cryptic form of *C. trachomatis* in infertile women suggesting probable role of these cHSPs in immunopathogenesis. Our results of *in vitro* stimulation of cervical mononuclear cells with cHSP60 and cHSP10 suggested that exposure to cHSPs could significantly affect mucosal immune function by increasing the release of Interferon-gamma (IFN-γ), Interleukin (IL)-10 and Tumor Necrosis Factor-alpha (TNF-α) in *C. trachomatis*-infected infertile women.

In addition, cHSP60 and cHSP10 induced apoptosis of primary cervical epithelial cell was studied. A DNA microarray study showed that 10 of 205 cellular genes related to apoptosis were upregulated in cHSP60 stimulated cells and 11 in cHSP10 stimulated cells whereas 6 and 4 genes were downregulated, respectively. Among these genes, the upregulation of caspase-3, -8 and -9 genes were confirmed in real-time reverse transcriptase (RT)-PCR and western blotting. The levels of pro-inflammatory cytokines IL-1β and IL-18 were also upregulated in cHSPs stimulated cells. Our results indicate that cHSP60 and cHSP10 were able to trigger apoptosis of primary cervical epithelial cells that are privilege target for chlamydial infection with concomitant production of proinflammatory cytokines. This mechanism may have a role in pathogenesis of infertility in women with persistent chlamydial infection.

Contents

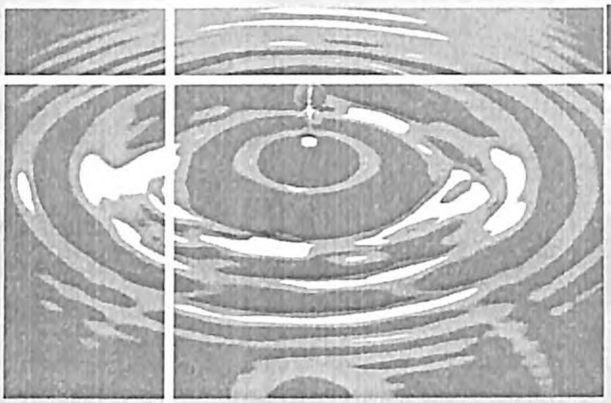
List of Tables

List of Figures

Abbreviations

A	Absorbance
α	Alpha
β	Beta
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CT	*Chlamydia trachomatis*
DAB	3, 3'- Diamino Benzidine
DC	Dendritic cell
DFA	Direct fluorescence assay
DEAE	Diethylaminoethyl
ddH_2O	Double Distilled water
DNA/RNA	Deoxyribose/Ribose nucleic acid
DMEM	Delbecco's Minimum Essential Media
dNTP	Deoxyribose nucleotide triphosphate
DTT	Dithiothreitol
EBs	Elementary Bodies
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
EMEM	Earle's Minimum Essential Media
FCS/FBS	Fetal calf/bovine serum
FITC	Fluorescin iso-thiocyanate
h	Hour/hours
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethgylpiperazine-N-2 ethane sulfonic acid
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horseradish peroxidase
HSP	Heat Shock Protein
γ	Gamma
IDO	2',3'-indolamine dioxygenase
IFN	Interferon
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
IPTG	Isopropyl-β-D-thio-galactosidase
Kb	Kilo base
LB	Luria Bertani medium
LGV	Lymphogranuloma venereum
M	Molarity
mg	Milligram
MHC	Major histocompatibility complex
MOMP	Major Outer membrane protein
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide
min/mins	Minute/Minutes
ml	Mililitre
mM	Milimolar
N	Normality

$NaHCO_3$	Sodium bicarbonate
ng	Nanogram
NO	Nitric oxide
°C	Degree Celsius
OD	Optical Density
ORF	Open reading frame
PAGE	Poly Acrylamide Gel Electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PBS-T	Phosphate buffered Saline with Tween-20
PCR	Polymerase chain reaction
pg	Picogram
PID	Pelvic inflammatory disease
PPLO	*Pleuropneumonia*-like organism
PVDF	Polyvinyl Difluoride
RBs	Reticulate Bodies
RFLP	Restriction fragment length polymorphism
RNase	Ribonuclease
rpm	Revolution per minute
RT-PCR	Reverse transcription- PCR
s/ secs	Second/ Seconds
SD	Standard deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Poly acrylamide Gel Electrophoresis
SE	Standard error
SPG	Sucrose phosphate glutamate
STD	Sexually transmitted diseases
STI	Sexually transmitted infection
TAE	Tris acetate EDTA
TEMED	N,N,N',N' tetramethyl ethylene diamine
Th	T-helper
TFI	Tubal factor infertility
TLR	Toll-like receptor
TMB	Tetramethyl benzidine
TNF	Tumor necrosis factor
Tris	Tris (hydroxymethyl) amino acid
U	Unit
UV	Ultra Violet
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
μm/ μM	Micromole / Micromolar



Chapter 1
Introduction

Introduction

Chlamydiae are gram-negative intracellular obligate bacterial pathogens causing major health problems. Traditionally, genus *Chlamydia* comprised of four species i.e. *C. trachomatis*, *C. psittaci, C. pneumoniae and C. pecorum* (Everett et al., 1999). *C. trachomatis* is the most common agent of bacterial urogenital infections responsible for cervicitis, endometritis and pelvic inflammatory disease (PID) in women; epididymitis and proctitis in men and arthritis in both men and women (Tavakoli et al., 2002). Also, *C. trachomatis* infection is thought to be the leading global cause of tubal infertility, ectopic pregnancy (Faro, 1991; Farquhar, 2005; Pellati et al., 2008) and failure of pregnancy after *in vitro* fertilization (Romero et al., 2004; Sulak, 2003). It has been reported that among all the preterm births, nearly 30% of deliveries may be associated with intrauterine infection (Ananth and Vintzileos, 2006; Romero et al., 2007). Moreover, *C. trachomatis* is an important risk factor for Human Immunodeficiency Virus (HIV) transmission (Ho et al., 1995; Laga et al., 1993) and for Human Papilloma Virus (HPV)-induced cervical neoplasia (Anttila et al., 2001). Other than genital complications, *C. trachomatis* is a major cause of trachoma, the leading cause of preventable blindness in humans (Solomon et al., 2006). *C. pneumoniae* causes respiratory diseases and has been associated with asthma and atherosclerosis (Belland et al., 2004; Campbell and Kuo, 2002; Hahn et al., 2002). *C. psittaci* and *C. pecorum* cause infections in birds, sheep, goat, cattles and swine. Zoonotic infections in humans are mainly caused by *C. psittaci* leading to pneumonia (Corsaro et al., 2003).

According to World Health Organization (WHO) report, 92 million new cases of genital *C. trachomatis* infections occur every year, highlighting the worldwide prevalence of *C. trachomatis* infections and also the economic burden on healthcare delivery (WHO, 2001;

Beagley and Timms, 2000). In India, a high prevalence (>30%) of *C. trachomatis* infection in symptomatic female patients (presenting with vaginal discharge and infertility) has been reported (Jalgaonkar et al., 1990; Joyee et al., 2004; Mittal et al., 1993b; Singh et al., 2002). In addition, a high percentage of *C. trachomatis* positivity has been reported in women with bad obstetric outcome including still birth, preterm delivery (Rastogi et al., 1999), spontaneous abortions (Rastogi et al., 2000) and infertility (Malik et al., 2006; Malik et al., 2009; Mittal et al., 1996).

C. trachomatis strain identification involves serotyping the Major Outer Membrane Protein (MOMP) and genotyping *ompA*, which encodes MOMP (Morre et al., 1998). On the basis of serotyping, *C. trachomatis* is divided into three groups of serovars: (i) Serovars A to C are endemic and the major cause of trachoma; (ii) Serovars D to K are sexually transmitted and often asymptomatic causing variety of urogenital complications in both men and women; (iii) Serovars L1 to L3 are also sexually transmitted but cause systemic Lymphogranuloma Venereum (LGV). Serovars A to K are known as the trachoma biovars and L1 to L3 as LGV biovars (Schachter and Caldwell, 1980). In India, the occurrence of serological variants of *C. trachomatis* serovar D (48%), serovar E (34%), serovar F (12%) and serovar I (6%) have been reported in infected urogenital samples (Singh V 2003). Strains of *C. trachomatis* have a highly conserved small genome of approximately 1 Mb and harbour 7-10 copies of plasmid of approximately 7 Kb (Stephens et al., 1998).

C. trachomatis strains are energy parasites in that they lack enzymes of the electron transport chain and thus, needs to acquire nutrients and adenosine triphosphate (ATP) from the host to promote its metabolism and replication (Hatch et al., 1982). They typically are characterized by a biphasic developmental cycle which alternates between an infectious,

extracellular form, the elementary body (EB) and a noninfectious, intracellular replicating form, the reticulate body (RB). The EBs attaches to a susceptible host cell and are endocytosed by phagocytosis. Inside the phagosome, EBs divides into RBs by binary fission and after multiple divisions, the RBs convert into EBs which are released on disruption of the host cells (Moulder, 1991; Wyrick, 2000). The whole developmental cycle takes around 48 to 72 h. Furthermore, the low nutrient availability, interferon (IFN)-γ mediated tryptophan starvation or other stressful conditions can trigger a persistent state with abnormal non-dividing RBs. These RBs can be reactivated to enter the developmental cycle when the conditions are again suited for growth (Beatty et al., 1994d).

C. trachomatis is a strong immunogen which stimulates both humoral and cell-mediated immunity (CMI) (Brunham and Rey-Ladino, 2005). The consequence of *C. trachomatis* infection depends on intricate balance between secreted cytokines by the lymphocytes and its interaction with host cells. Also, the persistent *C. trachomatis* infection remains a problem and often leads to immunopathogenesis (Kimani et al., 1996). Typically, *Chlamydiae*, T-helper (Th) 1 cells product IFN-γ is the most important factor in host defense while Th-2 cells activation product, IL-10 has been linked with disease susceptibility (Beatty et al., 1993). It is well known that *C. trachomatis* modulates immune system of the host for its own survival and induces persistent infections (Golden et al., 2000; Joyner et al., 2002). The single acute infection does not cause severe pathology associated with chlamydial disease but the recurrent reinfections are also common and responsible for persistent inflammation (Beatty et al., 1994b; Paavonen and Lehtinen, 1996). Therefore, induction of Th-1 or Th-2 type cell response may be an important determinant of chlamydial disease pathogenesis. Further, *in vitro*, studies have shown that the IFN-γ treatment of infected cells

limits the development of *C. trachomatis*. Such persistent infections not only exhibit highly unusual form of intracellular *Chlamydiae*, but also display induction of Chlamydial Heat Shock Protein (cHSP) 60 and reduction in the expression of other chlamydial antigens like MOMP and Lipopolysaccharide (LPS) (Beatty et al., 1994c; Kuipers et al., 2003). Therefore, further study is warranted to elucidate the actual role of cHSPs in the immunopathogensis of *C. trachomatis* infection in women.

In Chlamydiae, the 60-kDa cHSP60, or GroEL (Ct110), and the 10-kDa cHSP10, or GroES (Ct111), are genetically linked because they are encoded by genes arranged on the bicistronic groESL operon (Morrison et al., 1990). These two proteins bind to each other and prevent incorrect folding and denaturation, thus *C. trachomatis* is able to survive and persist in host cell and this may be maximized by higher expression of cHSP60 and cHSP10 (Karunakaran et al., 2003). Among a panel of 116 recombinant *C. trachomatis* proteins, cHSP60 has recently been identified as an immunodominant antigen, being a strong target of both humoral and CMI responses, whereas cHSP10 has been identified as a human T cell target (Follmann et al., 2008).

The cHSP60 released from the *C. trachomatis* infected cells is capable of eliciting potent localized proinflammatory immune response. Chronic or intermittent release of cHSP60 into extracellular milieu results in the production of antibodies against cHSP60 and other inflammatory mediators (Linhares and Witkin 2010). Several studies in women have identified a relationship between titers of serum immunoglobulin G (IgG) against the cHSP60 antibodies with chronic infection, salpingeal fibrosis, ectopic pregnancy, PID and tubal infertility (Domeika et al., 1998; Eckert et al., 1997; Gaur et al., 1999; Kinnunen et al., 2003; Ondondo et al., 2009; Tiitinen et al., 2006; Toye et al., 1993). It is reported that

because of the molecular mimicry and chronic antigenic stimulation, antibodies to cHSP60 in women with chlamydial related pathology also react with human HSP60 (hHSP60) (Patton et al., 1994; Sziller et al., 2008; Sziller et al., 1998; Yi et al., 1993). This eventually leads to loss of tolerance and the generation of immunity to conserved amino acid sequences that are also present in the homologous hHSP60. Moreover, high levels of anti-cHSP60 antibodies play a pivotal role in *C. trachomatis*-associated immune mediated inflammation leading to PID, fallopian tube obstruction and ectopic pregnancy (Brunham and Peeling, 1994; Paavonen, 1996b). Also, a significant association has been found between antibodies to anti-cHSP60 in serum and in follicular fluid of infertile women (Cortinas et al., 2004). The persistent inflammation of upper genital tract leads to low implantation rate as hHSP60 is expressed in early stage of embryogenesis and a cross-reacting antibody may induce destruction of the embryo (Jakus et al., 2008). Thus, detection of IgG antibodies to cHSP60 may indicate persistence of *C. trachomatis* in the upper genital tract with low implantation rates resulting from chronic inflammatory sequelae like PID and infertility.

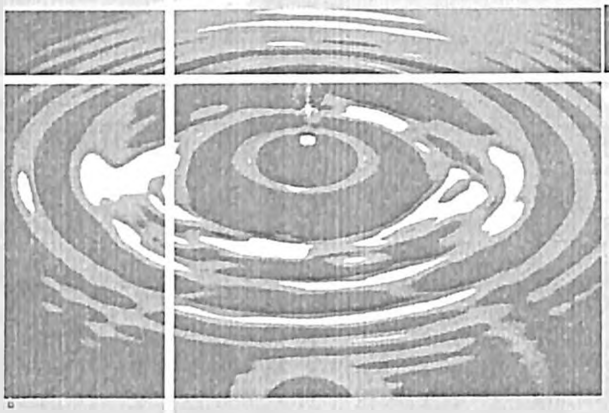
Also, a very strong association has been observed between chronic chlamydial infection and the presence of anti-cHSP10 antibodies (Brunham and Peeling, 1994; LaVerda et al., 2000; LaVerda et al., 1999). The presence of hydrosalpinx has been shown to be associated with an increased prevalence of antibodies to cHSP10 as compared with that of tubal factor infertility (TFI) patients without hydrosalpinx (Spandorfer et al., 1999). It has also been reported that the detection of anti-cHSP10 antibodies does not parallel that of anti-cHSP60 antibodies (Betsou et al., 1999). Betsou *et. al.* reported that there is cross-reactivity between cHSP10 and early pregnancy factor (EPF) in *C. trachomatis* infected women. Also, antibodies against cHSP10 may act as a marker for autoimmune responses to self-EPF

initiated by molecular mimicry (Betsou et al., 2003). This cross-reactivity may be associated with either failed pregnancy or infertility which suggests underlying inflammation induced upregulation of cHSP10 production and the recruitment of memory T cells to the site of upregulated cHSP10 presentation in infertile women (Lindquist and Craig, 1988; van Eden et al., 1998).

The survival or persistence of *Chlamydia* in infected cells is regulated by many factors including responses generated by host. The host inflammatory response is required for the elimination of infection and is also responsible for the pathogenesis of *C. trachomatis* (LaVerda et al., 1999; Stephens, 2003). Survival of the infected cells during chlamydial infection, followed by non-inflammatory "apoptotic death" could be beneficial to both the *C. trachomatis* and to some extent to the host. However, premature apoptotic death of *C. trachomatis*-infected cells limits the ability of pathogen to proliferate (Ying et al., 2007). *Chlamydia* has been described to either induce or inhibit host cell death and this may be due to the fact that chlamydial genes are expressed at different levels at different stages during the life cycle (Byrne and Ojcius, 2004; Miyairi and Byrne, 2006). Despite the mechanisms evolved by *C. trachomatis* to prevent host cells death, it has also been reported that cell death is induced at the end of developmental cycle (Jungas et al., 2004; Ying et al., 2006). *C. trachomatis*-derived antigens (viz; MOMP, cHSP60, cHSP10 etc.) are relevant for immune responses by *Chlamydia*-induced cell death. This may have impact on innate immune responses wherein pro-inflammatory mediators from dyeing cells are released which promote long-term tissue damage (Darville et al., 1997). The cHSP60 is known to be associated with outer membrane and can be exposed to extracellular milieu to induce immune activation at the site of infection. cHSP60 is also known to be associated with persistent models of *C.*

trachomatis infection leading to severe sequelae like PID and infertility. Further, Equils *et. al.* have shown that cHSP60 induces apoptosis in primary trophoblasts and JEG3 cell line through caspase dependent pathway. This further suggests contribution of cHSP60 in the pathogenesis for poor fertility and pregnancy outcome in women with persistent *C. trachomatis* infection (Equils et al., 2006). However, the role of cHSPs in the immunopathogenesis of *C. trachomatis* infection in women is not completely understood.

The proposed study was undertaken with the aim of studying the role of cHSP60 and cHSP10 in diagnosing *C. trachomatis* infection in fertile and subfertile women and also studying its role in immunopathogenesis. Therefore, for this study, (i) Seroprevalence of IgG antibodies to conserved regions of cHSP60 and cHSP10 was studied in symptomatic women. (ii) The possible association of the antibody titers to cHSPs with severity of diseases (cervicitis, PID and infertility) were evaluated. (iii) Serological responses to cHSPs in different clinical conditions (primary infertility, secondary infertility, cervicitis and discharge) were also evaluated to know which cHSP is more reliable for early diagnosis of *C. trachomatis* infection in women. Further, (iv) Full-length recombinant cHSP60 and cHSP10 were used to study the specific immune responses for better understanding the role of these two proteins in the immunopathogenesis and apoptosis of primary epithelial cells at the site of infection.



Chapter 2
Review of Literature

Review of Literature

Chlamydiae are gram-negative, eubacteria and obligate intracellular parasite in eukaryotic cells, which thrive in a host derived vacuole termed "Inclusion" (Abdelrahman and Belland, 2005). This phylogenetically well-isolated group of closely related bacteria constitutes the single family *Chlamydiaceae* of the order *Chlamydiales*, which form a separate phylum in the domain Bacteria, the *Chlamydiae.* Four families are recognized within the order *Chlamydiales* (Corsaro et al., 2003), with a total of six genera and 13 species **(Table 2.1)**, namely, family *Chlamydiaceae* (*Chlamydia* and *Chlamydophila*), family *Parachlamydiaceae* (*Parachlamydia acanthamoebae*, *Neochlamydia hartmannellae*, *Protochlamydia amoebophilia*), family *Simkaniaceae* (*Simkania negevensis*, *Fritschea bemisiae*), and family *Waddliaceae* (*Waddlia chrondrophila*) (Beagley et al., 2009).

The family *Chlamydiaceae* comprises the best known human and animal chlamydial pathogens. For a long time, the single genus, *Chlamydia*, was the only genus within this family. In 1999, Everrett *et al.* proposed splitting of this family into two families, *Chlamydia* and *Chlamydophila*, based on a range of phenotypic, bioecological, and genotypic data (Everett et al., 1999). This subdivision into the two genera has not been widely accepted by the chlamydial community (Schachter, 2001). A phylogenetic analysis based on 110 concatenated genes conserved in all *Chlamydia* genomes confirms and strengthens the close and linked evolutionary relationship among *Chlamydiae* **(Figure 2.1)**. In this tree, species *trachomatis, muridarum, pneumoniae* and *pecorum* cluster away from species *felis, caviae, psittaci* and *abortus*, further depreciating the value of 16S rRNA gene-based trees for the purpose of taxonomic classification in the *Chlamydiaceae* (Stephens et al., 2009). Chlamydial genome analysis revealed the evolution of *Chlamydiae* as early as 60-100 million

years ago (Stephens et al., 1998). But, due to common constraints and intracellular niche,

Table 2.1: Taxonomy of *Chlamydiae* (Beagley WK et al 2009)

Systematics	Natural host	Pathogenecity
Order: Chlamydiales		
Family I: Chlamydiaceae		
Genus I: *Chlamydia*		
C. trachomatis	Humans	Ocular, Urogenital infections
C. pneumonia	Humans, Horses, Koalas	Respiratory, Ocular, Urogenital infections
C. muridarum	Rodents	Respiratory, Ocular, Urogenital infections
C. suis	Swine	Enteritis
C. psittaci	Birds	Avian Chlamydiosis
C. abortus	Ruminants	Abortion
C. pecorum	Cattles, Sheep, Koalas	Enteritis, Abortion, Polyarthritis, Ocular, Urogenital infections
C. felis	Cats	Ocular, Urogenital infections
C. caviae	Guinea pigs	Ocular, Urogenital infections
Family II: Parachlamydiaceae		
Genus I: Parachlamydia		
P. acanthamoebae	Acanthamoeba	Endosymbiont
Genus II: Neochlamydia		
N. hartmannellae	Hartmannella	Parasite
Family III: Simkaniaceae		
Genus I: Simkania		
S. negevensis	Humans (?)	Respiratory infections
Genus II: Fritschea		
F. bemisiae	Insects	
F. eriococci	Insects	
Genus III: Rhabdochlamydia		
Rhabdochlamydia spp.		
Family IV: Waddliaceae		
Genus I: Waddlia		
W. chrondrophila	Cattle, Fruit bats (?)	Abortion

Chlamydiae genome reflected in the accumulation of mutations and gene loss while retaining their unifying biology. Therefore, the spectrum of divergence among *Chlamydiae* is without genetic difference (Stephens et al., 2009). With the acceptance of single *Chlamydia* genus, there are now nine recognized species namely *C. trachomatis*, *C. muridarum*, *C. suis*, *C. psittaci*, *C. pneumoniae*, *C. caviae*, *C. felis*, *C. abortus*, and *C. pecorum*.

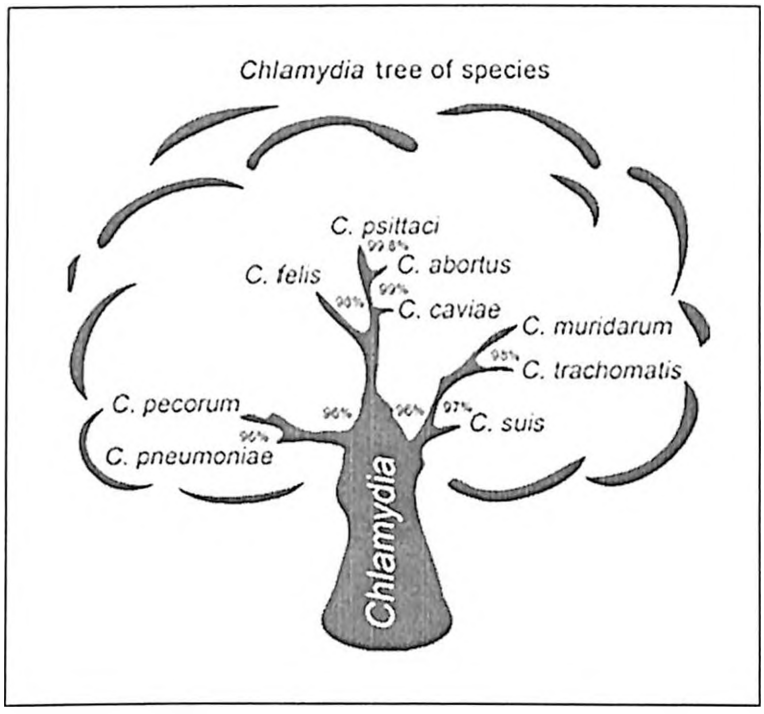


Figure 2.1: Different species of *Chlamydia* showing percent similarity of 16S rRNA gene.

Life-cycle of Chlamydia

The chlamydial developmental cycle consists of two distinct morphological forms, namely, (i) EB, which is the metabolically inactive, extracellular, infectious stage, which is designed for passage between suitable host cells, and (ii) RB, which is the metabolically active, replicating, intracellular form. The complete developmental cycle takes between 36 and 72 h, depending on the chlamydial species. Chlamydial infection is initiated by attachment of EBs to the susceptible host cells by either phagocytosis (Ward and Murray,

1984) or receptor mediated endocytosis (Hodinka et al., 1988) or via both mechanisms depending on chlamydial strain and host cell (Prain and Pearce, 1989). In epithelial cells, the inclusion does not acquire lysosomal markers but does fuse with trans-Golgi network-derived sphingolipid containing secretory vesicles (Hackstadt et al., 1995). Once entry occurs, the EBs becomes internalized in a double membrane inclusion after 4-8 h and does not enter early into the pathway of the golgi apparatus, avoiding phagolysosomal fusion. The EBs inside double membraned endosome multiply by binary fusion through several rounds of replication results in RBs after 8-12 h, then replication of RBs continues till 20 h. Further, RBs converting back to EBs and ready for exit from the host cell after lysis in 48-72 h (Hoare et al., 2008). Although, the developmental cycle is highly conserved across the chlamydial species, there are differences in the attachment, entry and survival of different species in mouse macrophages. Recently Rank *et. al.* showed intense infiltration of polymorphonuclear leukocytes (PMNs) in conjunctival epithelium after four day of *C. caviae* infection. These PMNs play an active role in detaching infected cells from the epithelium and die releasing organism but in the process, move to new tissue sites via fluid dynamics (Rank et al., 2008). *C. trachomatis* infects mannose receptor–positive mouse macrophages better than mannose receptor–negative cells whereas; *C. pneumoniae* infects mannose receptor–negative cells better than positive ones, whereas *C. psittaci* infects both cells types equally (Kuo et al., 2002).

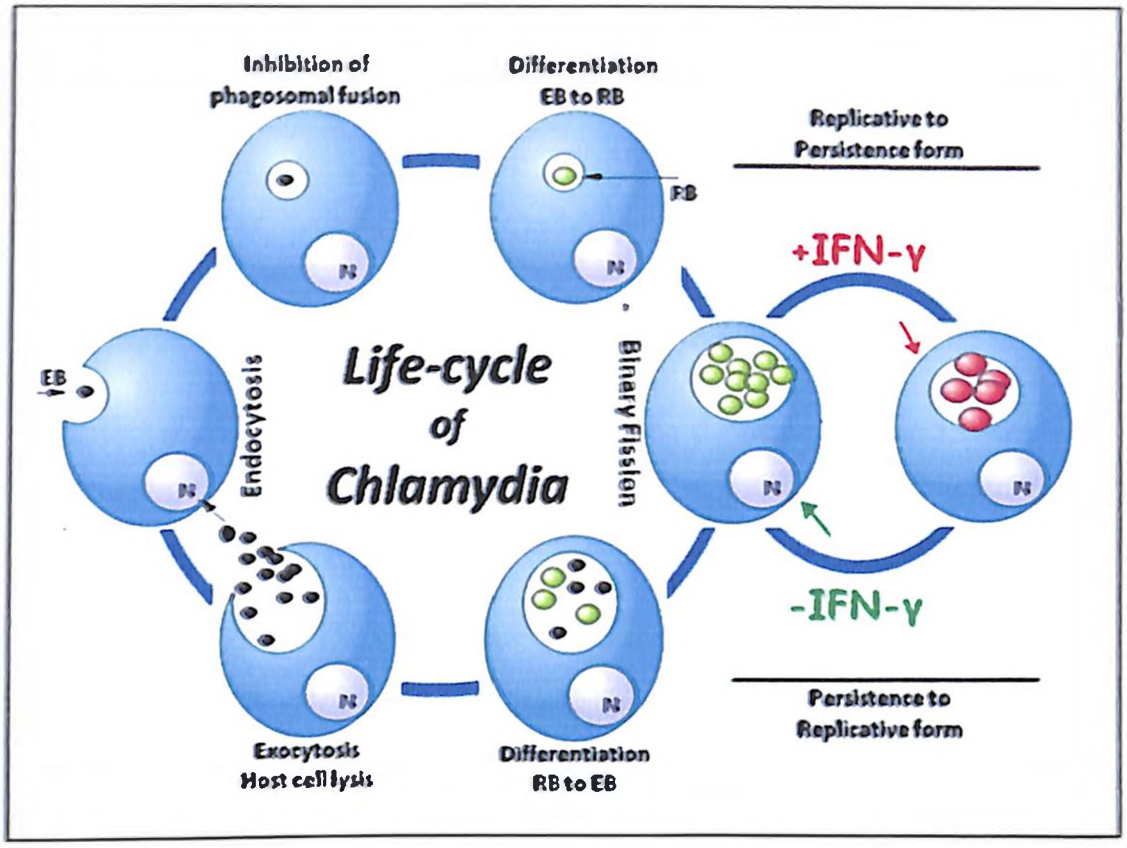


Figure 2.2: Life-cycle of *Chlamydia*. The *Chlamydia* Development cycle characterized by a metabolically inactive EB (black) differentiates into metabolically active RB (green) then again differentiates back to EB to initiate new round of infection. Persistence can follow the addition of IFN-γ, which induces non replicating atypical aberrant bodies (red) whereas resumption of growth and release of EB observed upon removal of stress (- IFN-γ) (Rottenberg et al., 2002).

The conversion of EB to RB then back to EB is considered as main replicative form but persistence phase also occurs during some stressful conditions such as nutrient depletion especially tryptophan starvation, iron depletion, low levels of IFN-γ and heat shock. Chlamydial persistence is defined as a viable but noncultivable growth stage resulting in a long-term relationship with the infected host cell. In persistence phase, the developmental cycle becomes stalled and infectious EBs is not produced. Stress induced atypical inclusions

are generally smaller in diameter and contain reticulate-like aberrant bodies, larger in diameter than typical RBs. Perhaps the best-studied model of in vitro persistence is that induced by treatment with IFN-γ. It is well established that IFN-γ controls the *in vitro* growth of *C. trachomatis* through production of the enzyme indoleamine-2,3-dioxygenase (IDO). Activation of IDO by IFN-γ leads to the degradation of tryptophan, and lack of this essential amino acid causes the death of *C. trachomatis* through tryptophan starvation (Beatty et al., 1994a). Further, it has been shown that genital, but not ocular, serovars of *C. trachomatis* can use indole as a substrate to synthesize tryptophan in the presence of IFN-γ, which might allow genital strains of *C. trachomatis* to escape IFN-γ-mediated eradication in the genital tract (Caldwell et al., 2003; Fehlner-Gardiner et al., 2002). Resumption of growth and the release of infectious EBs are observed upon removal of stress and after the reorganization of the aberrant form into a replicative organism **(Figure 2.2)**.

Chlamydia trachomatis Associated Clinical Complications

C. trachomatis infection causes most common bacterial sexually transmitted disease (STD) and infection of eye in humans (Gerbase et al., 1998). As many as 85 to 90 % of *C. trachomatis* infection in men and women are asymptomatic (Cecil et al., 2001) which can persist for several months if do not seek medical treatment (Stamm, 1999). Untreated infection in both sexes increases the risk of HIV (Fleming and Wasserheit, 1999; Galvin and Cohen, 2004) and HPV –induced cervical neoplasia (Anttila et al., 2001). The highest incidence of positive isolation of *Chlamydiae* is in the age group of less than 25 years (Barnes et al., 1990). The incidence gradually decreases with increasing age (Arno et al., 1994). According to the specificity of MOMP epitopes, *C. trachomatis* is currently divided into 19 serovars (Schachter J, 1999). Serovars A, B, Ba and C are the agents of trachoma, a

major cause of blindness in Africa, the Middle East, Asia and South America. Serovars D–K, including D, Da, E, F, G, Ga, H, I, Ia, J and K, are the most common sexually transmitted bacteria, and serovars L1, L2, L2a and L3 are the agents of transmission of LGV **(Table 2.2).**

Table 2.2: *Chlamydia trachomatis* serovars and their associated human diseases (Brunham and Ray-Ladino, 2005)

Serovar	Clinical manifestation	Complication
A-C	Keratoconjunctivitis	Scarring trachoma, blindness
D-K	Males: Urethritis, Proctitis	Epididymitis
	Females: Cervicitis, Urethritis, Proctitis	Endometritis, Salpingitis, Pelvic pain, Ectopic pregnancy, Perihepatitis (Fitz-Hugh-Curtis syndrome), Infertility
	Males and Females: Conjunctivitis	Reiter's syndrome, Reactive arthritis
L1-L3	Lymphogranuloma venereum: Inguinal syndrome, proctitis	Fibrosis, Rectal stricture

Trachoma

Trachoma is the world's leading cause of preventable blindness. The ocular infection manifests from mild conjunctival lesions (follicular conjunctivitis) to severe inflammatory forms that eventually lead to scarring and blindness (Skwor et. al., 2010). Severe forms develop through repeated or persistent infections by *C. trachomatis* serovars A, B, Ba and C (Abu el-Asrar et al., 2001; Taylor et al., 1982). Trachoma is endemic mainly in tropical and subtropical countries. Main reservoir of the organism is eye of the infected person, usually a

child, and transmission may be potentiated by flies that carry infected secretions from person to person (Emerson et al., 1999; Jones, 1974).

Sexually Transmitted Infection

In women, clinical manifestations of *C. trachomatis* infections include acute urethral syndrome, urethritis, bartholinitis, cervicitis, endometritis, perihepatitis (Fitz-Hugh–Curtis syndrome), and reactive arthritis (Brunham et al., 1984; Dieterle et al., 1998; Mardh et al., 1981; Stamm, 1999; Stamm et al., 1980). Asymptomatic and untreated chlamydial infection in women can lead to severe reproductive complications causing PID (De Punzio et al., 1995; Mardh, 1997; Paavonen, 1996a). Repeated infection with different or same serovars have been shown to increase the risk for subsequent infertility (Patton et al., 1990; Pavletic et al., 1999; Rank et al., 1995), ectopic pregnancy (Barlow et al., 2001; Cates and Wasserheit, 1991; Gerard et al., 1998), chronic pelvic pain (Westrom et al., 1992), tubal factor infertility (TFI) (Barlow et al., 2001; Punnonen et al., 1979; Rhoton-Vlasak, 2000) and spontaneous abortions (Quinn et al., 1987; Witkin and Ledger, 1992). Chlamydial infection during pregnancy is associated with a number of adverse outcomes including preterm labor, premature rupture of the membranes, low birth weight, neonatal death, postpartum endometritis (Andrews et al., 2000; Mardh, 2002) and may be transmitted to the infant during delivery causing conjunctivitis and nasopharyngeal infection (Jain, 1999).

In men, the most common clinical manifestation of *C. trachomatis* infection is nongonococcal urethritis. Other clinical syndromes in men include acute epididymitis, acute proctitis, acute proctocolitis, conjunctivitis, and Reiter's syndrome (Berger et al., 1978; Paavonen and Eggert-Kruse, 1999; Stamm et al., 1984). In fact, *C. trachomatis* causes approximately 35 to 50 percent of all cases of nongonococcal urethritis in heterosexual men.

Symptoms of nongonococcal urethritis may develop after an incubation period of 7 to 21 days and include dysuria and mild to moderate whitish or clear urethral discharge. In most cases, physical examination reveals no abnormalities other than the discharge. Male infertility, chronic prostatitis, and urethral strictures are possible results of infection. Both Reiter's syndrome (urethritis, conjunctivitis, arthritis, and mucocutaneous lesions) and reactive tenosynovitis or arthritis (without the other components of Reiter's syndrome) has been associated with genital *C. trachomatis* infection (Stamm, 1999).

LGV, is a systemic disease caused by invasive serovars (L1, L2 and L3) of *C. trachomatis*. LGV infects lymphatic and subepithelial tissues which spread exclusively through sexual contact. Although, it is an important pathogen in some developing countries and relatively uncommon in industrialized countries (Schachter J 1999). However, all the reported cases of LGV from Netherlands, France, Sweden, Canada and United States since 2003 have been caused by the L2 serovars, although there is some evidence that a number of genetically distinct strains of *C trachomatis* L2 are responsible for these outbreaks (Blank et al., 2005; French et al., 2005).

Chlamydial Genome Organization

Chlamydial genome comprises of closed circular double stranded DNA and all the strains of *C. trachomatis* and many of *C. psittaci* have 7-10 copies of plasmid. The chromosomal DNA is approximately 600-850 kb in size (Becker, 1978) that provided a much clearer picture on genome organization after the completion of chlamydial genome project. The first *Chlamydia* genome sequences of *C. trachomatis* (Stephens et al., 1998) and *C. pneumoniae* (Kalman et al., 1999) are available after the completion of *Chlamydia* genome project in 1998 (http://chlamydia-www.berkeley.edu:4231/ and GenBank accession no.

AE001273). These sequences provide insight into the pathogenic and metabolic pathways of *Chlamydia* and form a basis for further research to understand *Chlamydia* biology. The sequenced *C. trachomatis* genome serovar D consists of circular chromosome of 1,042,519 base pairs (bp), which is about one quarter of the size of *Escherichia coli* DNA (Stephens et al., 1998).

The whole genome is encoding about 894 protein coding genes with 58.7% A+T content. Plasmids of size 7,493 bp are detected initially in three strains from serovars B, C and L2 of *C. trachomatis*. In a later report, plasmids were detected in strains from serovars B, C, D, L1, L2, and L3. (Hyypia et al., 1984; Lovett et. al., 1980). It has been suggested that plasmid is essential for the survival of *C. trachomatis*. However, some reports suggest that it may not be essential. A strain of serovar L2 that lacks the plasmid and does not contain plasmid integrated into the chlamydial chromosome was isolated from the patient with proctocolitis (Peterson et al., 1990). *C. pneumoniae* does not have plasmid (Campbell et al., 1987) and strains of *C. psittaci* showed considerable diversity. Most strains of plasmid range from 6.2-7.9 Kb, but some strains lack plasmids (Joseph et al., 1986). Some plasmid-free isolates of *C. trachomatis* have been described, but these are exceedingly rare and the only viable clinical isolates described that are plasmid free belong to serotypes L2, D and E (Farencena et al., 1997; Peterson et al., 1990; Stothard et al., 1998).

Clustering by sequence similarity revealed that 256 chlamydial proteins (29%) belong to 58 families of similar genes within the genome (paralogs), a fraction similar to other bacteria with relatively small genomes such as the *mycoplasmas* and *Haemophilus influenzeae* (Brenner et al., 1995; Koonin et al., 1997; Tatusov et al., 1997). The limited and obligate intracellular growth of *Chlamydiae* and lack of any direct and indirect genetic

transfer method is also confirmed by absence of genes involved in DNA uptake and insertion sequences (Stephens, 1992). Also, chlamydial genes encoding proteins to synthesize amino-acids is greatly reduced. A large number of gene encoding different ABC transporters which are primarily involved in smaller peptides and amino acids (Stephens et al., 1998). Therefore, *Chlamydiae* have traditionally been described as energy parasites obtaining ATP from host cells (Hatch et al., 1982; Moulder, 1991) and the genomes of *C. trachomatis* confirmed the presence of two genes CT065 and CT495 which is homologous to genes encoding ATP transporting proteins from *Rickettsia prowazekii* (Andersson et al., 1998). This ability enables *Chlamydia* to obtain ATP from the host cells in the early and late stages of developmental cycle (Hatch et al., 1982). In Chlamydial genome, genes encoding the 60-kDa cHSP60 or GroEL (CT110) and the 10-kDa cHSP10 or GroES (CT111) are genetically linked because they are encoded by genes arranged on the bicistronic *groESL* operon (Morrison et al., 1990).

Cell-wall Structure

The *Chlamydia* cell wall consists of both genus-specific and species-specific structures. The LPS constitutes the component of the membrane common to all *Chlamydia* species (genus-specific) (Stuart et al., 1987). The membrane also contains various Outer Membrane Proteins (OMPs). The outer membrane protein is made up of MOMP of molecular weight 40 kDa, OMP2 of molecular weight 60 kDa and a third protein of molecular weight 60 kDa **(Figure 2.3)**. The most abundant of three proteins is MOMP which is found in all chlamydial membranes reported so far. It contains genus, species, subspecies and serovar specific antigenic determinants (Stephens et al., 1986; Ward, 1983). MOMP gene of the *C. trachomatis* serovar L2 was the first chlamydial gene to be cloned and sequenced (Stephens et al., 1986). The MOMP is the immuno-dominant antigen of *C. trachomatis* and

contains 4 variable domains (VDs) that are flanked and interspaced by 5 constant domains (CDs). Three of the variable domains (VD1, VD2, and VD4) are surface exposed and contains antigenic epitopes while the constant regions show cross-reacting, genus-specific epitopes (Stephens et al., 1988).

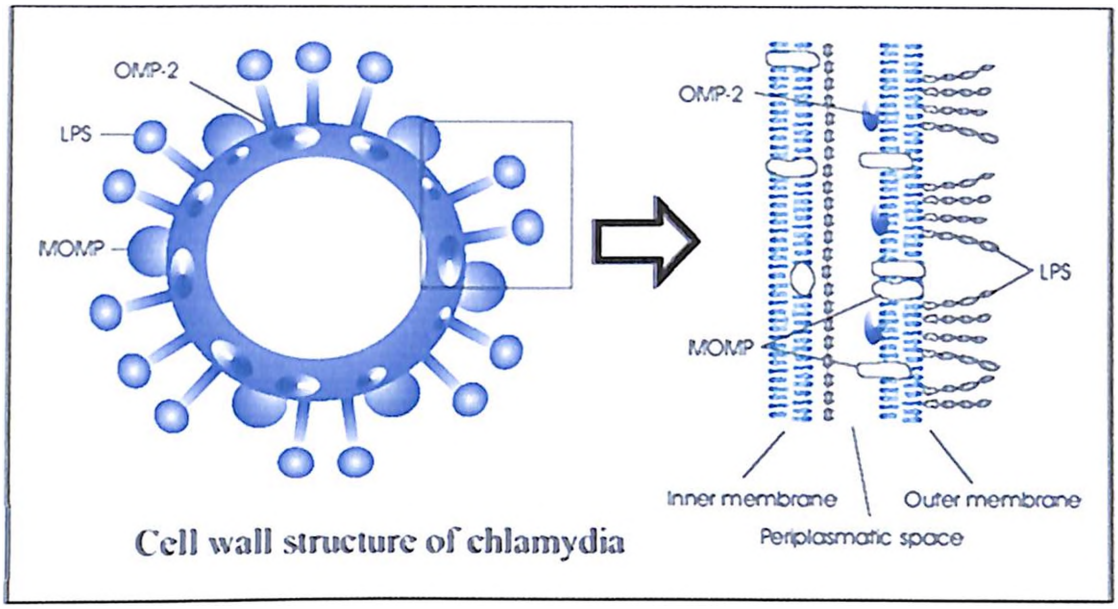


Figure 2.3: Cell wall structure of *Chlamydia*. The *Chlamydia* cell wall consists of both genus-specific and species-specific structures. The LPS constitutes the component of the membrane common to all *Chlamydia* species (genus-specific). The membrane also contains various OMPs.

Chlamydial Immunology

C. trachomatis stimulates both humoral and CMI responses. Immune system perturbations induced by *C. trachomatis* infact assist its own survival in infected host and induce persistence infection. Several immunological observations suggest that there is an ultimate relationship between *Chlamydia* and host immune system.

Innate Immunity

The mucosal epithelial cells of female reproductive tract serve as sentinels in immune protection and evolved to meet the unique requirements with sexually transmitted bacterial pathogens (Wira et al., 2005). The immune response to *C. trachomatis* is a co-ordinated event where innate immune cells, B cells, and T cells act in concert. The mucosal barrier acts as a first line of host defense during genital *C. trachomatis* infection. Shedding of the endometrial epithelium during certain stages of this estrus cycle can limit the ability of *C. trachomatis* to establish infection (Ramsey et al., 1999; Tuffrey et al., 1986). Natural antimicrobial peptide, Defensins, have been shown to inhibit *C. trachomatis* infection in urethral samples (Porter et al., 2005). *C. trachomatis* infection of both human and murine epithelial cells can induce the production of pro-inflammatory cytokines and chemokines such as interleukin (IL)-1, IL-6, tumour necrosis factor (TNF)-α, Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Johnson, 2004; Rasmussen et al., 1997) and IL-8 (Buchholz and Stephens, 2006). In addition, natural killer (NK) cells and phagocytic cells such as neutrophils, macrophages and dendritic cells (DCs), which are abundant in the genital mucosa (Parr and Parr, 1991) produce more inflammatory cytokines such as TNF-α, within the infected epithelial cells (Dessus-Babus et al., 2002). Lower levels of TNF-α and IL-6 have been correlated with decreased genital tract tissue pathology following *Chlamydia* genital infection (Darville et al., 2003).

Another major inflammatory cytokine produced by innate immune cells is IFN-γ, which can upregulate the phagocytic potential of macrophages, thereby promoting the engulfment and destruction of extracellular EBs (Zhong and de la Maza, 1988). Upregulation of inducible nitric oxide synthase in response to IFN-γ treatment can kill intracellular

Chlamydia organisms in infected cell lines (Chen et al., 1996; Igietseme et al., 1997). The production of IFN-γ and other pro-inflammatory cytokines in response to *C. trachomatis* infection is enhanced through the recognition of pathogen-associated molecular patterns (PAMPs). The best characterized receptors for PAMPs are the Toll-like receptors (TLRs). Although *C. trachomatis* LPS can be recognized by TLR4 (Prebeck et al., 2003) and cHSPs are known to be ligand for TLR2 and TLR4 (Bulut et al., 2002; Da Costa et al., 2004), TLR2 appears to be more essential for signaling pro-inflammatory cytokine production following *Chlamydia* infection (Darville et al., 2003; O'Connell et al., 2006). In addition to producing inflammatory cytokines and destroying *Chlamydia* organisms, a subset of phagocytes, the DCs, is also efficient at processing and presenting *Chlamydia* antigens to T cells (Steele et al., 2004). Recently, Agarwal *et. al.* shown that both plasmacytoid and myeloid DC subsets are attracted to the site of chlamydial infection with more prevalence of plasmacytoid DCs in inflammatory condition (Agrawal et al., 2009). The DCs therefore provide an essential link between innate and adaptive immunity.

Adaptive Immunity

Cells of the adaptive immune system are necessary to limit the infection and provide protection during a future encounter with *Chlamydia*. The concept of B-cells and *Chlamydia*-specific antibodies correlated with protective immunity in humans (Barenfanger and MacDonald, 1974; Jawetz et al., 1965). The monoclonal antibodies against MOMP could neutralize *Chlamydia* infection *in vitro* (Peeling et al., 1984; Peterson et al., 1991) and also provide protection against infection when passively administered to mice (Cotter et al., 1995). In addition, the Fc receptor mediated activities of antibodies also play an important role in combating infection (Moore et al., 2002; Moore et al., 2003).

During primary infection, B cell-deficient mice control *Chlamydia* genital infection as efficiently as wild-type mice. However, *Chlamydia* clearance following secondary infection is slightly delayed in the absence of B cells (Su et al., 1997; Williams et al., 1997), suggesting that B cells may play a role in the memory response to *C. trachomatis*, although this protective effect may not hold true for all *Chlamydia* serovars (Johansson and Lycke, 2001). A more definitive protective role for B cells was revealed by the observation that B cell-deficient mice depleted of $CD4^+$ T cells are completely unable to control secondary infection with *Chlamydia*, whereas wild-type mice depleted of $CD4^+$ T cells alone only exhibit a slight delay in clearing secondary infection (Morrison et al., 2000). This B cell-mediated protection is due to antibody production because passive transfer of immune serum or *Chlamydia*-specific monoclonal antibodies into B cell deficient, $CD4^+$ T cell-depleted mice rescues the ability of these mice to control secondary infection (Morrison and Morrison, 2005). The high susceptibility of the $CD4^+$ T cell-depleted, B cell-deficient mice to *Chlamydia* infection suggests that there is a synergy between B cells and T cells in immunity to *C. trachomatis*.

Although antibodies producing B cells are able to neutralize *Chlamydia* infectivity, they are unable to access the organisms that have established intracellular infection. At this stage, T-cells become crucial for recognizing infected cells and *Chlamydia* clearance. The first evidence of T-cells controlling *Chlamydia* infection was documented when nude mice, which lack T-cells establish chronic infection while wild type mice clear infection in 20 days (Rank et al., 1985). This was further proved when transfer of polyclonal *Chlamydia*-specific T-cells into *Chlamydia*-infected T-cell deficient mice facilitates bacterial clearance (Ramsey and Rank, 1991; Thoma-Uszynski et al., 1998). Furthermore, it has been demonstrated that

$CD4^+$ and $CD8^+$ T-cell clones can confer protection against *Chlamydia* infection when transferred to nude mice or immunocompetent mice (Igietseme et al., 1993; Igietseme et al., 1994; Roan et al., 2006; Roan and Starnbach, 2006; Starnbach et al., 1994; Su and Caldwell, 1995).

$CD4^+$ T-cells recognize antigens that are engulfed by professional antigen presenting cells (APCs) and the resulting peptides are presented to MHC class II molecule (Trombetta and Mellman, 2005). In contrast, $CD8^+$ T-cells typically recognize antigens that have access to the cytosol of infected cells and eventually presented to the MHC class I molecule (Cresswell et al., 2005). Therefore, chlamydial EBs and RBs in the extracellular space within tissues can be phagocytosed by professional APCs and serve as a source for $CD4^+$ T-cells antigens whereas chlamydial proteins or inclusion membrane proteins that have access to the cytosol serve as $CD8^+$ T-cell antigens.

Activated $CD4^+$ T-cells are important producers of effector cytokines into two major lineages Th1 and Th2 cells. Th1 cells produce IFN-γ that plays an important role in adaptive immunity as it can enhance the presentation of antigens to both $CD4^+$ and $CD8^+$ T-cells (Gaczynska et al., 1993; Steimle et al., 1994). $CD4^+$ T-cell derived IFN-γ appears to protect against infection as an IFN-γ-producing $CD4^+$ T-cell clone, but not an IL-4-producing $CD4^+$ T-cell clone, protected mice against *Chlamydia* genital infection (Hawkins et al., 2002). Th2 cells produce IL-4, IL-5 and IL-13 and do not appear to protect against *Chlamydia* infection and may even indirectly enhance *Chlamydia* load by inhibiting the development of protective Th1 responses (Brunham and Rey-Ladino, 2005). The inability to completely eliminate *Chlamydia* organisms from hosts with inadequate Th1 responses can result in continuous production of inflammatory cytokines which can lead to tissue destruction. The factors that

determine whether Th1 or Th2 response develops following *Chlamydia* infection is largely unknown, but experiments comparing immune responses against different species of *Chlamydia* have suggested that NKT cells may be an important determinant of the Th1/Th2 bias during *Chlamydia* infections (Joyee et al., 2007a).

In addition, $CD4^+$ T cells are crucial activators of other immune effectors like B-cells and $CD8^+$ T-cells as mice deficient in $CD4^+$ T-cells are more susceptible to *Chlamydia* infection than mice deficient in only B cells or $CD8^+$ T-cells (Morrison et al., 1995; Williams et al., 1997). Like $CD4^+$ T-cells, $CD8^+$ T-cells are also producers of IFN-γ that can contribute significantly towards controlling *C. trachomatis* infection. Adoptive transfer of IFN-γ-producing *Chlamydia*-specific $CD8^+$ T-cells into naive mice confers protection against *C. trachomatis* challenge whereas *Chlamydia*-specific $CD8^+$ T-cells unable to produce IFN-γ do not confer protection (Lampe et al., 1998). CD8+ T cell-dependent cytolysis is very specific for cells expressing the appropriate peptide-MHC class I complex, thereby ensuring the elimination of infected cells while sparing neighboring healthy ones. T-cell mediated lysis of *Chlamydia*-infected cells has been observed *ex vivo* (Roan et al., 2006; Starnbach et al., 1994). Therefore, the balance between protective versus pathological responses determines the final outcome of *Chlamydia* infection.

Laboratory Diagnosis for C. trachomatis Infection

Diagnosis of chlamydial infections include cell culture, enzyme-immuno assays (EIA), direct fluorescence assays (DFA), nucleic acid hybridization and amplification tests (NAHTs and NAATs, respectively), microimmunofluorescence (MIF) and enzyme-linked immuno assays (ELISA) (Battle et al., 2001). As *C. trachomatis* is an obligate intracellular bacterium, cell culture remains a method of choice for diagnosis. Although, cell culture has

near 100% specificity, it is not recommended for routine use because of its lack of sensitivity and technical complexicity (Black, 1997; Essig, A 2007). Since, cell culture detects only viable organisms, it is useful for antibiotic susceptibility testing thereby suggesting clinicians to accurate therapy (Warford et. al., 1999).

Antigen based detection methods DFA and EIA is very specific and rapid to perform but not suitable for large number of specimens (Black, 1997). EIA is more reproducible than DFA and its sensitivity is comparable to culture but lower than that of NAATs (Bebear and de Barbeyrac, 2009). Recently, a rapid or 'point of care' test was developed by the wellcome trust based on signal amplification test EIA for chlamydial LPS in a dipstick-type format (Mahilum-Tapay et al., 2007; Michel et al., 2006). NAHTs are based on DNA probing (with Pace 2, Gen Probe, Digene Hybrid Capture II) which is comparable to cell culture methods (Schachter J 2005). The sensitivity of Digene Hybrid Capture II test is substantially higher than that of Pace 2 test and is comparable to that of PCR (Schachter et al., 2006). These tests can be used with endocervical or urethral swabs but is not recommended for use with noninvasive specimens. It is also noteworthy that multiple site sampling for the diagnosis of *C. trachomatis* does not increase the sensitivity of diagnostic test (Dietrich et al., 2010).

Nevertheless, because of high sensitivity, specificity and their possible use of large range of sample types, NAATs can be considered as tests of choice for diagnosing *C. trachomatis* infection (Paavonen and Eggert-Kruse, 1999; Puolakkainen et al., 1998). The major targets for amplification-based tests are generally multiple copy genes, e.g. those carried by the cryptic plasmid of *C. trachomatis*, or gene products such as rRNAs. Several commercial NAATs, using different technologies: PCR and real-time PCR (Roche Diagnostics, Abbott, IL, USA); strand displacement amplification (Becton Dickinson, NJ,

USA); transcription-mediated amplification (Gen Probe); and nucleic acid sequence-based amplification (bioMerieux, Nancy L'Etoile, France) are available for diagnosis of *C. trachomatis* (Leber et. al., 2006). The goal for the future is to improve the laboratory tests, in particular DNA microarray technology seems to be promising for the diagnosis of *C. trachomatis*.

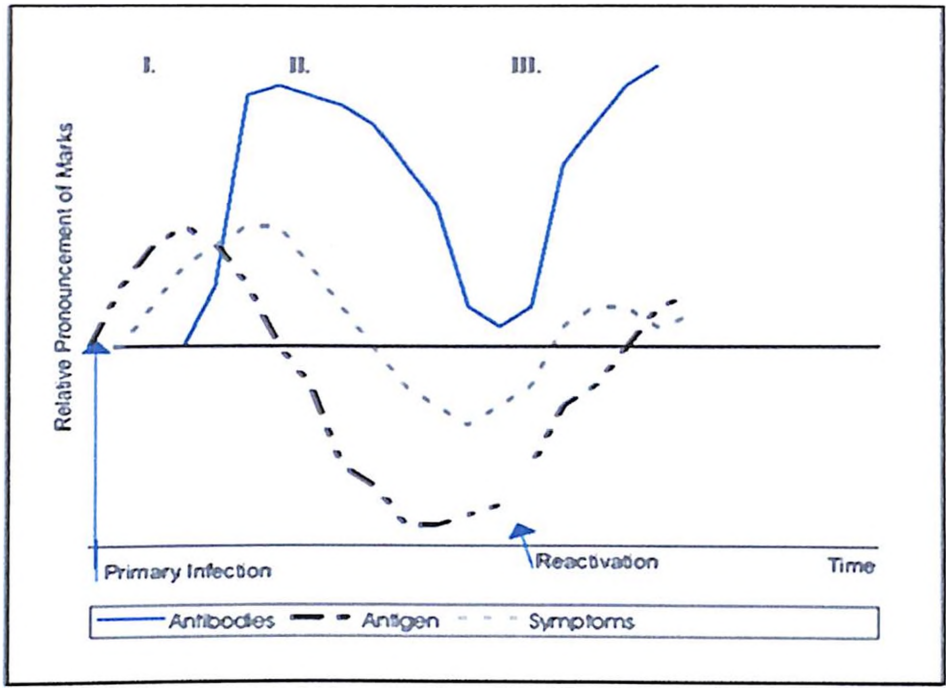


Figure 2.4: Chlamydial antibody levels and its importance in diagnosis. (I) Antigens and antibodies are present but symptoms does not appear in all cases, (II) When primary infection resolved and symptoms disappear, the antigens are no longer detectable but antibodies can be useful for diagnosis, (III) After reinfection the antibodies levels increases rapidly and linked to pathological sequelae.

Serologic tests like ELISA, are mainly used to detect acute infections but they are not useful in the diagnosis of chronic chlamydial infections because antibodies are long-lived and a positive result does not distinguish between current and past infection (Clad et al., 2000;

Tuuminen et al., 2000). *C. trachomatis* infection has received significant attention as a primary etiolgical factor responsible for PID, ectopic pregnancy and tubal infertility in women (Westrom, 1990; Wolner-Hanssen et al., 1990). These complications in women are strongly associated with the presence of antibodies to *Chlamydia* with infertility compared to control women (Moore et al., 1982; Mascellino et al., 2008). The clinical significance of anti-chlamydial IgG antibodies have strongly supported by elevated titers in women with tubal infertility and ectopic pregnancy (Shepard and Jones, 1989). Actually, the elevated anti-chlamydial antibody titers appear to persist for long period after the infection has cleared and consequently serology may not diminished chronic active infection from past infection (Keay et al., 1998; Moore et al., 1982). Although, IgG antibodies may denote previous resolved infection, the presence of IgA antibodies has been reported in some studies as marker of chronic infection and linked to pathological sequelae of upper reproductive tract (Chaim et al., 1992; Numazaki et al., 1996). Therefore, detection of anti-chlamydial IgG could serve as a promising tool for prognosis and diagnosis for sequelae of *C. trachomatis* infection **(Figure 2.4)**.

Chlamydial Heat Shock Proteins

In 1962, the first description of cellular heat stress response was identified serendipitously in fruit fly, *Drosophila melanogaster* that were inadvertently exposed to high temperatures and exhibited characteristic puffing pattern in salivary gland chromosome (Ritossa, F 1962). The first gene product of this chromosomal puffing was identified 12 years later and the term 'Heat Shock Protein' was coined (Tissieres et al., 1974). These genes, and the proteins encoded by them, are highly conserved and present in all cells in all forms of life and in a variety of intracellular locations. These include the cytosol of prokaryotes, and the

cytosol, nuclei, endoplasmic reticulum, mitochondria and chloroplasts of eukaryotes (Lindquist and Craig, 1988). Further, analysis of immune response to bacteria in 1970's identified a term 'Common Antigen' in many bacterial species (Kaijser, 1975). Subsequent work in patients infected with Mycobacterium exhibit significant antibody response to 65 kDa antigen which was identified as the molecular chaperone, HSP60 (Young et al., 1987). It is now fully understood that a number of heat shock protein from bacteria to protozoan parasites (HSP10, HSP60, HSP70 and HSP90) are potent immunogenic, active immunoregulators and inducers of cross-reactive immunity (van Eden et al., 2005).

In *Chlamydiae*, the 60-kDa cHSP60, or GroEL, and the 10-kDa cHSP10, or GroES, are genetically linked because they are encoded by genes arranged on the bicistronic groESL operon (Morrison et al., 1990). Numerous studies have suggested that both cHSP60 and cHSP10 are implicated in the induction of deleterious immune responses in human chlamydial infections. Thus, among a panel of 116 recombinant *C. trachomatis* proteins, cHSP60 (Ct110) has recently been identified as an immunodominant antigen, being a strong target of both humoral and CMI responses, whereas cHSP10 (Ct111) was identified as a human T cell target (Follmann et al., 2008). Also, in a population based study, serological association of antibodies to cHSP60 and cHSP10 antigens with female infertility has been established (Karinen et al., 2004). Interestingly, cHSP60 became duplicated at the origin of the *Chlamydiae* lineage presenting three distinct molecular chaperones, namely the original protein cHSP60-1 (Ct110), and its paralogous proteins cHSP60-2 (Ct604) and cHSP60-3 (Ct755) (Karunakaran et al., 2003). A recent study showed that cHSP60 copies have diversed functionally after the gene duplication events (McNally and Fares, 2007) **(Figure 2.5)**. Among the three cHSP60 genes, only the expression levels of cHSP60-1 (cHSP60) and

its cochaperone cHSP10 increase under heat-stress conditions and only the cHSP60 complements its function in thermo-sensitive mutation in HeLa cells under heat-stress conditions (Karunakaran et al., 2003).

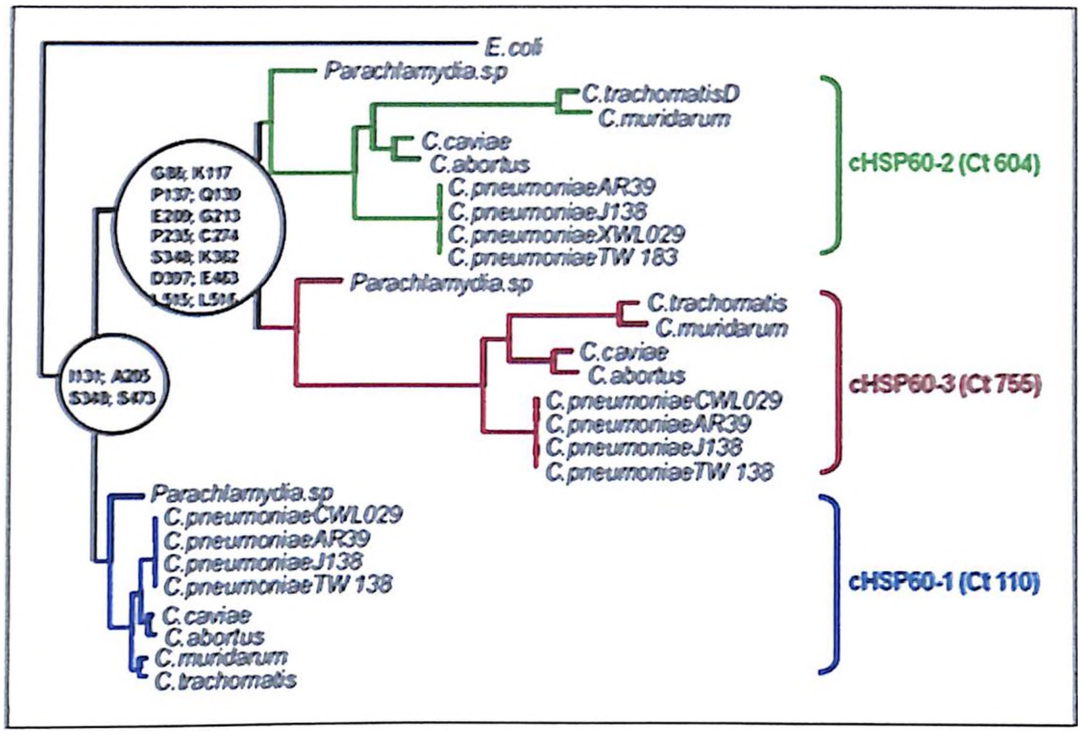


Figure 2.5: Divergence of cHSP60: Analysis of functional divergence type I in the multiple sequence alignment comprising sequence from cHSP60-1 (Ct110), cHSP60-2 (Ct604) and cHSP60-3 (Ct755) in *Chlamydiae*. The different cHSP60 paralogs are indicated and the sites detected with high posterior probabilities within the class of functional divergence type I are shown in each node corresponding to the gene duplication events (McNally and Fares, 2007).

Immunology of Chlamydial Heat Shock Protein

Immune responses to the cHSP60, have been associated with the sequelae of upper genital tract disease, including ectopic pregnancy (Sziller et al., 1998), PID (Domeika et al., 1998; Eckert et al., 1997; Kimani et al., 1996; Peeling et al., 1997), and TFI (Ault et al., 1998; Toye et al., 1993; Witkin et al., 1998b; Witkin et al., 1993). In general, serological reactivity to cHSP60 is low among healthy controls but increases stepwise as disease becomes more severe (Brunham and Peeling, 1994). Since purified cHSP60 elicits mononuclear cell inflammation and tissue damage in animal models of chlamydial infection, it has been hypothesized that the increased level of immune reactivity to cHSP60 contributes to the development of immune pathology (Morrison et al., 1989; Patton et al., 1994). Involvement of other chlamydial antigens (LPS, MOMP and OMP2) in immunopathological processes, to date, has been discussed controversially (Gerard et al., 2000; Thiel et al., 2000). Reports on the immunopathogenecity of HSP10 antigens from other microbial pathogens suggest that the HSP10 family of proteins are capable of eliciting chronic inflammation and delayed hypersensitivity. In particular, the immune response to the HSP10 homologues of *Mycobacterium leprae* and *Mycobacterium tuberculosis* have been shown to be prominent T-cell antigens and targets of serum antibody responses (Barnes et al., 1992; Kim et al., 1997). Both *M. leprae* and *M. tuberculosis* HSP10 elicit strong Th1 phenotype human T-cell responses, with the production of IL-2 and IFN-γ, consistent with a delayed type hypersensitivity response (Launois et al., 1995).

Several studies on the incidence of sexually acquired reactive arthritis have reported acute *C. trachomatis* infection in 42-69% of individuals immediately preceding the onset of arthritis (Keat et al., 1980; Vilppula et al., 1981). There are some evidences indicates that a

subset of T-cells, γδ T-cells, react with antigens on autologous cells in which heat shock antigens are induced (Rajasekar et al., 1990), and γδ T-cells isolated from the synovial fluid of patient with rheumatoid arthritis recognize mycobacterial HSP60 (Holoshitz et al., 1986). The association of γδ-phenotype T-cells with mucosal epithelial tissues and the mucosal site of *C. trachomatis* infection raise questions on the role of T-cell subsets specificity directed toward cHSP60 in mediating either protective or immunopathology responses during infection. Purified recombinant cHSP60 has been evaluated in animal models of chlamydial disease pathogenesis and shown to be a potent T-cell antigen capable of eliciting an intense inflammatory response when applied to the mucosal surfaces of the fallopian tube of immunologically primed animals (Morrison et al., 1989; Patton et al., 1994). These characteristics are highly suggestive of the chlamydial hypersensitivity antigens viz. cHSP60 and cHSP10 implicated in chlamydial immunopathology and tissue fibrosis (Morrison et al., 1989). These cHSPs are similar to the HSPs of human and other organisms (Shinnick, 1991; Wagar et al., 1990). Immune recognition of HSP60 may incite an auto-immune inflammatory response because of molecular mimicry among shared peptide sequences between the cHSP60 and hHSP60 (Cappello et. al., 2009; Campanella et. al., 2009). Relevant to this hypothesis, antibody responses to peptide epitopes from hHSP60 have been observed among women with *C. trachomatis*- associated ectopic pregnancy (Yi et al., 1993). However, the autoimmune responses to hHSP60 or immunopathological processes caused by overexpression of hHSP60 and/or cHSP60 are controversial (Larsen et al., 1994; Yokota et al., 2000).

The availability of recombinant cHSP60 has facilitated detailed immune-epidemiological studies among *C. trachomatis* -infected humans. Studies of antibodies to the

cHSP60 antigen among women with different sequelae to chlamydial infection show a consistent gradient in seroprevalence to the cHSP60: 16-25% of MIF antibody-positive fertile women have cHSP60 antibody, as 36-44% of women with *C. trachomatis* cervicitis, 48-60% of women with *C. trachomatis* PID, and 81-90% of women with *C. trachomatis* related fallopian tube obstruction (Brunham et al., 1992; Toye et al., 1993). Futhermore, among women with laparoscopically visualized chlamydial PID, those with high antibody titers to cHSP60 have had significantly more severe inflammatory manifestations (Brunham et al., 1985). The woman with antibody responses to the cHSP60 among those with the severe forms of *C. trachomatis* infection suggests that cHSP60 may induce the tissue damaging immune responses. It is clear that low level infections can produce relatively high quantities of HSP60 and that women who suffer from tubal factor infertility and ectopic pregnancy often have high levels of antibody to cHSP60 (Toye et al., 1993; Wagar et al., 1990). The question remains whether the higher frequency of anti-cHSP60 antibody is confounded merely by longer term exposure to chlamydial antigens, and hence more severe disease sequelae, or whether a specific immune reactivity to cHSP60 contributes to disease.

Studies have shown that treatment of *Chlamydia* infected cells with IFN-γ inhibit chlamydial development, while permitting expression of cHSP60 and downregulation of MOMP expression (Beatty et al., 1994a). Thus, in chronically or acutely infected individuals, continued cHSP60 expression secondary to the action of IFN-γ produced by CMI might ultimately drive chronic inflammatory responses associated with fibrosis and scarring, characteristic of the severe sequelae of chlamydial infection. Individuals able to resolve ocular chlamydial infection show enhanced lymphoproliferative response to cHSP60 compared to those with persistent infection, but the stimulated lymphocytes from both groups

were able to mount a comparable Th1 (IFN-γ) cytokine response (Bailey et al., 1995). Furthermore, IFN-γ response to cHSP60 are significantly associated with reduced risk of infection (Cohen et al., 2005), whereas repeated chlamydial infection or PID are associated with cHSP60- specific IFN-γ response (Debattista et al., 2002). Rcecently, Ondondo *et. al.* showed systemic and mucosal IFN-γ responses are correlated, with preferential systemic targeting of $CD4^+$ T-cells and CHSP60 response is largely $CD4^+$ T-cell mediated which follows discrete Th1 and Th2 pathways (Ondondo et al., 2009).

In another study, co-immunization with cHSP60 resulted in cross-reactive T-lymphocytes that proliferated in response to mouse HSP60 and which were characterized by a 12- fold increased ratio of IFN-γ to IL-10 production. Adoptive transfer of cHSP60 specific T-cells in primed mice produces high titers of self-HSP60 antibody (Yi et al., 1997). Therefore, it appears that switches in cytokine production may mediate the pathogenesis of cHSP60-associated immunopathology (Yi et al., 1997). Thus, individuals with scarring trachoma more frequently had cHSP60 antigen-specific IL-4 secreting peripheral blood lymphocytes than did matched community controls without trachoma scarring. Such individuals less frequently had cMOMP antigen- specific IFN-γ-secreting peripheral blood lymphocytes than did community controls (Holland et al., 1996). Therefore, cMOMP specific Th1 CD4 T-cells are important in immunity to *C. trachomatis* infection and disease and that cHSP60 specific Th2-like CD4 T-cells are associated with the pathological sequelae of persistent chlamydial infection. It seems that persistent *Chlamydiae* synthesize different amounts of chlamydial proteins (reduced amounts of MOMP and sustained amounts of cHSP60) (Beatty et al., 1993). This may account for the distinctive immunodominance of

cHSP60 as a serologic antigen observed in seroepidemiologic studies of individuals with complicated chlamydial infection (Brunham and Peeling, 1994).

In an earlier report suggests, immune sensitization to HSPs probably requires prolonged exposure of them at elevated concentrations (Witkin et al., 1998a) confirming women with recurrent infections have significant humoral and CMI responses towards cHSP10 and cHSP60. Although, co-expression of cHSP60 and cHSP10 are recognized feature (Dadamessi et al., 2005; Spandorfer et al., 1999), cHSP10 induces proliferation of cervical lymphocytes obtained from women with recurrent infections more significantly than cHSP60, thus showing its role can be more important than cHSP60 in the pathogenesis of chlamydial infections (Agrawal et al., 2007). Also, cHSP10 is associated with chronic genital tract infection and is homologous to human chaperonin (Cpn10) and EPF, a form of Cpn10 which is secreted especially at the start of pregnancy. It has been reported previously that infertility was associated with the presence of anti-cHSP10 and anti-EPF antibodies in serum (Betsou et al., 2003). A study by LaVerda *et. al.* demonstrated that women with acute infection and TFI recognized cHSP10 more frequently, with infertile women having greater seroreactivity to cHSP10 than acutely infected women. They also demonstrated that among women with similar exposure to *Chlamydiae*, serological responses were greater to cHSP10 in the TFI group than cHSP60 or cMOMP (LaVerda and Byrne, 1997).

Heat shock proteins of the families 60, 70, and 90 belong to the first proteins which are expressed by the zygote after fertilization (Bensaude et al., 1983). During the first trimester of pregnancy they can also be detected in the decidua (Neuer et al., 1997) and throughout the whole pregnancy in placental tissues (Ziegert et al., 1999). Autoimmunity to hHSP60 is not typically evident in women of reproductive age. During pregnancy an

established sensitization to cHSP60 in connection with overexpression of hHSP60 may cause reactivation of HSP60 sensitized lymphocytes with subsequent induction of inflammatory processes (Swanborg et al., 2006). At this stage, such inflammatory processes disturb immunoregulatory mechanisms that are responsible for implantation (immunologically conditioned impairment of implantation), or the embryo itself is target of destructive autoimmune responses because of its pronounced HSP expression (Neuer et al., 1999). In both the early phase and during the further pregnancy course the maternal deciduas and/or the embryo can be affected by HSP60-conditioned autoimmune reactions which may lead to an immunologically mediated rejection of the embryo (spontaneous abortion) (Witkin, 1999). Fallopian tube pregnancy is the most frequent form of ectopic pregnancy (EP). Scarring, fibroses, and necroses, caused by persistent inflammatory processes (Askienazy-Elbhar and Suchet, 1999) lead to extreme reduction of the tubal lumen and impair the function of the tubal ciliar epithelium. Overexpressed cHSP60 has a key function in the development of these immunopathological processes (Neuer et al., 2000). The combined determination of cHSP60 and *C. trachomatis* antibodies enable clear identification of ectopic pregnancy and are of high predivtive value (Sziller et al., 1998). In addition, Infertility because of total occlusion of the tubal lumen belongs to common sequelae of silent *C. trachomatis* infections in the upper genital tract (Ault et al., 1998) and the associated immunopathological or autoimmune processes induced by cHSP60 (Kinnunen et al., 2000). Antibodies to cHSP60 are strongly associated with TFI (Ault et al., 1998) and are of high predictive value. The combined detection of cHSP60 and *C. trachomatis* antibodies even increases sensitivity and specificity (Claman et al., 1997).

Chlamydial Heat Shock Proteins and Apoptosis

The term 'Apoptosis' derived from the greek for falling, as of leaves from a tree, was derived originally as a physiological or programmed form of cell death. Some bacterial pathogens modulate apoptosis of infected epithelial cells to accommodate the organism's life-cycle and facilitate infection. Hence, a large number of bacterial pathogens such as *Bordetella pertusis*, *Salmonella typhimurium*, *Shigella flexneri*, *Escherichia coli* and *Staphylococcus aureus* induce apoptosis of their infected host cells (Gao and Kwaik, 2000; Weinrauch and Zychlinsky, 1999).

In *Chlamydia*, which infect mainly epithelial mucosa modulate apoptosis of infected cells in two opposite directions; pro-apoptotic or anti-apoptotic (Perfettini et al., 2003). The resistance of *Chlamydia* infected cells against apoptosis triggered by external ligands may contribute to persistence while cell-death at the end of infection cycle would allow *Chlamydiae* to exit from infected cells and initiate new round of infection. Several strain of *C. trachomatis* protect infected cells against apoptosis due to external ligand like TNF-α, antibodies against Fas and the kinase inhibitor Staurosporine (Dean and Powers, 2001; Fan et al., 1998). The anti-apoptotic activity was observed due to inhibition of cyto C release from mitochondria which thus result in inhibition of apoptosome pathway leading to caspase-3 and caspase-9 activation. Moreover, epithelial cells display characteristic features of apoptosis during infection with the serovars of *C. psittaci* (Ojcius et al., 1998). But no cell death was observed during the early stage of infection by either *C. trachomatis* or *C. psittaci*, although nuclear condensation, a hallmark of apoptosis became prominent towards the end of the infection cycle (Gibellini et al., 1998; Perfettini et al., 2000). Thus, it seems protection

against apoptosis during early stage and cell death during late stage is controlled by *Chlamydia* proteins but not host protein (Ojcius et al., 1998).

Also, *Chlamydia* infected human monocytes are known to secrete IL-1β and IL-18 which require Caspase-1 for their maturation (Los et al., 1999). Further, inhibitors of Caspase-1 and Caspase-3 showed no effect on cell death during infection with *C. trachomatis* and *C. psittaci* suggesting that caspases do not participate in apoptosis (Ojcius et al., 1998; Perfettini et al., 2000). However, there are alternate mechanisms of apoptosis which can take place in the absence caspase activation. Like, a pro-apoptotic member of the Bcl-2 family, Bax, can induce cell death in absence of the caspase activation (Xiang et al., 1996). Activated Bax translocates to mitochondria in *C. trachomatis* infected cells that play a pivotal role in *Chlamydia*-induced apoptosis (Perfettini et al., 2002). Thus, Bax activation is known to lead either caspase-dependent or caspase-independent cell-death (Pastorino et al., 1998).

Chlamydia spp., produces large quantities of HSP60, which is reported to be localized in the cytoplasm and in the outer membrane of the host cell during persistent infection (Bavoil et al., 1990). Also, cHSP60 has an amino acidic sequence similar to its human counterpart, with the exclusion of the mitochondrial localization signal. It may be possible to correlate some cellular responses during *Chlamydia spp.* infections with the accumulation of cHSP60 in the cytoplasm of the host cell and its possible association with cleaved caspase-3 and/or Bax-family proteins. During chronic persistent infection, aberrant chlamydial forms produce large quantities of cHSP60 which can be transported to the cytosol of the host cell through a type III secretory apparatus (Clifton et al., 2004; Fields et al., 2003). cHSP60 could form a complex with cleaved caspase-3 and/or Bax and Bak, inhibiting apoptosis before cytochrome c release and caspase-3 activation (Di Felice et al., 2005) **(Figure 2.6)**.

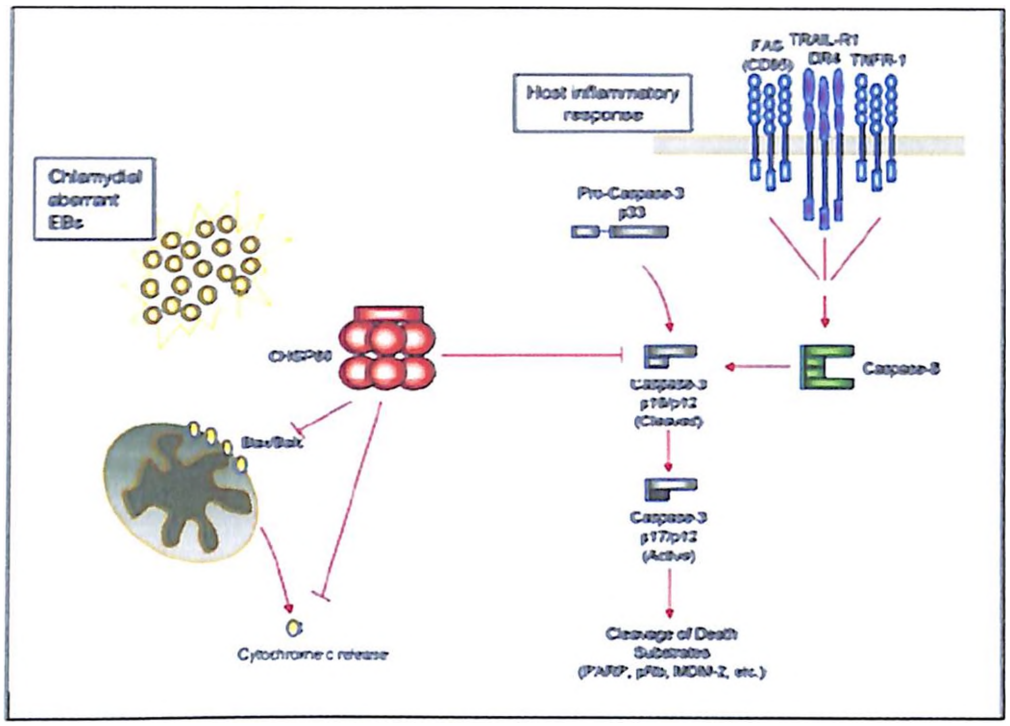
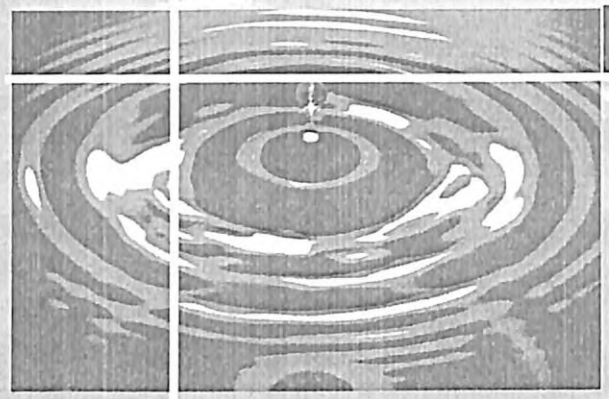


Figure 2.6: Schematic diagram showing probable role of cHSP60 in apoptosis. During persistent infection cHSP60 may interfere with apoptosis induced by host inflammatory response. During *Chlamydia* spp. persistent infection, aberrant EBs; (light green) produces large quantities of cHSP60 (red), which localizes in the cytoplasm and in the outer membrane of the infected cells. The presence of the bacterium induces a strong B and T CMI response. Apoptosis induced by activated lymphocytes is initiated by stimulation of the receptors Fas, TNFR-1 or TRAIL-R1 (blue). Their ligands are in the form of trimers, and when they bind, they induce trimerization of the receptors themselves. These in turn interact through adaptor proteins with caspase-8 (dark green) leading to caspase cascade activation with cleavage of death substrates. To elude the immune system, cytoplasmic cHSP60 may interact with cleaved caspase-3 (gray) in the cytoplasm, blocking caspase cascade activation. Furthermore, cHSP60 may bind to Bax or Bak, inhibiting cytochrome c (yellow) release from mitochondria (Di Felice et al., 2005).

Gaps in Existing Research

Female genital tract infection due to *C. trachomatis* is often asymptomatic. In such cases, a strong association between TFI and circulating cHSP60 and cHSP10 antibodies has been reported. Although the presence of cHSP antibodies has been reported previously by various workers worldwide. However, in India, seroprevalance of cHSPs and correlation of antibody titers in women with cervicitis/infertility is largely unexplored. Till now, it is also not clear that among cHSP60 and cHSP10, which cHSP is more reliable for early diagnosis and if cHSP60 can be used as a prognostic marker for sequelae of *C. trachomatis* infection in women. Further, numerous studies have suggested that cHSP-specific immune responses play an important role in immunopathogenesis associated with a chlamydial infection, but so far no study have assessed the immune response and expression of cHSP60 and cHSP10 at the actual site of infection. In addition, no study has been dedicated to cHSP60 and cHSP10 for their potential role in apoptosis of primary cervical epithelial cells that are privilege target for chlamydial infection.



Chapter 3
Aims and Objectives

Aims and Objectives

The aim of the study is to evaluate seroprevalence of cHSP60 and cHSP10 in symptomatic women and their probable role in the immunopathogenesis in *C. trachomatis* infected women. The specific objectives of the present study are defined as under.

1. Seroprevalence of antibodies to conserved regions of cHSP60 and cHSP10 in symptomatic women.

To study the seroprevalence of antibodies to cHSP60 and cHSP10, ELISA was performed in symptomatic *Chlamydia*-positive and -negative women. Further, antibody levels against cHSP60 and cHSP10 were evaluated with respect to antibodies to cMOMP in order to correlate and to study the possible association of cHSP60 and cHSP10 antibodies levels with severity of disease.

2. Diagnostic and prognostic efficacy of IgG antibodies to anti-cHSP60 and anti-cHSP10 in symptomatic women.

Sensitivity and specificity of cHSP60 and cHSP10 IgG ELISA was evaluated with respect to IgG-MOMP ELISA and also DFA and PCR in symptomatic *C. trachomatis* infected infertile and subfertile women.

3. Cloning and expression of cHSP60 (Ct110) and cHSP10 (Ct 111).

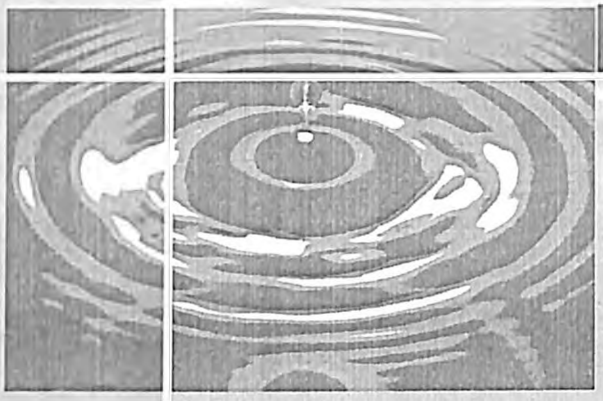
For expression of proteins (cHSP60 and cHSP10), full-length genes were amplified and products were cloned into pGEM-T vector. Further characterization of clones was done by restriction digestion and colony PCR and subsequently expression of recombinant cHSP60 and cHSP10 was done in protein expression vector.

4. Role of cHSP60 and cHSP10 in *in vitro* evaluation in the immunopathogenesis at the local site of infection in infertile women.

Cervical cells from *C. trachomatis*-infected infertile, fertile and uninfected women were collected and subsequently cells were *in vitro* stimulated with cHSP60 and cHSP10. Secreted cytokines (IFN-γ, IL-10, TNF-α, IL-13 and IL-4) levels in stimulated cervical mononuclear cell supernatants were evaluated by ELISA.

5. Role of cHSP60 and cHSP10 in the apoptosis of cervical epithelial cells.

The potential role of cHSP60 and cHSP10 in the apoptosis of primary cervical epithelial cells that are privileged target for *C. trachomatis* infection was evaluated. Cervical cells from healthy women were collected and *in vitro* stimulated with cHSP60 and cHSP10. A quantitative measurement of apoptosis was performed by cytofluorometry and also by microarray wherein the apoptosis related genes were analyzed. Up-regulated or down-regulated genes in microarray were subsequently validated with real-time PCR and western blotting. Levels of proinflammatory cytokines (IL-18 and IL-1β) were also determined by semi-quantitative RT-PCR.



Chapter 4
Seroprevalence of Antibodies to Conserved Regions of *Chlamydia trachomatis* Heat Shock Proteins 60 and 10

Seroprevalence of Antibodies to Conserved Regions of Chlamydia trachomatis Heat Shock Proteins 60 and 10

Introduction

Chlamydia trachomatis infection is the most prevalent sexually transmitted bacterial disease worldwide (Schachter J, 1999). Up to 80% of women with genital chlamydial infection are asymptomatic, and upper genital tract chlamydial infection is often silent clinically (Gaydos et al., 1998). Both symptomatic and asymptomatic women show a similar potential for tubal damage (Patton et al., 1989). Persistent antigen synthesis and an ineffective immune response contribute to chronic inflammation, tissue damage and immunopathology associated with salpingitis/infertility (Pal et al., 1998). Protective immunity to *C. trachomatis* infection is inadequate and repeated episodes of infection are common. Undetected and untreated chlamydial infection can ascend to the upper genital tract and this may lead to PID, Fallopian tube injury and infertility (Stamm, 1999). Several case-control studies and cohort analyses have reported a strong epidemiological, histological and serological correlation between *C. trachomatis* upper genital tract infection and PID (Brunham et al., 1992; Westrom et al., 1981). Also, a strong association between TFI and circulating chlamydial antibodies has been reported (Baehr et al., 1988; Moore et al., 1982).

Numerous seroepidemiological studies have shown a consistent immunopathogenic association between antibody responses to the cHSP60 and the development of PID, ectopic pregnancy and tubal infertility (Arno et al., 1995; Sziller et al., 1998; Toye et al., 1993). In *C. trachomatis*-induced disease, enhanced antibody and CMI responses to cHSP60 are detected (Ault et al., 1998; den Hartog et al., 2005; Kinnunen et al., 2000). This stress response is believed to interrupt the normal progression of RBs to infectious EBs, resulting in persistent

infection that may serve as an antigenic reservoir for potentially immunopathogenic anti-HSP immune system responses (Beatty et al., 1993; Beatty et al., 1994b).

In an experimental monkey model, repeated *C. trachomatis* inoculations resulted in extensive tubal damage and occlusion (Patton et al., 1987), which suggests the involvement of the host's immune response in tubal pathogenesis. Subsequent studies in monkeys (Patton et al., 1994) and in guinea pigs (Morrison et al., 1989) 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 (Betsou et al., 1999) and tubal occlusion (LaVerda et al., 2000). In addition, co-expression of cHSP10 with cHSP60 has been reported (Morrison et al., 1990); however; detection of anti-cHSP10 antibodies does not parallel that of anti-cHSP60 antibodies (Betsou et al., 1999; Dadamessi et al., 2005), and the latter are reported to be an independent marker.

In India, approximately 10 million asymptomatic cases of genital chlamydial infection are reported in the sexually active age groups of the general population (Joyee et al., 2004). Cervical *C. trachomatis* infection has also been found to be associated with bad obstetric outcome, including still birth, preterm delivery (Rastogi et al., 1999), spontaneous abortions (Rastogi et al., 2000) and infertility (Malik et al., 2006; Malik et al., 2009; Mittal et al., 1993b). Also, using conventional serological and antigen detection assays, a high prevalence of chlamydial infection in symptomatic women (upto 40% in cervicitis and 36% in infertility) has been reported (Jalgaonkar et al., 1990; Kapur et al., 2006; Mittal et al., 1993a; Mittal et al., 1993b; Singh et al., 2002; Bhujuwala, et al., 1982; Ray, et al., 1993). In another serological study, Joyee *et. al.* have reported 58.7% IgG positivity in *C. trachomatis*

infected patient's sera and suggested the usefulness of serology instead of PCR to trace chlamydial etiology (Joyee et al., 2007b).

Inspite of high positivity of *C. trachomatis* infection in symptomatic women in India, no study was undertaken to assess the seroprevalence of cHSP60 and cHSP10 and risk of developing sequelae to *C. trachomatis* infection. Therefore, in order to assess the significance of the possible association of the response to cHSP60 and cHSP10 with disease progression, seroprevalence of *C. trachomatis* infection was studied. Further, the severity of disease was also assessed by measuring antibody levels against cHSP60 and cHSP10 with respect to anti-cMOMP. This will help in providing a clinically useful antibody screening test for predicting or confirming PID and infertility in *C. trachomatis*-infected women.

Materials

Conserved sequence of *C. trachomatis* peptides were used as antigens for cHSP60 (^{151}SANNDAEIGNLI1^{62}) (LaVerda and Byrne, 1997), cHSP10 (^{79}SGQELTVEG^{87}) (Meikle et al., 1994) and cMOMP (^{288}SATAIFDTTLNPTIAGAGDVKT GAEGQLG^{317}) (Yuan et al., 1989) and were synthesized from Techno Concept, India. Materials (NaCl, KCl, Na_2HPO_4 and K_2HPO_4) used for the preparation of PBS and TMB were purchased from Sigma Aldrich. Horse radish peroxidase (HRP)-conjugated rabbit anti- human IgG antibodies were purchased from Bangalore Genei, India. Taq polymerase, dNTPs and $MgCl_2$ were from Invitrogen, USA. Microtiter plates (96-well) for ELISA were purchased from Greiner.

Experimental Methods

Study population

A total of 255 women (aged 16 to 45 years) attending the outpatient department of Safdarjung 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/infertility. The study was approved by the hospital ethical committee and informed consent was obtained from each patient.

Collection of samples

The vulva was examined for evidence of lesions and vaginal/cervical discharge. The cervix was inspected for ulcers, warts, ectopy, erythema, discharge or any other abnormalities. After cleaning the cervix with cotton swab (Hi Media, Mumbai, India), two cervical swabs were collected in sterile vials containing phosphate-buffered saline (PBS) from patients for the diagnosis of *C. trachomatis* and other sexually transmitted infection pathogens. The cervical canal was wiped clean and a cytobrush was placed within the endocervical canal so that cells from the endocervical region and the zone between the endocervical and ectocervical regions (transformation zone) could be obtained (Mittal et al., 1993a). The cytobrush was then held in a sterile centrifuge tube containing phosphate-buffered saline (PBS) with penicillin (100 U/ml), streptomycin (100 µg/ml) and glutamine (100 µg/ml). Swabs collected from patients were stored at 4°C and transported to the laboratory within 1 h for diagnosis of *C. trachomatis* and other STD pathogens. Non-heparinised venous blood was drawn; the serum was separated and then stored at –70°C for the detection of antibodies against cMOMP, cHSP60 and cHSP10.

Detection of *C. trachomatis* and Other STD Pathogens

Five-millimetre spots were made on clean glass slides using endocervical swabs. Samples were stained with flourescein isothiocyanate (FITC)- conjugated monoclonal antibodies to cMOMP using *C. trachomatis* Direct Specimen Test kit (Microtrak, Syva

Corporation, Palo Alto, CA, USA) according to the manufacturer's instructions. In principle, this kit involves the use of FITC labeled monoclonal antibodies directed against cMOMP which is a common protein antigen located in the outer membrane of the cell wall in all *C. trachomatis* serotypes. This FITC labeled antibody binds specifically to *C. trachomatis* present in the methanol-fixed smears previously applied to a well on a microscope slide. When viewed under a fluorescent microscope, *C. trachomatis* exhibits a bright apple-green fluorescence which is either EBs or RBs and contrast with the reddish-brown colour or counter-stained material. A sample was considered to be positive when at least 10 EBs were detected by this DFA (Singh et al., 2003; Vats et al., 2004). Samples were simultaneously confirmed for positivity by PCR analysis.

Gram-stained cervical smears were examined for the presence of yeast cells (Candidiasis) and clue cells in vaginal smears for the diagnosis of bacterial vaginosis. Gram stains showing predominance of *Lactobacillus* morphotype were interpreted as normal and those showing *Gardnerella* morphotype or mixed flora were interpreted as consistent with bacterial vaginosis. Wet mount microscopy was performed for the diagnosis of *Trichomonas vaginalis*. For detection of *Neisseria gonorrhoeae* cervical specimens were incubated at 35°C in humidified CO_2 incubator for 48 h on Thayer Martin medium. Colony growth was noted and *N. gonorrhoeae* was identified on the basis of gram-stained smears. *Pleuropneumonia*-like organism (PPLO) broth was used for the identification of *Mycoplasma hominis* and *Ureaplasma urealyticum* by diluting the cervical samples in arginine-containing and urea-containing liquid media, respectively, thereafter incubating the media at 37° C (Reddy et al., 2004).

Polymerase Chain Reaction for Detection of *C. trachomatis*

Endocervical samples were subjected to DNA extraction using QIAamp Viral RNA mini Kit (Qiagen, CA, USA) according to manufacturer's instuctions. In brief, samples were centrifuged at 14,000 rpm for 20 min and supernatant was discarded. The pellet was resuspended in appropriate volume of lysis buffer. The samples were passed through columns provided in the kit and DNA was finally eluted in 30-50 µl of TE buffer. Concentration of DNA was quantified spectrophotometrically at 260 nm (Biometra, USA). This DNA was used as a template to screen for presence of *C. trachomatis* and subsequent detection of cHSP60 gene.

DNA from endocervical samples were tested for chlamydial positivity by PCR analysis using *C. trachomatis* plasmid (pCT) specific primers (George et al., 2003). The sequences for primers were: forward primer 5'- CTA GGC GTT TGT ACT CCG TCA-3' and reverse primer 5'- TCC TCA GGA GTT TAT GCA CT -3'; Each reaction contained, in a total of 25 µl, 5 µl DNA, 2.5 µl 10X PCR buffer (200mM Tris-HCL [pH 8.4], 500 mM KCl), 0.5 µl 10 mM dNTP mixture, 0.8 µl 50 mM $MgCl_2$, 3 µl of each primer (5pM/ µl), 0.1 µl Taq Polymerase (5U/ µl) (Invitrogen). All reaction tubes were subjected to cycling parameters for pCT amplification at 94 °C / 1 minute, 55 °C / 1.5 minutes, 74 °C / 2 minutes for 40 cycles. All PCR reactions were performed on DNA Eppendorf Mastercycler personal Thermal Cycler (Eppendorf GmbH, Hamburg, Germany). All amplification products were electrophoresed on 1.2% (w/v) agarose gel and ethidium bromide (EtBr) (0.5 pg/ml) stained gels were visualized on Alpha Imager gel documentation system (AlphaInnotech, San Leandro, USA).

Detection of Antibodies Against cMOMP, cHSP60 and cHSP10

ELISA was used to detect IgG antibodies to *C. trachomatis*-specific antigens (cMOMP, cHSP60 and cHSP10). Briefly, 2.5 μg antigens was coated on microtiter 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 1 in 10,000 dilution of HRP-conjugated rabbit anti-human IgG antibody was added and incubated for 1 h at room temperature. After final washing TMB substrate was added to achieve colorimetric detection of each plate at optical densities read at 450 nm. Positive samples were defined as those yielding an absorbance (A) value at least two standard deviations (SDs) above the mean value obtained from the panel of samples taken from the negative subjects [mean +2SD of the negative samples] i.e. A_{450} > 0.72 (cMOMP), > 0.522 (cHSP60) and > 0.49 (cHSP10) (Domeika et al., 1998; Yi et al., 1993).

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-square (χ^2) test. Significance of antibody titres was calculated by independent *t*-test.

Results

Diagnosis of *C. trachomatis* and Other STD Pathogens in the Cervix

Cervical *C. trachomatis* infection was diagnosed in 75 (29.4%) patients using DFA and all samples were further confirmed to be positive using PCR which detected 200 bp amplicon of pCT **(Figure 4.1)**. None of the *C. trachomatis*-infected patients were found to be infected with other STD pathogens. Among the *C. trachomatis*-negative patients, 11 (6.1%)

were infected with *Candida spp.* 17 (9.4%) showed *Bacterial vaginosis*, 13 (7.2%) were infected with for *M. hominis*, and 48 (26.7%) with *U. urealyticum*. No cases of *N. gonorrhoeae* or *T. vaginalis* were detected.

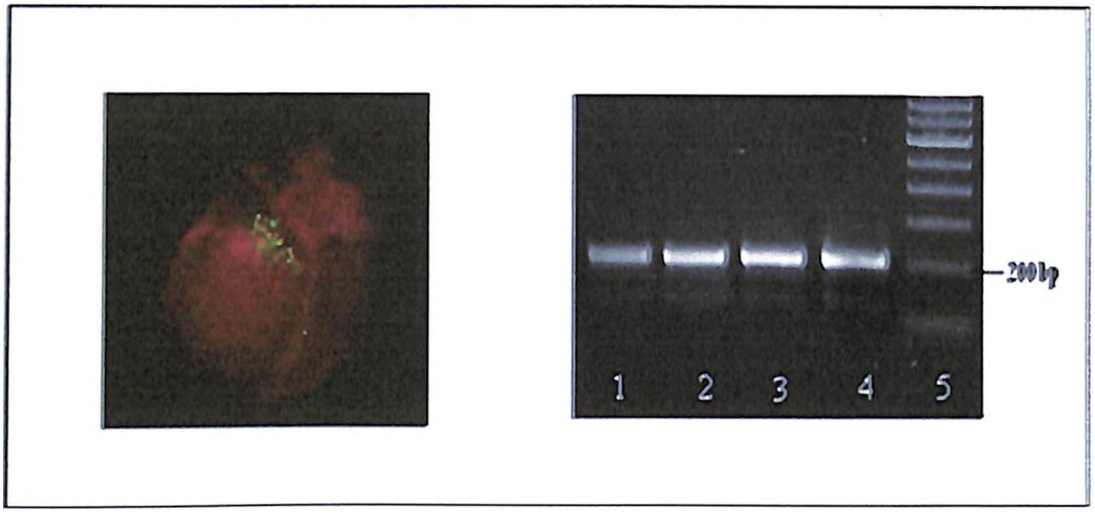


Figure 4.1: Representative figure showing presence of chlamydial elementary bodies in clinical sample by DFA (100X magnification); (b) Representative 2 % agarose gel electrophoresis of pCT PCR 200 bp amplicon showing PCR positivity for detection of *C. trachomatis* in endocervical swabs collected from patients. Lane 1, 2 and 3 are *C. trachomatis* detected in patient samples, Lane 4 is positive control, Lane 5 is 100 bp DNA Ladder.

Detection of Antibodies Against cMOMP, cHSP60 and cHSP10

ELISA 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); **(Figure 4.2)**. 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-cMOMP, 48 (64.0%) for anti-cHSP60 and 46 (61.3%) for anti-cHSP10.

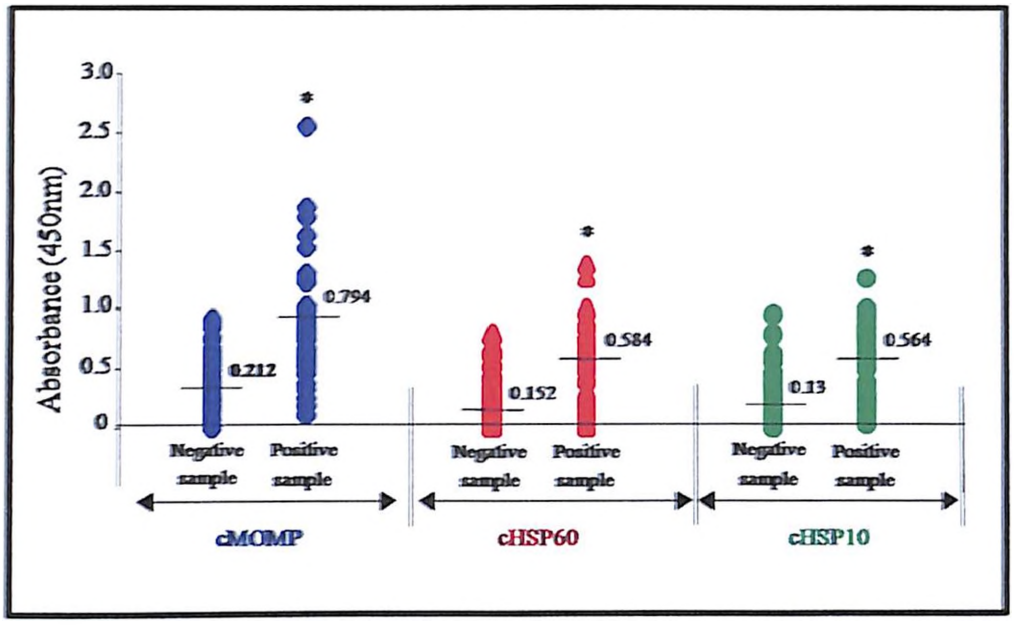


Figure 4.2: Quantitation of chlamydial antigen-specific antibodies in human sera by ELISA using peptide-specific sequence of antigens. Median values of anti-cMOMP, anti-cHSP60 and anti-cHSP10.

Antibody Titres for anti-cMOMP, 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-cMOMP 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 ($P < 0.05$) **(Figure 4.3)**.

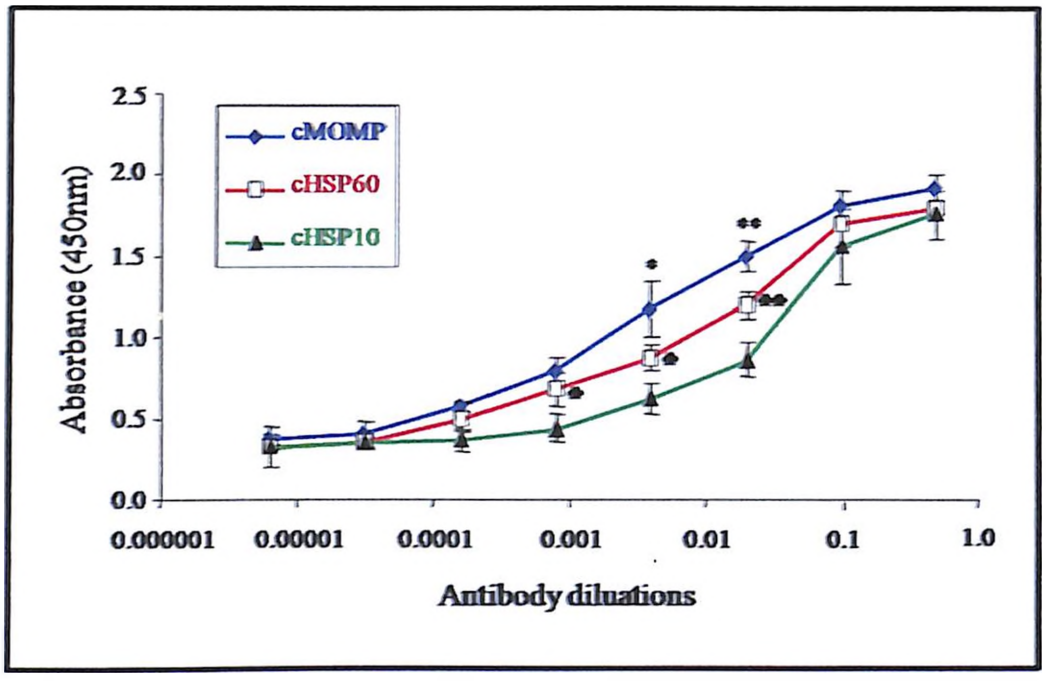


Figure 4.3: Positive patients' sera (N=75) were diluted and ELISAs of cMOMP, cHSP60 and cHSP10 were expressed as mean (A values) ± standard deviation. Statistically significant differences in A values between anti-cMOMP and anti-cHSP60 were $P < 0.01$ at 1:50 and $P < 0.05$ at 1:250). Antibody titres of anti-cHSP60 were significant at dilutions of 1:50 ($P < 0.01$) and at 1:250 and 1:1250 ($P < 0.05$).

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-cMOMP and anti-cHSP60 in the *C. trachomatis*-positive patients (**Figure 4.4a**). In the *C. trachomatis*-negative patients an insignificant correlation (R= –0.002) was observed between anti-cMOMP and anti-cHSP60 **(Figure 4.4b)**. A positive correlation (R= 0.286; $P < 0.05$) was seen between anti-cMOMP and anti-cHSP10 in *C. trachomatis*-positive patients (**Figure 4.4c**), but an insignificant correlation (R= 0.11) was seen in *C. trachomatis*-negative patients **(Figure 4.4d)**.

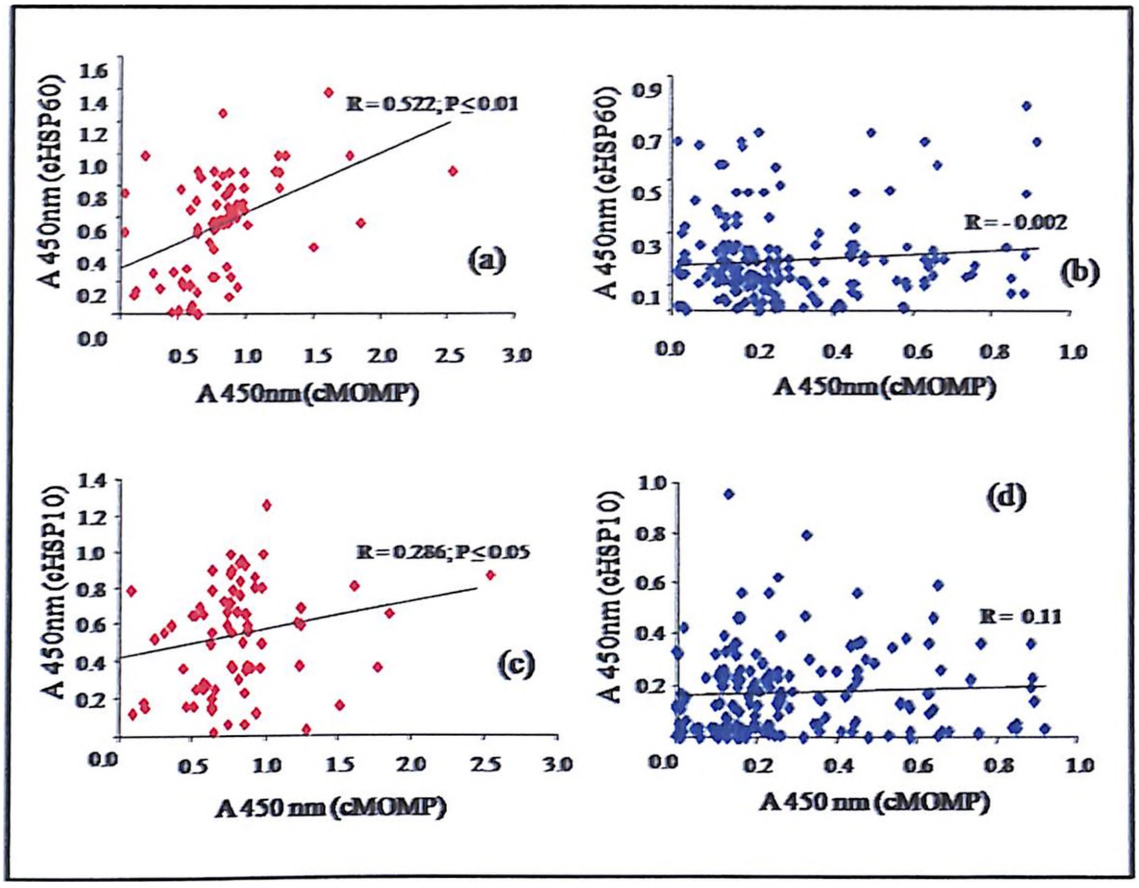


Figure 4.4: Scatter plot showing the correlation of the serological response to immunogenic chlamydial antigens: (a) anti-cMOMP versus anti-cHSP60 in positive patients; (b) anti-cMOMP versus anti-cHSP60 in negative patients; (c) anti-cMOMP versus anti-cHSP10 in positive patients; (d) anti-cMOMP versus anti-cHSP10 in negative patients.

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 **(Figure 4.5a)**, but a negative correlation (R= –0.098) was found in *C. trachomatis*-negative patients **(Figure 4.5b)**.

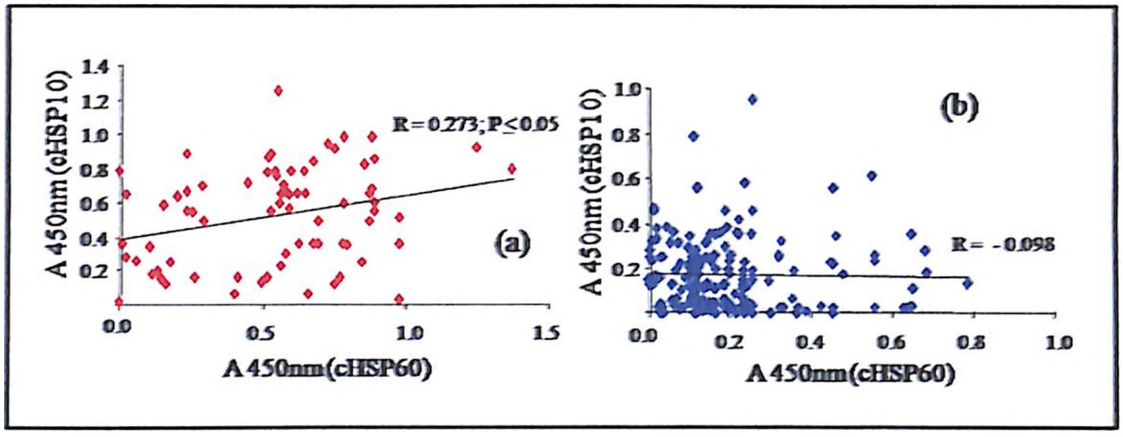


Figure 4.5: Scatter plot showing the correlation between the chlamydial heat shock proteins. (a) A significant positive correlation was seen between anti-cHSP60 and anti-cHSP10 in infected patients, and (b) a negative correlation between the two in uninfected patients.

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. Similar observations were made with cHSP10; however, titres were not as well demarcated **(Figure 4.6)**.

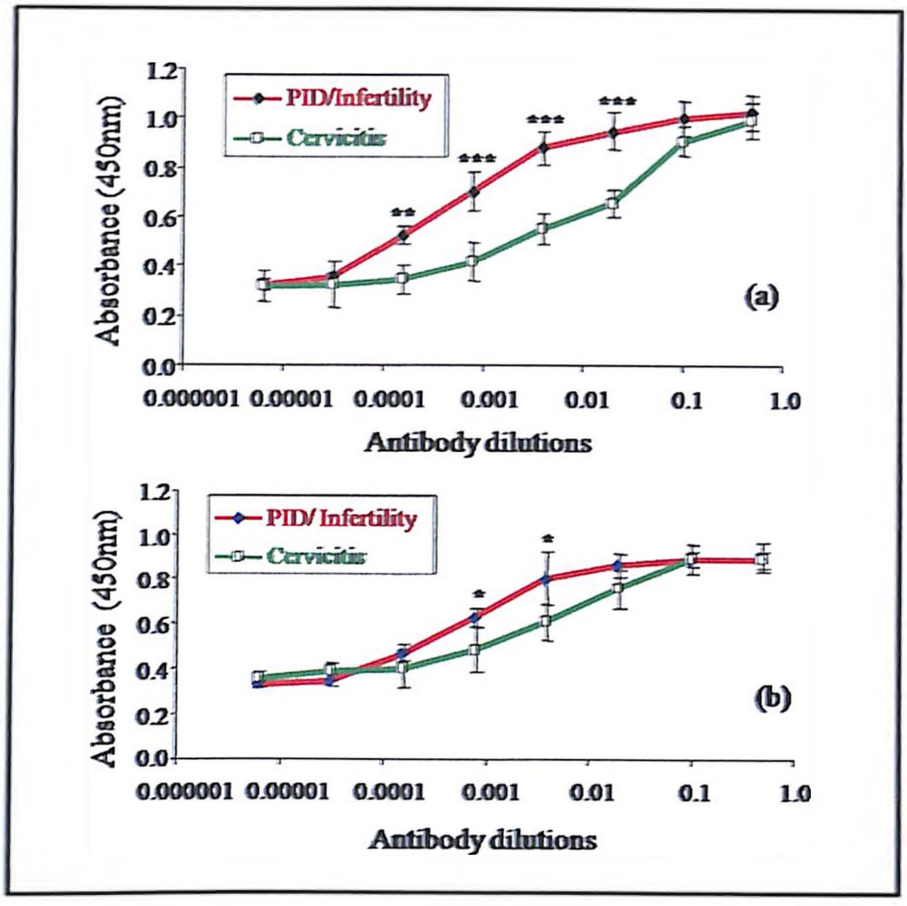


Figure 4.6: Sera (N=75) positive for cHSP60 and cHSP10 were diluted and antibody titres were compared between PID/infertility and cervicitis cases: a) *A* values for anti-cHSP60 showed statistically significant differences at 1:50, 1:250 and 1:1250 ($P < 0.001$) and at 1:6250 ($P < 0.01$); b) A values for anti-cHSP10 showed significant differences at 1:250 and 1:1250 ($P < 0.05$).

Discussion

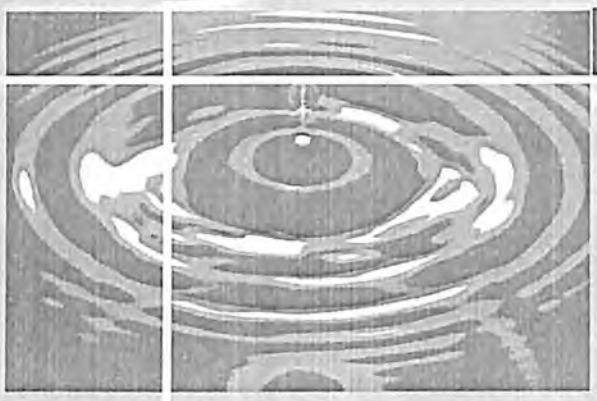
Upper female genital tract infection due to *C. trachomatis* is often asymptomatic. Immunity to cHSP60 and cHSP10 is associated more typically with chronic upper genital tract infection and fallopian tube damage than it is with acute infection of the lower genital tract (Arno et al., 1995; Betsou et al., 1999; LaVerda et al., 2000). The results of the present study agreed with previous data which showed 23–30% incidence of chlamydial infection in

the lower genital tract in Indian women (Mittal et al., 1993a; Mittal et al., 1993b; Singh et al., 2002; Singh et al., 2003). Various serological studies suggest that the host's immune response is directed predominantly against immunodominant proteins such as cMOMP and cHSP60 (Igietseme and Murdin, 2000; Sharma et al., 2004). In persistent chlamydial infection the ratio between cHSP60 and cMOMP increases dramatically in vitro; however, the production of cHSP60 antibodies may predominate over the production of cMOMP antibodies, which plays a major role in protective immunity against the initial stages of infection (Beatty et al., 1994b; Peeling et al., 1997).

The results of the present study suggest that cMOMP may be more immunogenic than cHSP60 in *C. trachomatis*-positive individuals. Similar observations have been reported in another study comparing cMOMP and cHSP60 antibody titres (Kinnunen et al., 2002). Many reports suggest that seropositivity for cHSP60 antibodies is associated with the increased prevalence of histological salpingitis, pelvic adhesions and a past history of PID (Arno et al., 1995; Ault et al., 1998; Cerrone et al., 1991; den Hartog et al., 2005; Sziller et al., 1998). Furthermore, it has been reported that the ratio between cHSP60 and cMOMP increases dramatically in subclinical chlamydial infection (Beatty et al., 1994c). Co-expression of cHSP60 and cHSP10 is a recognized 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 earlier reported (Morrison et al., 1990). This suggests that cHSP60 and cHSP10 may express simultaneously during chlamydial infection, which confirms previous findings (Dadamessi et al., 2005). Immune sensitization of the host to specific regions of the cHSP60 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 (Ault et al., 1998; den Hartog et al., 2005; Sziller et al., 1998). hHSP60 is a highly conserved protein that shares 48% amino acid sequence identity with cHSP60 (Kaufmann, 1994), and specific antibodies have been found to cross-react with hHSP60, which suggests that these antibodies may play a role in disease pathogenesis (Domeika et al., 1998). During rapid chlamydial cell growth or differentiation, or following environmental stress such as inflammation, hHSP60 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 (Yi et al., 1993). 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 (Ault et al., 1998; den Hartog et al., 2005).

In the present study, high cHSP60 and cHSP10 antibody titres were detected in PID patients. Similar results were obtained in a study comprised of 306 patients, which showed a significant correlation between cHSP60 antibody, PID and occluded fallopian tubes (Eckert et al., 1997). Although in our study, anti-cHSP60 titres were higher than anti-cHSP10 titres in PID/infertility patients, however, the association between cHSP10 seropositivity and TFI was greater in *C. trachomatis*-positive patients than in *C. trachomatis*-negative patients (Betsou et al., 1999; LaVerda et al., 2000). This implies that seropositivity for both cHSP60 and cHSP10 may be a useful marker for disease severity.



Chapter 5
Evaluation of *Chlamydia trachomatis* Specific HSP60 as Prognostic Marker in Infertile Women

Evaluation of Chlamydia trachomatis Specific HSP60 as Prognostic Marker in Infertile Women

Introduction

Genital *C. trachomatis* infection is known to be a major cause of female infertility, secondary to tubal damage. In majority of the women, long-term persistent *C. trachomatis* infection is often asymptomatic or clinically silent (Gaydos et al., 1998). Chronic chlamydial infection may affect female upper genital tract leading to secondary tubal infertility, PID, chronic pelvic pain, salpingitis and ectopic pregnancies (Cohen and Brunham, 1999). A WHO study documented that 18%–20% of infertile women are infected with *C. trachomatis* worldwide (WHO Task Force 1995) and in India, 28%–30% of infertile women were reported to be *C. trachomatis* infected, which is quite high in terms of world scenario (Malik et al., 2006; Mittal et al., 1993a; Mittal et al., 1993b; Singh et al., 2002; Singh et al., 2003).

A large proportion of *C. trachomatis*-infected women develop asymptomatic upper genital tract disease, as evidenced by genus-specific IgG antibodies, which are detected significantly more often in sera of subfertile women with distal tubal pathology than in those without (den Hartog et al., 2005). In addition, both cHSP60 and cHSP10 have been implicated in the development of female infertility, since circulating antibodies to these proteins are more common in infertile women than in fertile controls (Arno et al., 1995; Brunham et al., 1985; Claman et al., 1997; Eckert et al., 1997; Land and Evers, 2002; LaVerda et al., 2000; Toye et al., 1993). Moreover, cHSP60 serum antibodies have been shown to be the best predicting factor for TFI (Tiitinen et al., 2006). A significant association has been found between antibodies to cHSP60 in serum and in follicular fluid of infertile women (Cortinas et al., 2004). Women with tubal infertility have also been shown to

recognize cHSP10 more frequently than actively infected women, perhaps as a consequence of repeated or persistent chlamydial exposure (LaVerda et al., 1999). In an animal model, cHSP60 has also been implicated in urogenital tract disease leading to infertility (Higgins et al., 2005). High seroprevalence of both anti-cHSP60 and anti-cHSP10 antibodies were detected in *C. trachomatis*-infected women and elevated antibody titre of IgG against cHSP60 and cHSP10 were detected in patients with infertility (details mentioned in Chapter-4). High seroprevalence may suggest that cHSP-specific immune responses play an important role in immunopathogenesis associated with a *C. trachomatis* infection. However, no study has assessed the serological response in different clinical conditions viz. infertility, cervicitis and discharge in *C. trachomatis* infected women. Owing to the significant association between the serological responses of HSPs with the progressive disease, cHSP60 and cHSP10 antibodies levels were evaluated in *C. trachomatis*-infected fertile and infertile women to know which cHSP is more reliable for prognosis of *C. trachomatis* infection in women.

Materials

As mentioned earlier in this Chapter 4.

Experimental Methods

Study population

A total of 198 women (age group = 16–50 years) attending the outpatient department of Safdarjung Hospital, New Delhi, India, for gynecological complaints (cervical discharge, lower abdominal pain, cervicitis and infertility) were enrolled for the study after informed written consent and approval of Hospital Ethical Board Committee. After detailed and

careful physical examination, symptomatic patients were characterized into the following groups:

Group 1: Infertile females (72)

Group 1A: Primary infertility (30)

Group 1B: Secondary infertility (42)

Group 2: Fertile females (126)

Group 2A: Patients having vaginum discharge (94)

Group 2B: Chronic cervicitis patients (32).

Inclusion Criteria

Fertile women (asymptomatic women and women with mucopurulent cervicitis) were those having given birth within the last 4–12 months. Infertile women were identified as those who lacked recognized conception after 18–24 months of regular intercourse without the use of contraception. The infertile group included women who were referred for diagnostic laparoscopy and women whose infertility was of unknown reasons. The fertile group consisted of women who had no clinical history of a past infection, whereas the infertile group had a clinical history of a past infection for more than 3 years.

Exclusion Criteria

Patients taking oral contraceptives, having a positive urine pregnancy test, recent antibiotic therapy, history of a recently treated sexually transmitted infection other than *C. trachomatis* and genital tuberculosis were excluded from the study. Further, women with male factor-related infertility, endometriosis or any other factors, such as hormonal imbalances which could be attributed as the cause for infertility, were also excluded. Patients

with overlapping group characteristics were also excluded for the purpose of keeping the groups well segregated.

Collection of Samples and Laboratory Diagnosis

As described earlier in Chapter-4

Serological Detection of Antibodies Against cMOMP, cHSP60 and cHSP10

ELISA was performed for the detection of IgG antibodies to *C. trachomatis* using specific antigens against cMOMP, cHSP60 and cHSP10 as described in earlier. ELISA-positive samples were defined as those yielding an optical density (OD) value that was at least 2 SD above the mean values with the panel of samples from negative subjects (PCR and DFA negative), i.e., OD at 450 nm > 0.72 (Sziller et al., 1998).

Statistical Analysis

Kruskal-Wallis nonparametric test was used to compare continuous variables among multiple groups and Mann-Whitney U-test was used for comparing two groups. Data were analyzed using the SPSS Statistical package. The results presented with 95% confidence interval (CI) and P-value < 0.05 was considered to be statistically significant.

Results

STD Pathogens in the Cervix of Patients

Among the enrolled patients, none of the *C. trachomatis*-infected women was found to be infected with other STD pathogens. However, in *C. trachomatis*-uninfected women, 11 (6.1%) were found to be infected with *Candida spp.*, 17 (9.44%) with *Bacterial vaginosis*, 13 (7.22%) for *M. hominis*, 48 (26.67%) for *U. urealyticum* and none with *N. gonorrhoeae* and *T. vaginalis* in the cervix.

C. trachomatis Reinfection

C. trachomatis-reinfected cases were significantly higher in Group 1B (82.6%) and Group 2B (64.28%) in cHSP60-positive cases (**Table 5.1**). It was observed that *C. trachomatis* antibodies were present more frequently among younger women (57.8% age 16–27 years) than older groups (42.2% age 28–50 years).

Table 5.1: Percent positivity of *Chlamydia trachomatis* in different groups of patients (N=198)

Group	Number of Patients	Age, Median year (range)	CT IgG MOMP positive	cHSP60 IgG positive	cHSP10 IgG positive	cHSP60 positive cases	
						Primary infection	Reinfection
Group 1A	30 (38.46)	30 (23-38)	6 (20) [17.28-22.72]	9 (30.0) [28.16-31.84]	8 (26.67) [28.16-35.84]	7 (77.8) [d]	2 (22.2)
Group 1B	42 (53.85)	32 (21-50)	24 (57.14) [a] [50.37-63.62]	23 (54.76) [b] [51.16-52.36]	17 (40.48) [a] [35.34-45.62]	4 (17.39)	19 (82.6) [f]
Group 2A	94 (78.33)	23 (16-39)	23 (24.47) [18.08-30.85]	18 (19.15) [16.63-21.66]	23 (24.47) [20.43-28.51]	17 (94.4) [e]	1 (5.55)
Group 2B	32 (26.67)	27 (17-40)	11 (34.38) [26.6-42.14]	14 (43.75) [c] [38.57-48.92]	9 (28.13) [23.41-32.84]	5 (35.7)	9 (64.28) [g]

Note: Closed brackets ( ): percentages, square brackets [ ]: 95% confidence intervals, CT: *C. trachomatis*, MOMP: major outer membrane protein, cHSP: Chlamydia heat shock protein, Group 1A: primary infertility, Group 1B: secondary infertility, Group 2A: discharge, Group 2B: cervicitis [a] $P < 0.05$ as compared to group 1A (CT MOMP); [b] $P < 0.01$ as compared to Group 1A (cHSP60), $P < 0.01$ as compared to Group 1B (cHSP10); [c] $P < 0.01$ as compared to Group 2A (cHSP60), $P < 0.05$ as compared to Group 2B (cHSP10); [d] $P < 0.05$ as compared to Group 1B (primary infection); [e] $P < 0.01$ as compared to Group 1B and 2B (primary infection); [f] $P < 0.01$ and [g] $P < 0.05$ as compared to Group 1A and 2A (reinfection).

Seroprevalence of *C. trachomatis* Infections

In group 1, anti-cHSP60 specific antibodies were present in 54.76% (95% CI; 51.16% – 52.36%) of the secondary infertile patients and 30.0% (95% CI; 28.16% – 31.84%) of the primary infertile patients; however, in Group 2 antibodies were present in 43.75% (95% CI; 38.57% – 48.92%) of patients having cervicitis and 19.15% (95% CI; 16.63% – 21.66%) of the patients having discharge (Table 5.1). Anti-cHSP10 ELISA showed seroprevelence of 40.48% (95% CI; 35.34%–45.62%) in secondary infertile patients (group 1B), which is significantly higher than group 1A **(Table 5.1)**.

Sensitivity and Specificity of cHSP ELISA

In comparison to IgG MOMP ELISA the sensitivity and specificity for cHSP60 and cHSP10 ELISA was 90.91% and 75.6% and 89.47% and 73.87%, respectively, in secondary infertile women. Detection of anti-cHSP60 antibodies had a sensitivity of 67.33% and a specificity of 90.67% in secondary infertile women on considering DFA and PCR as test standard **(Table 5.2)**. In cervicitis patients, both the ELISA's showed 79.17% and 68.18% sensitivity and 77.78% and 88.89% specificity, respectively, which was higher than those in patients with discharge.

Table 5.2: Sensitivity and specificity of anti-cHSP60 and anti-cHSP10 ELISA in four different groups compared to IgG MOMP ELISA and DFA & PCR as test standard.

Group	IgG MOMP ELISA								DFA & PCR							
	cHSP60				cHSP10				cHSP60				cHSP10			
	Sen.[a]	Spe.[b]	PPV[c]	NPV[d]	Sen.[a]	Spe.[b]	PPV[c]	NPV[d]	Sen.[a]	Spe.[b]	PPV[c]	NPV[d]	Sen.[a]	Spec.[b]	PPV[c]	NPV[d]
Group 1A	50.0	73.1	22.2	90.5	60.0	88.4	65.2	85.9	60.0	76.0	33.3	90.48	45.45	78.05	21.74	91.43
Group 1B	90.91	89.47	83.33	94.44	75.6	73.87	44.44	75.4	67.33	90.67	63.48	83.68	60.0	79.37	52.94	76.0
Group 2A	52.63	88.41	55.56	87.14	50.0	79.20	37.5	86.4	54.55	85.37	33.33	93.33	20.0	72.0	12.50	81.82
Group 2B	79.17	77.78	82.61	73.68	68.18	88.89	88.24	69.6	61.0	92.57	67.14	83.33	55.0	71.2	88.9	90.9

Note: Direct flouresence assay (DFA); Polymerase chain reaction (PCR); Major outer membrane protein (MOMP); Chlamydia heat shock protein (cHSP); Group 1A = Primary Infertility; Group 1B = Secondary Infertility; Group 2A = Discharge; Group 2B = Cervicitis; [a]Sensitivity; [b]Specificity; [c]Positive Predictive Value; [d]Negative Predictive value. All figures are in percentage.

Discussion

Undiagnosed or untreated cervical *C. trachomatis* infection takes a chronic course. The pathogen ascends via the endometrial cavity to upper genital tract and may lead to various immunopathological events such as infertility or ectopic pregnancy (Cohen and Brunham, 1999). *C. trachomatis*-infected infertile women in Indian population are reported to be quite high, and among infertile couples from rural areas of north India, 46.5% had primary infertility and 53.5% secondary infertility (Malik et al., 2006; Mittal et al., 1993a; Mittal et al., 1993b; Singh and Dhaliwal, 1993; Singh et al., 2002; Singh et al., 2003). Therefore, screening of women at risk of developing infertility is highly recommended and a predictive serological test for the early detection is essential.

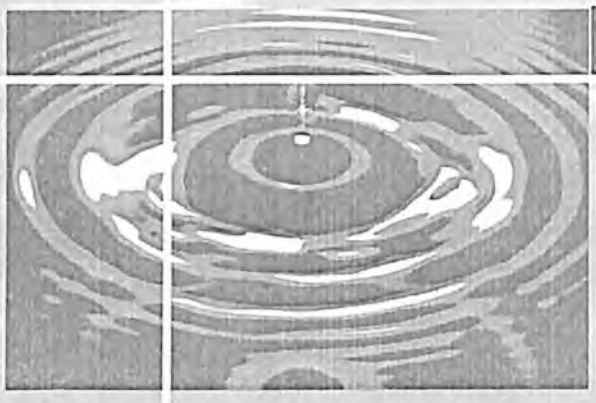
Immunity to the cHSP60 is more typically associated with a chronic upper gential tract infection and fallopian tube damage than with an acute infection of the lower genital tract suggesting that cHSP60 plays an important role in the immunopathogenesis of *C. trachomatis* infection (Clad et al., 2003; Claman et al., 1997; Cortinas et al., 2004; den Hartog et al., 2005; Tiitinen et al., 2006). Chronic *C. trachomatis* upper genital tract infection or reinfections may incite an autoimmune response through molecular mimicry in host that may lead to tissue damage as chlamydial growth is inhibited by the acquired immune response (Cohen and Brunham, 1999). Moreover, induction of HSPs interrupts the normal progression of reticulate bodies to infectious elementary bodies, resulting in a longer-term persistent infection. Such persistent infections may serve as antigenic reservoirs for potential immunopathogenic anti-HSP immune system responses (Beatty et al., 1994b).

The present investigation showed that women who were associated with *C. trachomatis* secondary tubal infertility were more likely to have anti-cHSP60 (54.8%, 30.0%) or cHSP10 (40.5%, 26.7%) antibodies than women having primary infertility. Our data suggest that in infertile *C. trachomatis*-infected women in India, cHSP60 antibodies are more likely to be associated with secondary infertility than cHSP10 antibodies. These findings are concordant with the pervious findings of Clad *et. al.*, where it was reported that cHSP60 IgG were more frequent in patients with occluded tubes (Clad et al., 2003).

In the present study, IgG cMOMP ELISA was considered as standard test, and calculated the sensitivity and specificity of cHSP's ELISAs with respect to cMOMP ELISA as *C. trachomatis* immunodominant MOMP is a target of neutralizing antibodies as well as a serotyping antigen (Cohen and Brunham, 1999). In addition, specific MOMP variants are associated with upper genital tract infection and cMOMP IgG and IgA levels were reported

to be higher in patients having occluded tubes (Clad et al., 2003; Dean et al., 1995). In the present investigation,. cHSP60 and cHSP10 ELISA showed high level of sensitivity and specificity in the secondary infertile (90.9%; 75.6% and 89.5%; 73.8%, respectively) and cervicitis (79.2%; 68.2% and 77.8%; 88.9%, respectively) patients in comparison to patients having primary infertility and discharge. Our data depict that cHSP60 ELISA have higher sensitivity and specificity in secondary infertile women than cHSP10. In previous study on considering PCR of biopsy tissue as a test standard of comparison, cHSP60 ELISA demonstrated 43% sensitivity and 100% specificity (Chernesky et al., 1998).

Additionally, in our study in secondary infertile women, 82% patients had reported to have chlamydial reinfection while patients with discharge and primary infertility had primary *C. trachomatis* infection. Our data suggest the importance of long term chlamydial reinfection in the pathogenesis of infertility in infected individuals. This suggested that repeated or chronic exposure to the cHSP60 was required for development of specific immunity to HSP60 epitopes shared between *C. trachomatis* and human. This study revealed that cHSP60 antibodies are more reliable serological marker for the sequelae of infertility in subfertile patients. Thus, an early detection of anti-cHSP60 antibodies can be routinely used as useful predictive and diagnostic marker for *C. trachomatis*-induced infertility. It will also improve cost effectiveness of screening strategies in infertility clinics in India. However, follow-up investigation would be required for the comprehensive prognostic information of anti-cHSP60 assay.



Chapter 6
Cloning, Expression and Purification of *Chlamydia trachomatis* Heat Shock Proteins 60 and 10

Cloning, Expression and Purification of Chlamydia trachomatis Heat Shock Proteins 60 and 10

Introduction

The primary structures of HSPs are highly conserved during evolution, suggesting that they serve similar and very important functions in all organisms, from bacteria to human (Lindquist and Craig, 1988). The chaperonin HSP60 or GroEL and co-chaperonin HSP10 or GroES constitute the GroE chaperone machine, which take part in the process of folding and assembly of proteins. The GroE chaperone interact with unfolded or partially folded protein subunits and facilitates correct folding by recognizing intermediates exposing hydrophobic surfaces (Hartl and Hayer-Hartl, 2002; Houry, 2001). Because of their chaperonin reactions and importance of GroEL as a major antigen of pathogenic bacteria, genes encoding these proteins have been cloned from a wide variety of bacteria. Cloning and sequencing of these genes help in studies directed at characterization of protein structure and function (Zeilstra-Ryalls et al., 1991) as well as in comparative studies on the regulation of heat-shock-gene expression (Segal and Ron, 1996).

In *Chlamydiae*, the 60-kDa cHSP60, or GroEL, and the 10-kDa cHSP10, or GroES, are known to be genetically linked because they are encoded by genes arranged on the bicistronic groESL operon (Morrison et al., 1990). Numerous studies have suggested that both cHSP60 and cHSP10 are implicated in the induction of deleterious immune responses in human chlamydial infections. Thus, among a panel of 116 recombinant *C. trachomatis* proteins, cHSP60 (Ct110) has been identified as an immunodominant antigen, being a strong target of both humoral and cell-mediated immune responses, whereas cHSP10 (Ct111) has been identified as a human T cell target (Follmann et al., 2008).

The retrieved sequence of cHSP60 from *Chlamydia* genome database and NCBI showed identical with sequence of cHSP60 amplified from *C. trachomatis* infected women. There are two other ortholog of cHSP60 copies in *Chlamydiae* species have diverged functionally after the gene duplication events but cHSP60 was found more abundant and immunodominant than the other two (McNally and Fares, 2007). The transcription of cHSP60 gene in *C. trachomatis* has been shown to be immediately induced in infected cells exposed to higher temperature at 45°C (Karunakaran et al., 2003), however, its specific role in different clinical conditions are not clear. Also, role of cHSP10 in the pathogenesis of genital *Chlamydia* infection is poorly understood. Therefore, cHSP60 and cHSP10 gene was cloned to understand the specific role of these antigens in chlamydial disease pathogenesis.

Materials

Agarose, BSA, Glycine, Glutamine, HEPES, Tris base, RPMI-1640, Penicillin, Kanamycin sulphate, Ponceau S, Diaminobenzamide, phenyl methyl sulfonyl fluoride (PMSF), Coomassie, and other fine chemicals were purchased from Sigma Chemicals, USA. Luria-Bertinni (LB) agar and broth were purchased from HiMedia, India. Taq polymerases, dNTPs, MgCl2, DNA ladder, Isopropyl-β-D-thiogalactoside (IPTG) were from Invitrogen, USA. Ni-NTA purification system, pQE-60 expression vector, plasmid extraction midi kit and gel extraction kit were purchased from Qiagen, CA, USA. pGEM®-T vector systems were purchased from Promega, USA. All restriction enzymes used were purchased from New England Biolabs. Primers for cHSP60 gene and plasmid targeting *C. trachomatis* were synthesized by Microsynth, Switzerland.

Experimental Methods

DNA Extraction

DNA was extracted from endocervical swab samples from *C. trachomatis* infected women as mentioned in Chapter 4.

Amplification of cHSP60 Gene

A unique primer set with their restriction endonuclease sites, against cHSP60 sequence (GenBank accession no. M58027) was designed for the amplification of cHSP60 gene. The sequences of oligonucleotide primers used were: (Forward primer- 5'-TCC CcccgggA TGG TCG CTA AAA ACA TTA AA-3' and Reverse primer- 5'-ACG CgtcgacTT AAT AGT CCA TTC CTG CGC C-3' with restriction endonuclease sites, *XmaI* and *SalI* respectively at 5'-end). Standardization of the optimum condition for cHSP60 gene PCR was performed initially. Reaction cocktails with gradually increasing concentrations of 1.5 mM to 3.5 mM $MgCl_2$ and annealing temperatures (50°C to 67°C) were used to determine the best amplification conditions through gradient PCR. The PCR reaction mixture (50 µl) consisted of 2 µl (1 µg/ml) of chlamydial genomic DNA, 5 µl 10X PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 1.0 µl 10 mM dNTP mixture, 0.75 µl 50 mM $MgCl_2$, 3 µl of each primer (5 pM/µl), and 0.2 µl Taq DNA Polymerase (5 U/µl). Samples were subjected to 40 cycles of DNA amplification in a thermocycler (Eppendorf mastercycler) as follows: 1 min (except for the first cycle, which was 5 min) of denaturation at 95°C, 1 min of primer annealing to template at 58°C, and 2 min of primer extension at 72°C. After the last cycle, all samples were incubated for 10 min at 72°C to ensure that the final extension step was complete. Amplified PCR product was confirmed on 1% agarose gel stained with ethidium bromide and visualized under UV transilluminator. The band of interest was cut from the gel with a scalpel

and the DNA was purified using gel extraction kit according to manufacturer's instruction (Qiagen, CA, USA).

Sensitivity limits of cHSP60 gene PCR reaction was determined by using serial dilutions (1:2) of DNA template as 25 ng/ml as starting concentration for all PCR reactions. Amplified PCR products were electrophoresed on 1 % (w/v) agarose gels and EtBr (0.5 pg/ml) stained gels were visualized on Alpha Imager gel documentation system.

Specificity of PCR products was confirmed by restriction digestion analysis. Restriction digestion map of cHSP60 gene sequence was generated from online tool (http://tools.neb.com/NEBcutter2/index.php). Ten microlitres of cHSP60 PCR product were subjected for restriction endonuclease digestion using *EcoR1* and *HindIII*. A 20 µl cleavage reaction was performed using 5 mM $MgCl_2$, and 10 U of restriction enzyme for 10 h at 37°C. Reactions were terminated at 65°C for 30 min and digested products were analysed by agarose gel electrophoresis as mentioned above.

Ligation

The purified PCR product of cHSP60 was ligated with pGEM®-T Vector (Promega). The ligation reaction mixure contains ligation Buffer (2X) 5µl; each insert (75 ng) 1 µl; pGEM-T Easy vector (50 ng) 1µl; ligase 1µl, make upto 10µl with nuclease-free dH_2O. The reaction mixture was incubated at 4°C for overnight. Following day, *E. coli* DH5α competent cells were prepared and the ligated product was transformed and subjected to ampicillin resistant (Amp^r) selection.

Competent Cell Preparation

The competent cells were prepared as described by Cohen *et al.*, with a slight modification (Cohen et al., 1972). This procedure yielded $5x10^6$ to $2x10^7$ transformed

colonies per µg of supercoiled plasmid from batches of competent bacteria. The host cell culture was streaked on a LB agar plate from the frozen glycerol stock. A single colony was inoculated into 5 ml LB tube. 500 ml of the overnight grown culture was further inoculated into 50 ml (1:100) LB and allowed to grow for 2-3 h until A_{600} reached 0.4-0.5. The culture was chilled on ice, transferred to ice cold 50 ml polypropylene tubes and centrifuged at 4000 rpm for 10 min. The supernatant was decanted and the pellet was resuspended gently in 20 ml of ice cold sterile 0.1 M $CaCl_2$ and incubated on ice for 30 min. The cells were then centrifuged at 4,000 rpm for 10 min. The pellet was resuspended in 1 ml of ice cold 0.1 M $CaCl_2$. Chilled glycerol was added to the cells to a final concentration of 20%. About 200 ml aliquots were taken for checking the viability, contamination and efficiency of transformation. The rest of the suspension was kept at 4°C for 12-24 h to enhance the competency of the cells and then stored in aliquots of 200 ml at -70°C (Dagert and Ehrlich, 1979).

Transformation of Competent Cells

Transformation of the competent *E. coli* cells was performed according to procedures described by Sambrook and Russel, 2001. Briefly, a 100 ml aliquot of competent cells was thawed on ice and 1 ml of the cHSP60 clone was added to it, mixed by tapping and incubated on ice for 30 min. The cells were subjected to heat shock at 42°C for 1.5 min in a water bath and were immediately chilled on ice for 1-2 min. Further, 850 ml of LB was added to the cells and the cells were incubated at 37°C for 1 h in a shaker incubator with constant shaking. The cells were pelleted and resuspended in 100 ml of LB and plated on LB agar plates containing 100 mg/ml of ampicillin, 10 ml of 0.8 M IPTG and 50 ml of 20 mg/ml of X-gal

and incubated at 37°C for 12 h for selection of recombinant clones (white) over non-recombinant clones (blue).

Screening of the Transformants

The presence of the ligated plasmid product was confirmed by mini-preparations of plasmid DNA. The DNA of the construct thus obtained was restriction enzyme digested with *XmaI* and *SalI* as described above and checked on agarose gel for the right size of insert of cHSP60 gene. The orientations of positive colonies were further determined by DNA sequencing on an automated DNA sequencer (ABI3770, Applied Biosystems, Fostercity, CA, USA).

Expression of cHSP60 and cHSP10

The 6x-His affinity tag system was used for the expression and purification of cHSP60 gene. The system provide high level expression of tagged proteins in *E. coli* using pQE vectors that is based on the T5 promoter and two lac operator sequences which increase lac repressor binding and ensure efficient repression of the powerful T5 promoter. Cloned plasmids from the overnight cultures of DH5α were isolated and subjected to restriction digestion with *BamH1* and *EcoRI*: distilled water (dH2O) 9 µl; Buffer (10X) 1.5 µl; Plasmid 4 µl *BamH1/EcoRI* 0.5 µl; incubated at 37°C for 1 h. The digested product (1655 bp) was cut from the gel with a scalpel and the DNA was purified with QIAGEN gel extraction kit as described above. The pQE-60 vector (Qiagen) was chosen to append a tag of six histidines (6xHis) to the C-termini of recombinant proteins for large-scale purification via nickel chelate affinity chromatography. Both the amplified insert and the pQE-60 vector were cut with appropriate restriction enzymes (*XmaI* and *SalI*), ligated with T4 DNA ligase and transformed into M15(pREP4)-competent *E. coli* cells (Qiagen) using standard protocol

($CaCl_2$ treatment and heat shock) as mentioned above. Recombinant gene containing full-length gene sequence of cHSP10 was obtained as kind gift from Dr Sylvette Bas at Department of Genetics and internal Medicine, University Hospital, Geneva, Switzerland. These clones were further propagated in LB, expressed and purified as follows.

Identification of Bacterial Colonies Containing Recombinant cHSP60 Plasmids

The transformed cells were plated onto 1.5% agar containing kanamycin (25 mg/ml) and ampicillin (100 mg/ml). Colonies were screened for the presence of an insert of the predicted size by DNA amplification using colony PCR. Overnight grown colonies were picked up in separate vials containing 10 µl of nuclease free water. Further, vials were incubated at 95^0C and centrifuged at 2000 g for 10 min. Supernatants were used as template for amplification of cHSP60 gene using primers and conditions as mentioned above. Bacteria from each tested positive colonies were grown overnight in 1.5 ml of Luria broth (LB) medium containing kanamycin (25 mg/ml) and ampicillin (100 mg/ ml) and glycerol stocks were prepared.

Induction of cHSP60 and cHSP10

To ensure that each protein (cHSP60 and cHSP10) is produced with adequate quantities, a small inoculate of glycerol stock was added to 25 ml of LB containing kanamycin (25 mg/ml) and ampicillin (100 mg/ml) and was incubated overnight at 37°C. This seeding culture was added to 500 ml of LB that also contained kanamycin and ampicillin, incubated at 37°C for about 1 h. Further, cells were induced with different concentrations (0.5 to 3 mM) of IPTG for about 3.5 h with vigorous shaking. Cultures of *E. coli* were pelleted by using an eppendorf centrifuge at 4,000 g for 20 min at 4°C. Pellets were washed in PBS and frozen at -20°C until used. Pellets were thawed and resuspended in lysis

buffer (50 mM Na_2HPO_4/NaH_2PO_4 buffer [pH 8.0], 300 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, 20 mM 2-mercaptoethanol, and 1 mg of lysozyme/ml). They were sonicated for 6 x 10 s on ice to ensure complete resuspension. Cells lysate was then centrifuged at 10,000 g for 30 min at 4°C to pellet cell debris.

Purification of cHSP60 and cHSP10

Fast protein liquid chromatography equipment (Pharmacia) was used to purify proteins under native conditions by nickel chelate affinity chromatography. Briefly, one bed volume (0.6 ml) of Ni-NTA resin was filled in glass column and equilibrated with 10-15 bed volume of equilibration buffer (20 mM Tris/HCl, 200 mM NaCl, 10 mM Imidazole, and pH 7.5). Further, protein supernatant was allowed to pass through the column. Then, washing was performed to remove unbound protein with 10 bed volume of washing buffer at different concentration (60-150 mM) of imidazole. After washing, specifically bound proteins were eluted with 10 bed volume of elution buffer (20 mM Tris/HCl, 200 mM NaCl, 200 mM Imidazole, and pH 7.5). A typical flow rate of 0.35 ml/min was maintained. The protein concentration was determined with the Bradford assay (Sigma). Purified proteins were subsequently characterized by SDS-PAGE and immunoblotting, separated into aliquots, and frozen at -80°C.

Polyacrylamide Gel Electrophoresis

A 10% and 12% separating gel for cHSP60 and cHSP10 respectively was used for the electrophoretic analysis of proteins. For stacking of proteins 5% gel was used. Protein samples for SDS-PAGE were prepared by the addition of the 6X loading buffer to the final concentration of 1X, boiled for 5 min followed by centrifugation at 12,000 g for 5 min at room temperature. Proteins were analyzed by SDS-PAGE according to method of Laemmli

at a constant voltage of 85V in a Bio-Rad mini gel apparatus (Laemmli, 1970). The resolved proteins were visualized by staining the gels with Coomassie Brilliant Blue R-250 for 30 min followed by destaining the gel to remove excess stain.

Western Blot Analysis

Western blot analysis was performed following the method described by Towbin H *et al.*, (Towbin et al., 1979). 2 mg of purified protein was resolved on SDS-PAGE and transferred to PVDF at a constant current of 100 ampere for 2 h. Blocking was done by using 2% BSA in PBST for 3 h. For probing the blot with antibody, the membrane was incubated for 2 h at room temperature with anti-cHSP60 monoclonal antibody (1:500) in PBST buffer (pH 7.4) containing 1% BSA. The membrane was washed and then incubated for 1h in conjugated anti-rabbit IgG (1:2500). The protein bands were visualized using the peroxidase substrate 3, 3'- diaminobenzidine (DAB) and H_2O_2.

Results

Amplification of cHSP60 gene

Amplicon of cHSP60 genes (1655 base pairs) was PCR amplified from genomic DNA of *C. trachomatis*- infected endocevical cells using the gene specific primers and was analyzed on 1% agarose gel stained with ethidium bromide. The sensitivity of cHSP60 gene PCR was determined by amplification of 1655 bp amplicon visualized on agarose gel. Amplicon was visualized with a starting concentration of DNA at 50 ng/ml **(Figure 6.1).** Specificity of PCR products was confirmed by restriction digestion analysis using restriction enzyme site for *Hind II*. Appearance of three fragments (895 bp, 403 bp and 337 bp) of *Hind II* digested PCR products was considered positive for confirmation of cHSP60 gene **(Figure 6.2).**

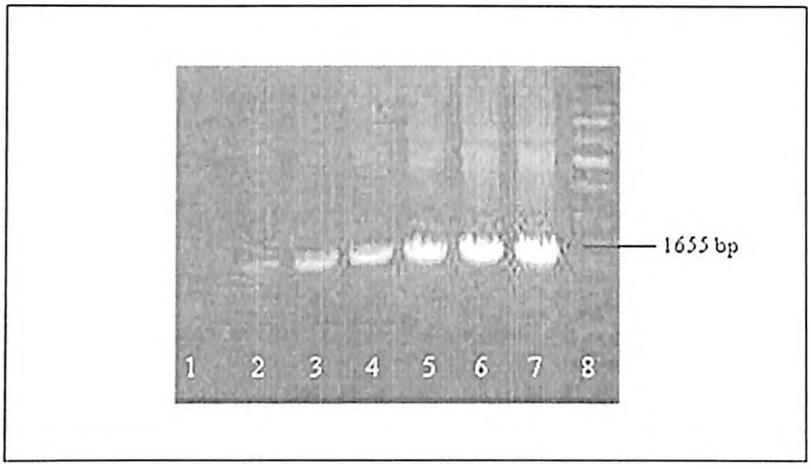


Figure 6.1: Representative agarose gel showing PCR amplification and detection limit of cHSP60 gene in endocervical samples of *C. trachomatis*-positive patients. Lanes 1-7 showing increasing concentrations of DNA template in the multiplication of 2 from starting concentration of 25 ng/ml in the lane 1; Lane 8 is 500 bp DNA ladder.

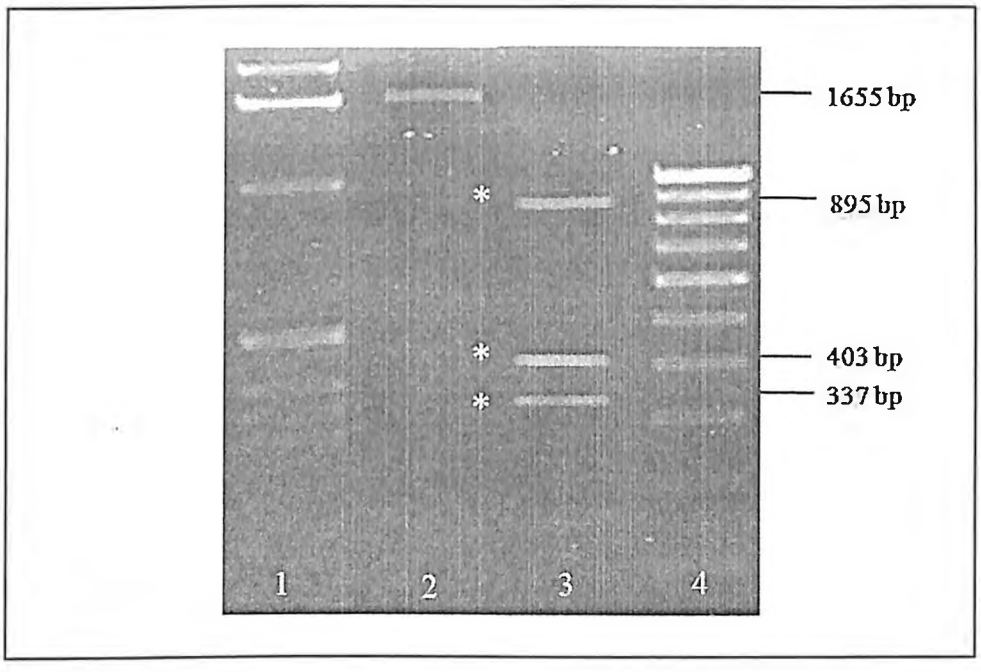


Figure 6.2: Specificity of cHSP60 PCR products by restriction digestion analysis. Lane 1; 1 kb DNA ladder, Lane 2; cHSP60 gene PCR product, Lane 3; *HindII* digested fragments of length 895 bp, 403 bp and 337 bp of cHSP60 gene, Lane 4; 100 bp DNA ladder.

Selection of cHSP60 clones

Transformed cells containing cHSP60 gene into pGEM-T vector producing white colonies on agar plate were screened using gene specific primers by colony PCR **(Figure 6.3)** and positive clones were confirmed by size of plasmid on 0.5 % agarose gel **(Figure 6.4)**. Further, single pass automated sequencing of the positive clone was performed using gene specific primers. Results showed that the sequence generated was homologous to CT serovar D by BLAST searches of the databases. BLAST analysis revealed 100% homology for cHSP60 with that of reported sequences in NCBI.

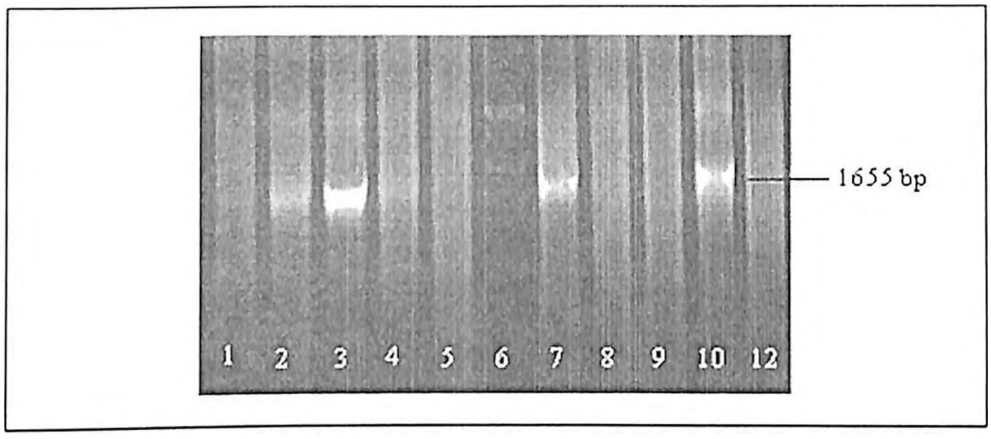


Figure 6.3: Selection of transformed positive clones containing cHSP60 gene in pGEM-T vector from white colonies grown on agar plate cHSP60 gene specific colony PCR. Lanes 2,3,7 and 10 showing positive clones, Lane 6; 500 bp DNA ladder.

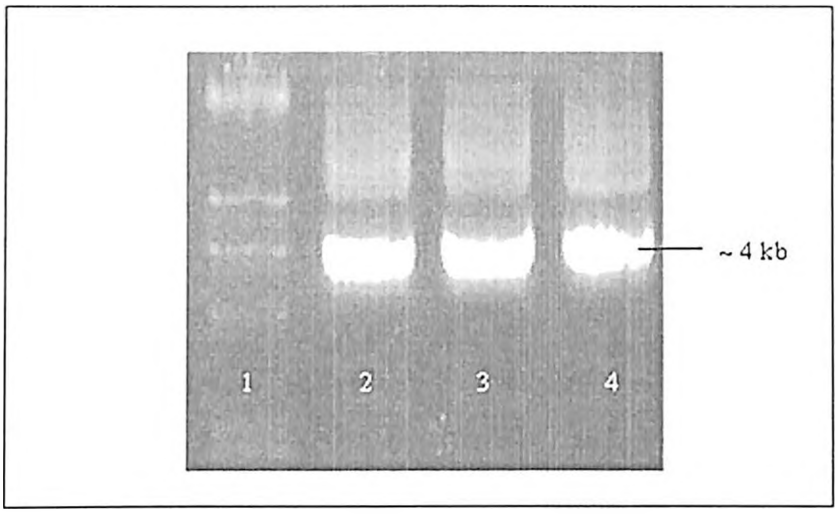


Figure 6.4: Confirmation of positive clones using plasmid extraction. Lane 1; Plasmid DNA ladder, Lanes 2-4; showing size of plasmid containing cHSP60 gene and pGEM-T vector.

Clones of cHSP60 in expression vector were selected on amp^r and ken^r supplemented agar plate and screened for the presence of cHSP60 gene using colony PCR and positive clones were further confirmed by restriction digestion analysis using suitable restriction enzymes whose sites were present in regions flanking the multiple cloning sites and in the PCR amplicons of cHSP60. Restriction digestion with *BamH1* and *EcoRI* yielded positive clones with fragments (linear DNA, 5 kb) and (3.8 kb, 800 bp and 432 bp) respectively **(Figure 6.5)**

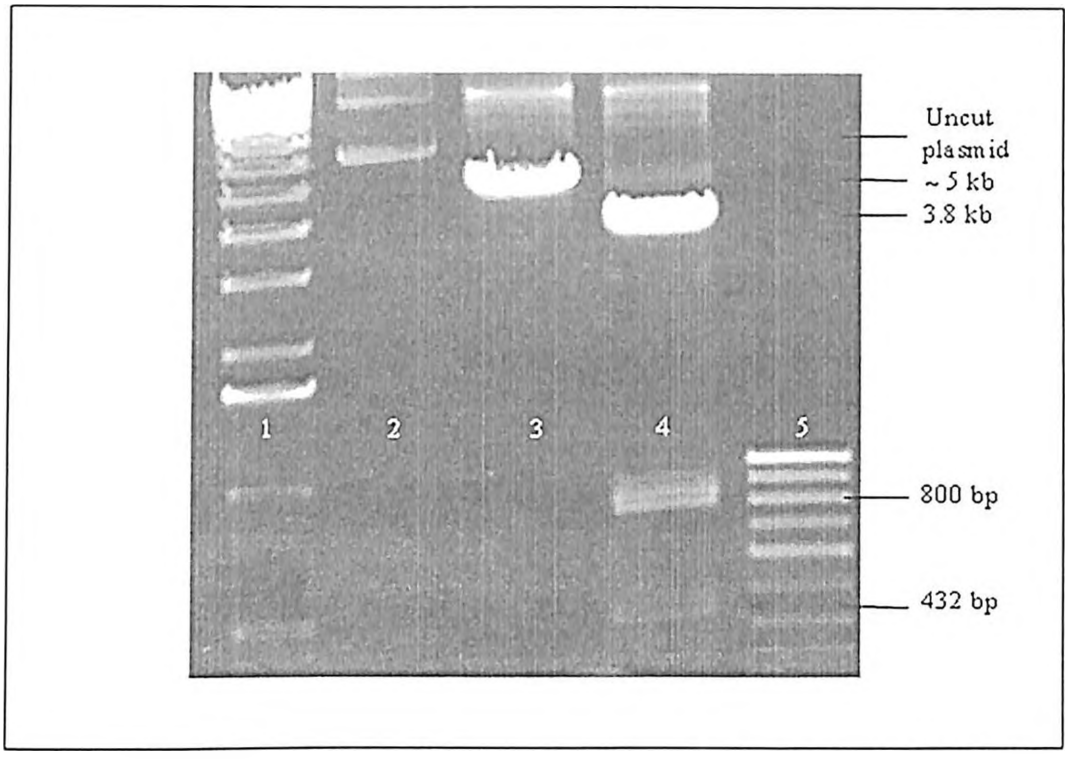


Figure 6.5: Characterization of expression vector carrying cHSP60 gene by restriction digestion. Lane 1; 1 kb DNA ladder, Lane 2; uncut plasmid carrying cHSP60 gene, Lane 3; linear DNA after *BamH1* digested product of plasmid giving size of ~5 kb, Lane 4; Plasmid digested with *EcoR1* giving products of size ~3.8 kb, 800 bp and 432 bp, Lane 5; 100 bp DNA ladder.

Expression of cHSP60 protein

Different concentration (0.5 mM to 2.0 mM) of IPTG induction of recombinant clones showed higher expression of a 60 kDa protein than uninduceed control **(Figure 6.6)**. Presence of cHSP60 proteins were further confirmed by western blot analysis using monoclonal antibody against cHSP60 **(Figure 6.7)**.

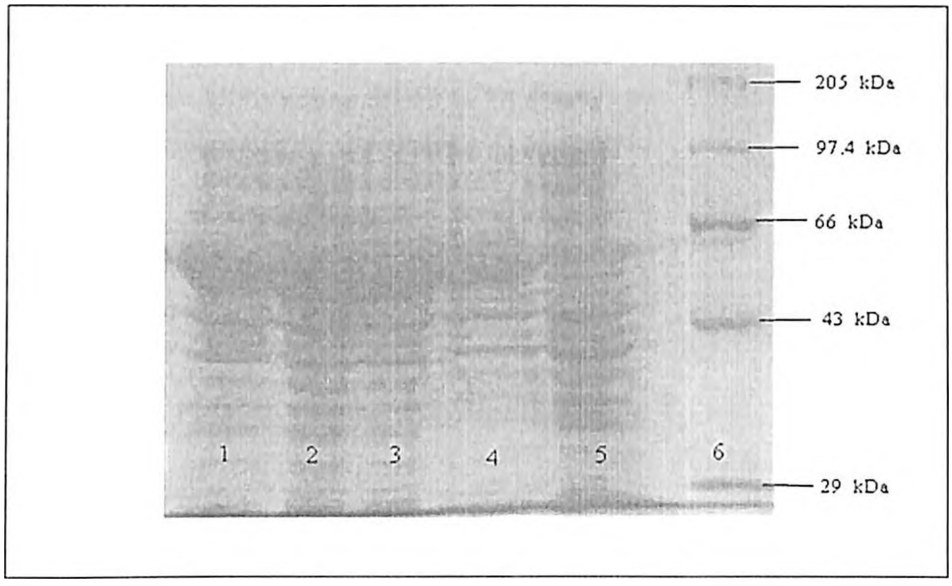


Figure 6.6: 10% SDS-PAGE analysis of 6x his-tagged cHSP60 induced with different concentrations of IPTG (Lane 1; 0.5 mM, Lane 2; 1.0 mM, Lane 3; 1.5 mM, Lane 4; 2.0 mM, Lane 5; uninduced), Lane 6; High molecular weight protein marker.

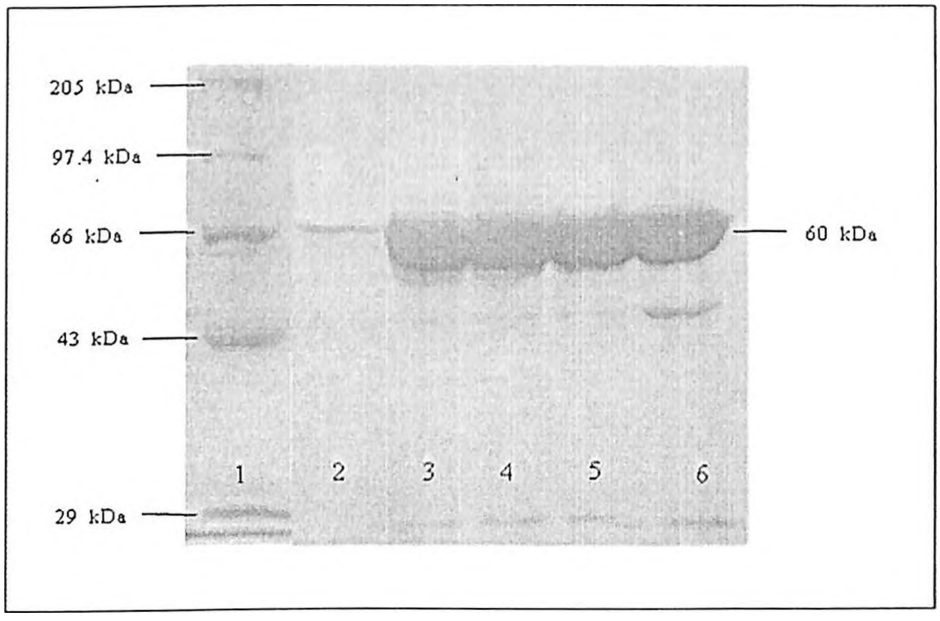


Figure 6.7: Characterization of protein using western blotting with monoclonal antibody against cHSP60. Lane 1; Protein marker, Lanes 2-6; Detection of cHSP60.

Purification of cHSP60 and cHSP10

Several elution fractions of Ni-NTA chromatographic purification of cHSP60 at different concentrations of imidazole were analyzed and 60 kDa single band purified protein was shown on 10% SDS-PAGE **(Figure 6.8 a)**. Similarly, purified protein band of cHSP10 was visualized on 12% SDS-PAGE **(Figure 6.8 b)**.

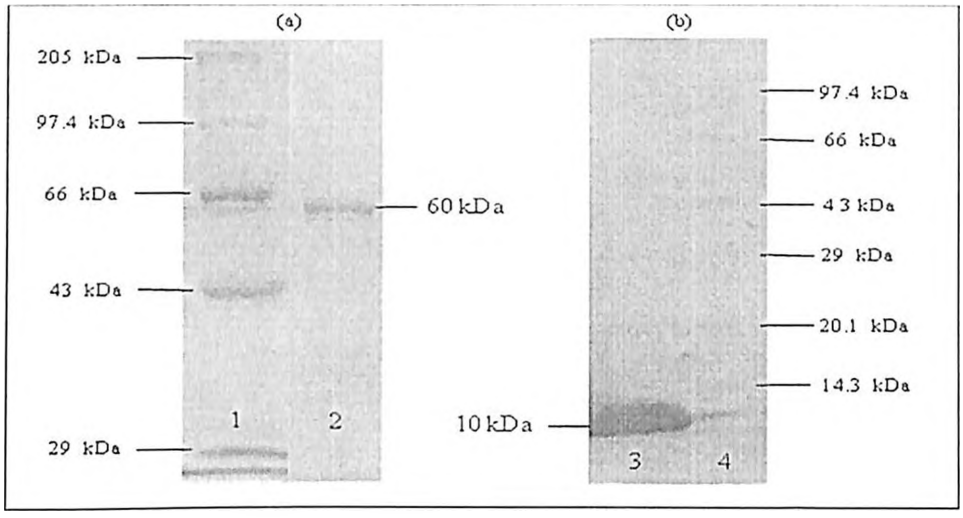


Figure 6.8: SDS-PAGE analysis shown single-band purified protein of cHSP60 and cHSP10. (a) 10 % SDS-PAGE showing Lane 1; High molecular weight protein marker and Lane 2; single band purified cHSP60. (b) 12 % SDS-PAGE showing Lane 3; single band purified cHSP10 and Lane 4; Low molecular weight protein marker.

Discussion

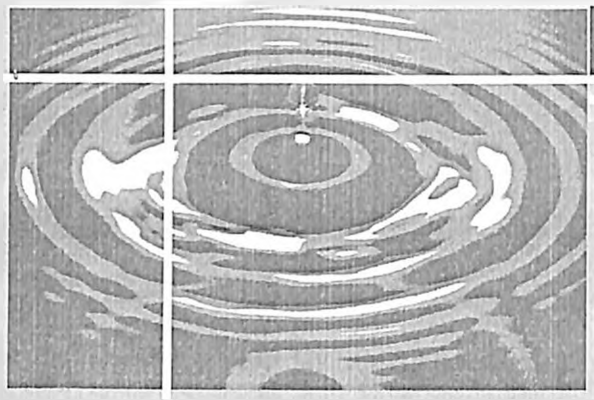
Heat shock proteins are highly conserved proteins present in almost all prokaryotic and eukaryotic organisms. The 60-kDa HSPs have been extensively studied for their chaperone function in protein folding and their cooperation with other chaperones in cellular trafficking. More than 150 homologues of HSP60 sequence are currently available with pair-wise similarity at the amino-acid level extending from 40 to 100% (Brocchieri and Karlin, 2000). In Chlamydiae, the cHSP60 (Ct110) and cHSP10 (Ct111) are encoded by genes

arranged on the bicistronic groESL operon (Morrison et al., 1990). In a panel of 116 recombinant *C. trachomatis* proteins, cHSP60 has recently been identified as an immunodominant antigen, being a strong target of both humoral and cell-mediated immune responses (Follmann et al., 2008). In addition, cHSP60 is highly homologous and conserved throughout the evolution. It belongs to a group of chaperone proteins ranging in molecular weight from 15 to 110 kDa which are produced in response to infection, stress, inflammation and other cellular insults (Engel et al., 1990). cHSP60 exhibit greater than 80% homology between *Chlamydia spp.*, 60% identity with other bacteria and 50% homology with hHSP60 and exhibits immuno-regulatory properties primarily by inducing proinflammatory response (Zugel and Kaufmann, 1999).

The open reading frame of cloned cHSP60 gene consists of 1635 bp and encoded a 58.2 kDa protein. The cHSP60 comprised of 548 amino acids with theoretical isoelectric point of about 4.68. The full-length cHSP60 gene was first cloned in pGEM-T vector and subsequently cloned into the QIAexpressionist pQE-60 expression vector and then transformed into *E. coli* M15 cells. The expression of cHSP60 protein was induced by IPTG for 30 min to 5 h, a large quantity of 6x-His tagged protein was detected in an about 3 h. High level expression of 6x-His-tagged cHSP60 and cHSP10 in E. coli in pQE vectors is based on the T5 promoter transcription-translation system. pQE plasmid belong to the pDS family of plasmids (Bujard et al., 1987) and was derived from plasmids pDS56/RBS11 and pDS781/RBS11-DHFRS (Stuber et al, 1990). The bacterial cultures were lysed and expression of cHSP60 was analyzed by gel electrophoresis followed by immunoblotting with anti-cHSP60 monoclonal antibodies. Both 6x-His-tagged cHSP60 and cHSP10 were purified using single step affinity nickel-nitriloacetic acid column chromatography (Porath et al.,

1975) and further used for stimulation studies. The *Chlamydia* genome database (http://chlamydia-www.berkeley.edu) containing both nucleotide and amino acid sequences are available.

In this study, PCR amplicons of cHSP60 were cloned into pGEM®-T vector systems and were further characterized by restriction digestion, colony PCR and western blotting. Clones of cHSP10 were a kind gift from Dr Sylvette Bas, University of Geneva, Switzerland. Further cHSP60 and cHSP10 clones were expressed and purified. Both purified proteins, cHSP60 and cHSP10 were subsequently used in this study for understanding their role in immunopathogenesis of *C. trachomatis* infection.



Chapter 7
Role of Chlamydial Heat Shock Proteins 60 and 10 in Immunopathogenesis of *Chlamydia trachomatis* Infection in Women

Role of Chlamydial Heat Shock Proteins 60 and 10 in Immunopathogenesis of Chlamydia trachomatis Infection in Women

Introduction

The immune responses to cHSP60 have been associated with the immunopathogenesis of *C. trachomatis*- associated ectopic pregnancy and tubal infertility (Brunham et al., 1992; Toye et al., 1993). The stress response in *Chlamydia* RBs is characterized by cHSP60 induction and by reduction in MOMP and LPS levels, as shown in an *in vitro* model of persistent infection (Beatty et al., 1993; Beatty et al., 1994b). This stress response is believed to interrupt the normal progression of RBs to infectious EBs, resulting in a longer-term persistent infection. Such persistent infections may serve as antigenic reservoirs for potentially immunopathogenic anti-cHSP immune system responses (Beatty et al., 1994d). The cHSP10 is genetically linked to cHSP60; the two proteins bind to each other and prevent incorrect protein folding and denaturation. Thus, the pathogen's ability to survive stressful environmental conditions and persist in the host is maximized by cHSP60-cHSP10 expression.

The development of infertility is reported to be due to enhanced immune responses to *C. trachomatis* (Debattista et al., 2003; Witkin et al., 2000). cHSP60 and cHSP10 antibodies seem to perform well in predicting TFI (Ault et al., 1998; Betsou et al., 1999; Dadamessi et al., 2005; den Hartog et al., 2005; LaVerda et al., 2000; Neuer et al., 1997; Spandorfer et al., 1999). CMI responses to cHSP60 were demonstrated in women with PID and TFI which showed IgG antibodies to cHSP60 are significantly more common as compared to women without tubal pathology (Cohen et al., 2005; Debattista et al., 2002; Kinnunen et al., 2002; Kinnunen et al., 2003; Tiitinen et al., 2006; Witkin et al., 1994). However, these studies were

restricted to the peripheral immune responses. In an another study done in lab, cHSP60 and cHSP10 specific proliferative responses were evaluated and suggested the probable role of cHSPs in modulation of mucosal immune responses (Agrawal et al., 2007). Overall these studies suggest cHSPs specific CMI responses plays an important role in the immunopathogenesis associated with chlamydial infection. Hence, it is worthwhile to further study the cytokines released by cervical mononuclear cells that are in direct contact with the pathogen and their modulation with cHSPs which play a crucial role in the modulation of mucosal immune responses leading to pathogenesis.

The role of Th1/Th2 responses in the genital tract during *Chlamydia* infection is considered to be crucial for controlling the duration of infection and subsequent tubal pathology. Indeed, Th1 cells produce IFN-γ that promotes the destruction of *Chlamydia* (Beatty et al., 1993) but can also promote inflammatory damage and fibrosis (Rottenberg et al., 2002) whereas Th2 cells produce IL-4, IL-5, and IL-13 believed to be critical for defense against extracellular pathogens. The secreted TNF-α and IL-10 was examined because their levels have been reported to be high in cervical secretions of *C. trachomatis* infected infertile women (Reddy et al., 2004).

Further, cHSP60 and cHSP10 mRNA and protein expressions were also analyzed in cervical cells that had previously encountered *C. trachomatis* in fertile and infertile women to better understand the role of these two proteins in the immunopathogenesis at the site of infection. Further, cHSP60 and cHSP10 specific cytokine responses by cervical mononuclear cells in women representing different clinical conditions, i.e. *Chlamydia* positive fertile and infertile women was characterized for understanding their role in immune responses at the site of infection.

Materials

RPMI-1640 and fetal calf serum (FCS) were obtained from Sigma-Aldrich and PAA, Austria respectively. RNeasy Mini Kit was from Qiagen, CA, USA. SuperScript™ First-Strand Reverse Transcriptase kit and Trizol, *Taq* polymerase, dNTPs, MgCl2, DTT, Superscript II reverse transcriptase, oligo dT20, RNasin were from Invitrogen, USA. DyNAmo™ SYBR® Green qPCR Kit was purchased from Finnzymes, Espoo, Finland. All HPLC purified primers were commercially synthesized from MWG-Biotech AG, Ebersberg, Germany. All ELISA kits for the quantitation of cytokines were purchased from ebiosciences, San Diego, CA, USA. Inactivated CT EBs were purchased from Antigenix America.

Experimental Methods

Study population

A total of 368 patients attending the gynaecology OPD, Safdarjung Hospital, New Delhi, India for gynecological complaints (cervical discharge, cervicitis and infertility) were enrolled in the study. The study received approval by the hospital's ethics review committee. Thirty-nine healthy age-matched controls attending the family-planning department for birth-control measures and with no previous history of any STD were also enrolled. At recruitment, a detailed clinical questionnaire was administered to each patient for collecting information on reasons for referral, gynecology history including menstruation, symptoms of genital and urinary tract infection, obstetric and medical histories. Patients with positive urine pregnancy test, recent antibiotic therapy and history of recently treated STI and genital tuberculosis were excluded from the study.

Collection of Samples

Collection of cervical samples was performed as described in Chapter 4.

Laboratory Diagnosis

Diagnosis of other STD pathogens and presence of *C. trachomatis* using pCT PCR and DFA was performed as described in Chapter 4.

Purification and Culture of Cervical Mononuclear Cells

Cervical specimens were vortexed before the removal of cytobrush. Mononuclear cells were separated by Ficoll-Paque density gradient centrifugation. The mononuclear cells were counted on haemocytometer and samples containing less than one million cells/ml were excluded. The mononuclear cells were washed three times with PBS and suspended in RPMI-1640 containing 5% FCS. Briefly, endocervical mononuclear cells were cultured in round-bottomed 96-well plates (5×10^4 cells/well) in a total volume of 200 µl and subsequently stimulated with cHSP60 (2 µg/ml), cHSP10 (3 µg/ml) and *C. trachomatis* EBs ($5x10^5$ per ml). Optimum concentrations of antigens were determined in preliminary experiments as optimum concentrations giving maximal proliferation post stimulation.

Nucleic Acid Preparation and Real-Time Quantitative RT-PCR

Total RNA was extracted from cervical lavage cells using a RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). All RNA preparation was confirmed to be DNA-negative by PCR targeting the host actin gene in the absence of reverse transcription. The integrity of each RNA preparation was checked by analysis on ethidium bromide-stained formaldehyde-agarose gels (Gerard et al., 2002; Gerard et al., 2001). cDNA was prepared using a standard reverse transcription reaction kit. Relative mRNA levels from the cHSP60 and cHSP10 genes were assessed in cDNA preparations from *C. trachomatis*-infected cells using SYBR green-

based real-time RT-PCR assays. The primers used for cHSP60 were [(forward primer)5' -TCA CTC TAG GGC CTA AAG GAC G- 3' and (reverse primer) 5' -TCA TGT TTG TCG GCA AGC TC- 3'] as described previously [Gerard et al., 2004]. Primer sequences for cHSP10 were [(forward primer) 5' -AAG GGC AGC AAC TTC CTT TT- 3' and (reverse primer) 5'-TCG CTC ATT TGA ACG ATG AC- 3'] designed using the online software tool Primer3, based on information from the chlamydial genome sequencing project. Specificity of the primers showed that they only amplified the *C. trachomatis* genes indicated, i.e. they did not amplify other coding sequences from other organisms. All assays were performed in triplicate using a Realplex Eppendorf Cycler (Eppendorf AG, Hamburg, Germany) and the data were normalized to the levels of chlamydial 16S rRNA as described (Gerard et al., 2002; Gerard et al., 2004). Data were calculated using Realplex software (Eppendorf AG).

Flow Cytometry Analysis

Cervical epithelial cells (0.5×10^5) were analyzed by intracellular staining after permeabilizing the plasma membrane with ice-cold methanol for 10 min. Cells were then blocked with 5% BSA in PBS and 0.05% Tween-20 (PBS-T) for 1 h and incubated with anti-cHSP60 monoclonal antibody (A57-B9; Alexis Corp., Lausen, Switzerland) for 30 min on ice. After washing with PBS-T, cells were incubated with goat anti-mouse IgG-FITC (BD Biosciences, San Jose, Calif., USA). After washing with PBS-T, cells were fixed with 3.7% (vol/vol) paraformaldehyde. Cells were then co-labeled with anti-cHSP10 monoclonal antibody (serovar A/HAR-13; StressGen Biotechnologies Corp., Victoria, BC, Canada) and phycoerythrin-conjugated anti-mouse IgG (EBioscience Inc., San Diego, Calif., USA). Appropriate isotype-matched IgGs were used as negative controls to rule out nonspecific fluorescence. After a final washing with PBS, containing 2% fetal bovine serum and 0.1%

sodium azide, cell fluorescence was analyzed on a fluorescence-activated cell sorter (Calibur Flow Cytometer; BD Biosciences, San Jose, Calif., USA). 10,000 events were collected and analyzed.

Antibody Assays

Cervical washes of patients and controls were assayed for presence of IgG antibodies to cHSP60 and cHSP10 using ELISA was performed as described in Chapter 4.

Cytokine Assays using ELISA

Quantification of IFN-γ, IL-10, TNF-α, IL-13 and IL-4 in the supernatant of cervical mononuclear cell cultures of samples after 72 h stimulation with proteins was performed by commercially available ELISA kits, in accordance with the manufacturer's instructions. The minimum detectable cytokine concentrations for these assays were IFN-γ-(4 pg/ml), IL-10-(2 pg/ml), TNF-α-(4 pg/ml), IL-13-(4 pg/ml) and IL-4-(2 pg/ml).

Statistical analysis

Statistical analyses were performed with GraphPad Prism software (version 5.0). Since the distributions in cytokine production were not normal, differences between two groups were evaluated using Mann-Whitney *U* test. A comparison between two groups was made only when the Kruskal-Wallis test yielded a statistically significant result. Categorical variables were compared using the Chi-square (χ^2) test. Correlation was tested with Spearman's correlation coefficient. $P < 0.05$ was considered to be statistically significant.

Results

Study population

Cervical *C. trachomatis* infection was diagnosed by DFA/PCR in 174 patients. Thirty-one of these patients were found to have *Bacterial vaginosis*, or to be co-infected with

either *Candida spp.*, *T. vaginalis*, *M. hominis*, *U. urealyticum* or *N. gonorrhoeae* in the cervix and were thus excluded from the study. Ten *Chlamydia*-positive patients were excluded, as the count of mononuclear cells in the cervical cells was less than 1 million cells/ml and epithelial cells were present. Based on diagnosis the women were divided into three groups. Group I (n = 39) comprised of uninfected healthy controls with no infertility problem; Group II (n = 63) comprised of *Chlamydia* positive women with no infertility problem; Group III (n = 70) comprised of *Chlamydia*-positive women with infertility and who had laparoscopic or hysterosalpingographic evidence of tubal damage. Candidates were considered infertile if they had regular unprotected intercourse for at least 2 years without conception. The median ages of women in each group were comparable **(Table 7.1).**

Table 7.1: Prevalance of cHSP60 and cHSP10 specific antibodies in study population

Groups	Age Median (range)	cHSP60-IgG	cHSP10-IgG
		Number (%)	
Group I (n = 39)	25 (21-37)	3 (8)	2 (5)
Group II (n = 63)	27 (23-43)	15 (24) [a]	13 (21) [b]
Group III (n = 70)	29 (21-42)	35 (50) [c,e]	26 (37) [d,f]

(n) represents number of patients

[a]$P = 0.04$ as compared to Group I; [b]$P = 0.03$ as compared to Group I; [c]$P < 0.0001$ as compared to Group I; [d]$P < 0.0001$ as compared to Group I; [e]$P = 0.002$ as compared to Group II; [f]$P = 0.04$ as compared to Group II where, Group I-Healthy women with no infertility problem, Group II- *Chlamydia* positive women with no infertility problem, Group III- *Chlamydia* positive women with infertility, Categorical variables were compared using the $\chi 2$ test.

Relative Expression of cHSP60 and cHSP10 Genes in *C. trachomatis*-Infected Cervical Cells

To investigate whether mRNA expression of cHSP60 and cHSP10 genes might be different between fertile and infertile women, their relative transcript levels were assessed using quantitative real-time RT-PCR analysis. After normalization to the level of chlamydial 16S rRNA, a higher transcript level of both cHSP60 ($P = 0.007$) and cHSP10 ($P = 0.0006$) was found in the infertile group in comparison to the fertile group (**Figure 7.1**).

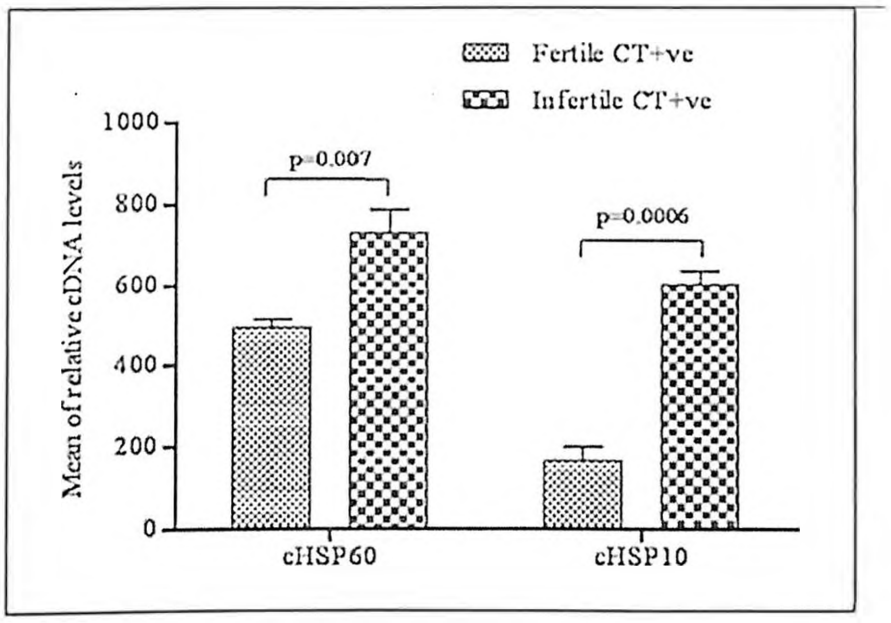


Figure 7.1: Real time RT-PCR assay targeting relative transcript levels from the cHSP60 and cHSP10 genes in RNA/cDNA prepared from CT-infected cervical epithelial cells from each of the 7 fertile and 7 infertile women. Data shown were normalized to the level of *C. trachomatis* 16S rRNA in each sample, and mean value in each group was calculated. Assays were repeated 3 times with each sample run in triplicate, and all repeats gave essentially identical results. Bars represent standard error. CT = *C. trachomatis*.

Intracellular Levels of cHSP60 and cHSP10 in *C. trachomatis*-Infected Cervical Cells

To investigate whether intracellular cHSP60 and cHSP10 were also over-expressed in infertile women, their expression level was examined by flow cytometry analysis. In contrast to mRNA levels, cervical epithelial cells collected from the *Chlamydia*-infected fertile group showed significantly higher expression of both cHSP60 ($P = 0.0006$) and cHSP10 ($P = 0.0041$) in comparison to the infertile group **(Figure 7.2)**.

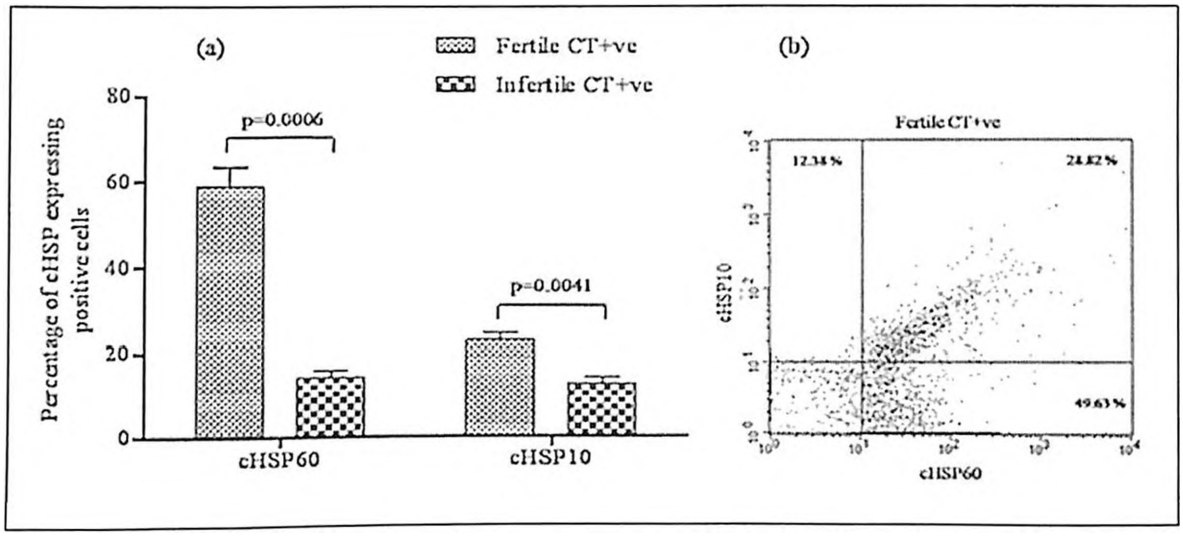


Figure 7.2: Flow cytometric assay targeting intracellular levels of cHSP60 and cHSP10 in *C. trachomatis* -infected cervical epithelial cells from each of the 7 fertile and 7 infertile women. Cells were simultaneously stained with monoclonal antibodies of cHSP60 and cHSP10 conjugated with FITC and phycoerythrin, respectively. Data shown were calculated as the mean percentage of cell population in each group. Appropriate isotype-matched control antibodies were used to rule out nonspecific fluorescence. **(a)** Percentage of cells stained with either cHSP60 or cHSP10. Bars represent standard error. **(b)** Representative figure (dot plot) showing intracellular levels of cHSP60 and cHSP10 in infected cervical epithelial cells of fertile women. CT = *C. trachomatis*.

However, when only cHSP positive cells were considered, the mean percentage of cells co-expressing both cHSP60 and cHSP10 was much higher (*P = 0.0006*) in the infertile group than in the fertile group of infected women **(Figure 7.3)**.

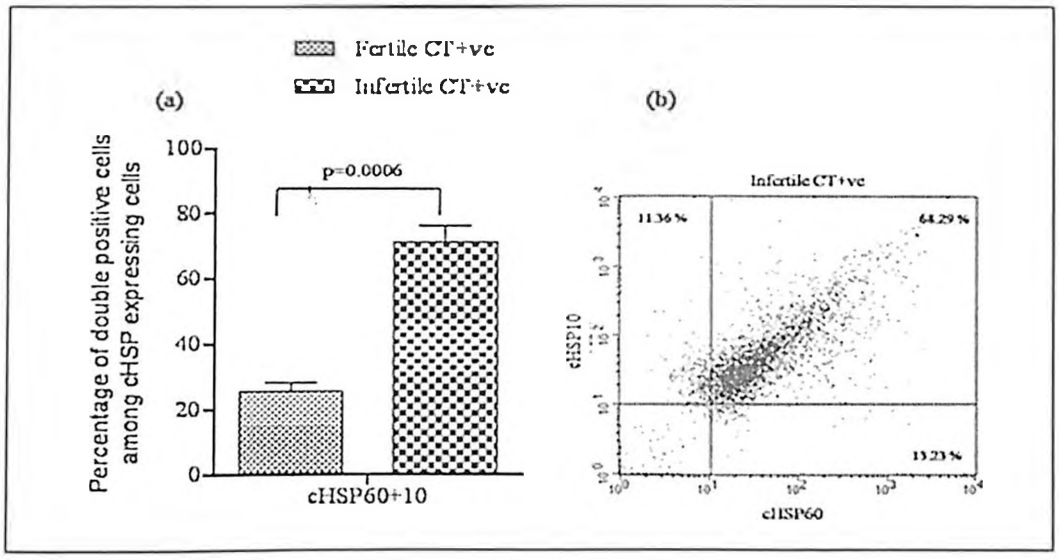


Figure 7.3: Flow cytometric assay targeting intracellular levels of cHSP60 and cHSP10 in *C. trachomatis* -infected cervical epithelial cells from each of the 7 fertile and 7 infertile women. Cells were simultaneously stained with monoclonal antibodies of cHSP60 and cHSP10 conjugated with FITC and phycoerythrin, respectively. Data shown were calculated as the mean percentage of cell population in each group. Appropriate isotype-matched control antibodies were used to rule out nonspecific fluorescence. **(a)** Percentage of double-positive cells (both cHSP60 and cHSP10) among cHSP-expressing cells. Bars represent standard error. **(b)** Representative figure (dot plot) showing intracellular levels of cHSP60 and cHSP10 in infected cervical epithelial cells of infertile women. CT = *C. trachomatis*.

Detection of Antibodies

ELISA results showed that the prevalence was significantly higher for IgG antibodies to both cHSP60 (*P = 0.04 & P < 0.0001*) and cHSP10 (*P = 0.03 & P < 0.0001*) in Group II

and III respectively as compared to Group I. In Group III the prevalence was significantly higher for IgG antibodies to both cHSP60 and cHSP10 as compared to Group II (*P = 0.002* & *P = 0.04* respectively) **(Table 7.1).** Correlation between cHSP60 and cHSP10 specific IgG antibodies were significant (*P = 0.04*, r = 0.26 & *P < 0.0001*, r = 0.6) in both Group II and Group III respectively **(Figure 7.4).**

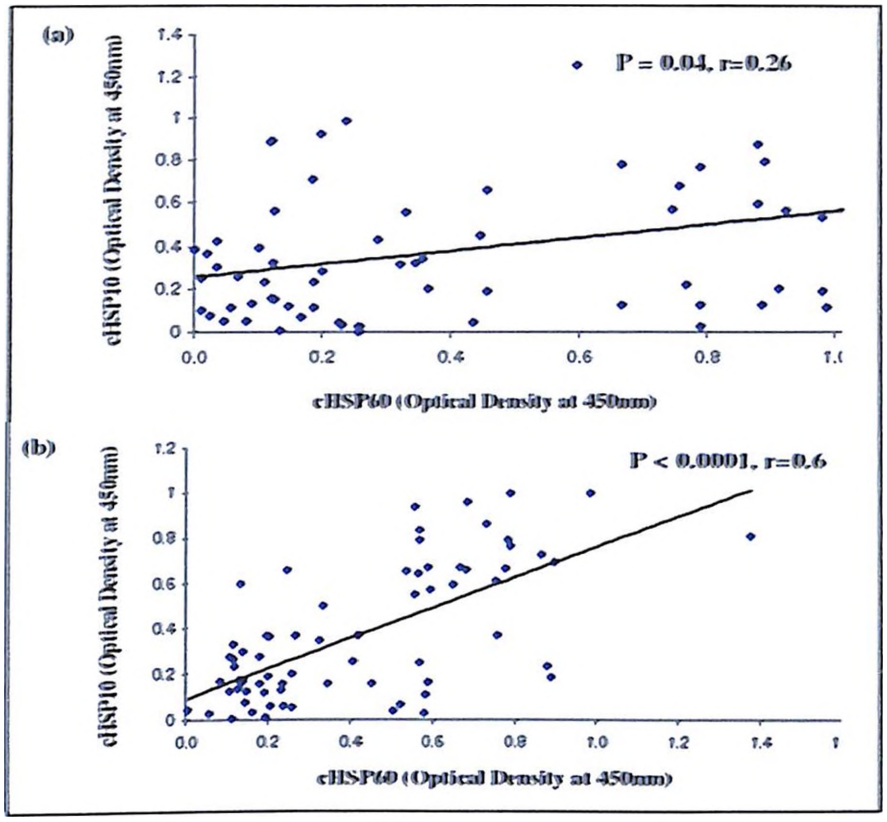


Figure 7.4: Correlation of anti-cHSP60 and anti-cHSP10 IgG antibodies in cervical washes of patients. Scatter plot showing the correlation between anti-cHSP60 and anti-cHSP10 IgG antibodies among patients groups (a) Group II and (b) Group III. A significant correlation was observed in Group II as well as Group III where, Group II (n = 63) – *Chlamydia* positive women with no infertility problem. Group III (n = 70) – *Chlamydia* positive women with infertility. Correlation was tested with Spearman's correlation coefficient.

Cytokine Production by Stimulated Cervical Mononuclear Cells

IFN-γ levels were significantly higher after stimulation with both cHSP60 cHSP10 and CT EBs in Group II (*P = 0.04, 0.02 & 0.04* respectively) and in Group III (*P < 0.0001*) as compared to Group I. Significantly higher levels of IFN-γ were observed after stimulation with both cHSP60 (*P = 0.006*) cHSP10 (*P = 0.04*) and *C. trachomatis* EBs (*P = 0.02*) in Group III when compared with Group II **(Figure 7.5a)**. Similarly when IL-10 levels were compared significant difference was observed after stimulation with both cHSP60, cHSP10 and CT EBs between Group II (*P = 0.03, 0.04* & non significantly respectively) and Group III (*P = 0.0005, 0.0007 & 0.0004* respectively) as compared to Group I and IL-10 was significantly higher (*P = 0.04*) in Group III as compared to Group II after cHSP60, cHSP10 and CT EBs stimulation **(Figure 7.5b)**. IL-13 levels were low (close to the minimum detectable cytokine concentrations) and no significant difference was observed after stimulation with both cHSP60 and cHSP10 in any group. The IL-13 levels were significantly (*P = 0.0006*) higher in Group III when stimulated with CT EBs as compared to Group I **(Figure 7.5c)**.

TNF-α levels were significantly higher after stimulation with cHSP60, cHSP10 and *C. trachomatis* EBs (*P < 0.0001, P = 0.02 & P = 0.02* respectively) in Group III as compared to Group I whereas there was no significant difference (*P = 0.07, 0.25 & 0.3* respectively) in Group II as compared to Group I. The TNF-α levels were significantly higher (*P = 0.0008*) in Group III when stimulated with cHSP60 as compared to Group II. The TNF-α levels were higher although not significant (*P = 0.1*) in Group III when stimulated with cHSP10 and CT EBs as compared to Group II **(Figure 7.5d)**.

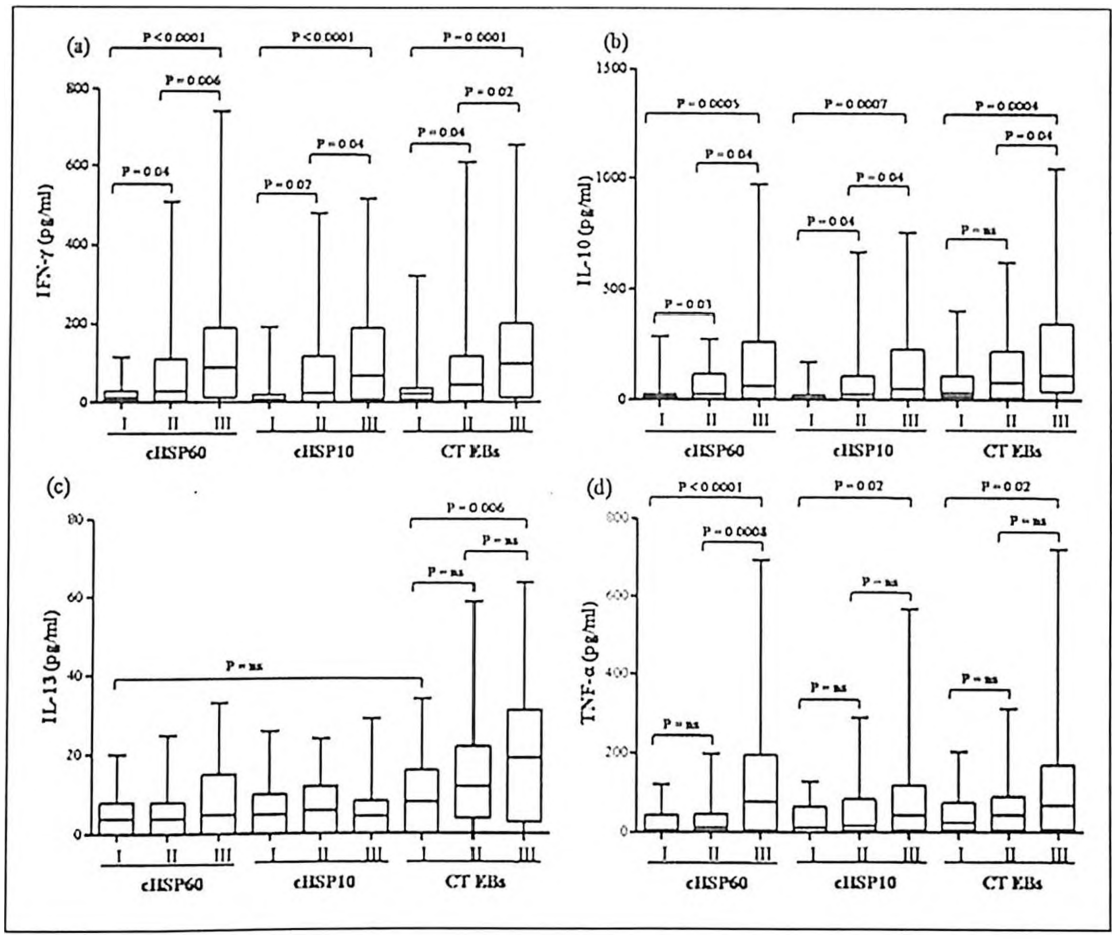


Figure 7.5: Cytokine concentrations after stimulation with cHSP60 and cHSP10: Box plot representing concentrations of (a) IFN-γ (b) IL-10 (c) IL-13 (d) TNF-α in supernatants of cervical mononuclear cells after stimulation with cHSP60, cHSP10 and CT EBs. A significant increase in levels of IFN-γ, IL-10 and TNF-α was observed after stimulation with cHSP60 and increased IFN-γ and IL-10 levels were observed after stimulation with cHSP10 in Group III as compared to Group I and Group II. The horizontal line in the middle of the box is the median value of the responses and the lower (upper) is the 25th (75th) percentile. I, II and III represent Group I, Group II and Group III respectively where, Group I (n = 39) – Healthy women with no infertility problem. Group II (n = 63) – Chlamydia positive women with no infertility problem. Group III (n = 70) – Chlamydia positive women with infertility. ns – Not significant. Mann-Whitney *U*-test was used for comparing two groups.

There was no detectable IL-4 production in any culture supernatant after stimulation with cHSP60, cHSP10 and CT EBs. IFN-γ and IL-10 levels after stimulation with both cHSP60 and cHSP10 were significantly correlated in Group III ($P < 0.0001$, r = 0.54 and $P = 0.004$, r = 0.33 respectively). Median and range for cytokine concentration in all the groups after cHSP60 and cHSP10 stimulation are mentioned in **Table 7.2**.

There was high standard error for cytokines as one or two samples showed very high concentration of cytokines. However, these high values did not affect the median value as well as the significance of the results when data was analyzed excluding these values.

Table 7.2: Concentration (pg/ml) of cHSP60 and cHSP10 specific cytokines in different groups.

Groups	IFN-γ			IL-10			IL-13			TNF-α		
	cHSP60	cHSP10	CT EBs	cHSP60	cHSP10	CT EBs	cHSP60	cHSP10	CT EBs	cHSP60	cHSP10	CT EBs
	Median (range) in pg/ml											
Group I (n = 39)	11 (0-115)	8 (0-193)	20 (0-321)	15 (0-289)	8 (0-174)	36 (0-404)	4 (0-20)	5 (0-26)	8 (0-34)	6 (0-121)	14 (0-128)	26 (0-203)
Group II (n = 63)	28 (0-508)	24 (0-480)	44 (0-611)	27 (0-278)	26 (0-671)	81 (0-623)	4 (0-25)	6 (0-24)	12 (0-59)	14 (0-199)	19 (0-289)	44 (0-311)
Group III (n = 70)	90 (0-743)	67 (0-516)	98 (0-655)	63 (0-973)	52 (0-758)	113 (0-1044)	5 (0-33)	4.5 (0-29)	19 (0-64)	77 (0-694)	45 (0-566)	69 (0-722)

(n) represents number of patients

Group I-Healthy women with no infertility problem

Group II-Chlamydia positive women with no infertility problem

Group III-Chlamydia positive women with infertility

Discussion

Disease stages developed upon infection with *Chlamydia* are mediated by the immune responses. Immunity to cHSP60 and cHSP10 is associated more typically with the chronic upper genital tract infection than it is with acute infection of the lower genital tract (Arno et al., 1995; LaVerda et al., 2000). To provide some insight regarding the role of cHSP60 and cHSP10 in the inflammatory process of the genital tract leading to infertility, cHSP60 and cHSP10 mRNA and protein expression were examined in the cervical cells of *C. trachomatis*-infected fertile and infertile women. The transcript levels of cHSP60 and cHSP10 genes were significantly higher in the infertile group than in the fertile group of women, whereas the percentages of cells expressing cHSP60 or cHSP10 were higher in the fertile group than in the infertile group of women. These discrepancies between mRNA and protein levels may be explained if both groups of patients differed in their chlamydial metabolic activity. Additionally, quantification in each sample was done by expressing the ratio of the transcript levels of cHSP60 and cHSP10 versus the level of 16S rRNA used as an internal standard transcript, which is one of the most prominent genes expressed when elementary bodies transform into actively replicating reticulate bodies (An et al., 1992; Burton et al., 2006; Engel and Ganem, 1987; Mathews et al., 1999). In contrast, the 16S rRNA level is low in persistent forms of *Chlamydiae* known to be nonreplicative or to replicate slowly (Rottenberg et al., 2002). Therefore, differences in the transcript levels of cHSP60 and cHSP10 between fertile and infertile groups of women were probably accentuated if *C. trachomatis* was actively replicating in the fertile women but was in a persistent form in the infertile women.

The presence of an abnormal cryptic form of *C. trachomatis* in the infertile women might also explain their lower number of cHSP60- and cHSP10-positive cells. Indeed, models of persistent chlamydial infection have demonstrated the release of cHSP60 into the extracellular milieu (Equils et al., 2006). The fact that the fertile group had no clinical history of past infection whereas the infertile group of women had a clinical history of past infection for more than 3 years supports the hypothesis of a persistent chlamydial infection in the infertile women. This hypothesis is also supported by earlier studies in our laboratory reporting an increased level of IFN-γ in the genital tract (Reddy et al., 2004) and an elevated release of IFN-γ by cervical mononuclear cells upon exposure to cHSP60 and cHSP10 in infertile women infected with *C. trachomatis*, thereby mediating chlamydial persistence (Beatty et al., 1994a).

The percentage of cells co-expressing both cHSP60 and cHSP10 was determined because antibodies to both cHSP60 and cHSP10 were found to be more common in the female partners of subfertile couples (Karinen et al., 2004), and their combined detection was reported to be a very specific predictor of *C. trachomatis*-associated infertility (Dadamessi et al., 2005). Results showed the percentage of cervical epithelial cells co-expressing both cHSP60 and cHSP10 to be significantly higher in the infertile women than in the fertile women, agreeing with the findings above as well as with those of our previous study showing a higher cHSP60- and cHSP10-specific IgG antibody response in the cervical washes of the infertile group as compared to the fertile group (Agrawal et al., 2007). The exact mechanisms whereby HSPs contribute to infertility and problems in reproduction are not known. However, cHSP60 has been shown to induce apoptosis in primary human trophoblasts and

placental fibroblasts, which could provide a mechanism of pathogenesis for poor fertility and pregnancy outcome in women with persistent chlamydial infection (Equils et al., 2006).

In this study, higher cHSP60 and cHSP10 specific IgG antibody responses in the cervical washes of infertile group were detected as compared to fertile group. These results were consistent with the previous reports where recurrent infection group showed high prevalence of cHSP specific IgG and IgA antibodies (Agrawal et al., 2007). Further, significant correlation of cHSP60 and cHSP10 IgG antibodies suggests that co-expression of cHSP60 and cHSP10 occurs at the site of infection too. This observation again adds to the previous reports suggesting cHSP10 is co-expressed with cHSP60 (Morrison et al., 1990). Also, cHSP60 and cHSP10 specific cytokines in cervical mononuclear cells were evaluated and it was found that IL-10 levels were more prominent when stimulated with both cHSP60 and cHSP10 in the infertility group as compared with other groups. Our data is consistent with the previous studies in which cHSP60 specific higher IL-10 levels in PBMCs have been reported in infertile women (Kinnunen et al., 2003). Cohen *et. al.* had recently demonstrated that cHSP60 specific IL-10 production by PBMCs act as a risk factor for *C. trachomatis* infection in humans (Cohen et al., 2005). The enhanced levels of IL-10 may not act as anti-inflammatory mediator and might be involved in prolonging the infection by exerting immunostimulatory effects (Conti et al., 2003). Overall, these results allow hypothesizing the role of IL-10 in fibrosis and tubal infertility. Indeed, IL-10 has been earlier shown to be involved in fibrosis in several human diseases (Martinez et al., 1997; Sato et al., 2001; Yang et al., 1999). Further, an association of fibrosis with cHSP60 and cHSP10 specific antibodies has been reported in infected animals (Higgins et al., 2005).

Furthermore, higher levels of both the cytokines IFN-γ and TNF-α were detected in infertility group than in fertile group after stimulation with cHSP60 and cHSP10. IFN-γ production has been identified as one of the main factors in protective immunity (Wang et al., 1999) and is also important in the development of chronic chlamydial infection (Beatty et al., 1994b). IFN-γ delays the developmental cycle of *Chlamydia* so that chlamydial RBs persist longer, which might result in persistent unapparent infection and also, play a role in immunopathogenesis by promoting inflammatory damage and fibrosis (Rottenberg et al., 2002). In addition, increased levels of IFN-γ have been reported in the endocervical secretions of *C. trachomatis* positive infertile women (Reddy et al., 2004). TNF-α which displays anti-chlamydial properties are also known to play an important role in the initiation of inflammatory response (Shemer-Avni et al., 1988). In the mouse genital tract, infertility associated with endometriosis has been shown to be related to the production of TNF-α (Darville et al., 2000). In human, both IFN-γ and TNF-α have been reported to be associated with infertility (Kwak-Kim et al., 2003; Naz et al., 1995; Ng et al., 2002). Proinflammatory cytokines are also known to drive the lipid peroxidation of the spermatozoa plasma membrane to levels that can affect the sperm fertility capacity (Martinez et al., 2007). In addition TNF-α and IFN-γ have effects on sperm motility, viability, membrane integrity and lateral head displacement, suggesting poor fertilizing potential of human spermatozoa during inflammatory conditions (Estrada et al., 1997). Previous evidence suggests that the concurrent immunization with cHSP60 switches the cytokine production of hHSP60 responding T cells to dominant production of proinflammatory IFN-γ (Yi et al., 1997) showing that cHSP60 can break the tolerance of autoreactive cell reactions and lead them to participate in the inflammatory reactions during chlamydial disease. Hence from the present

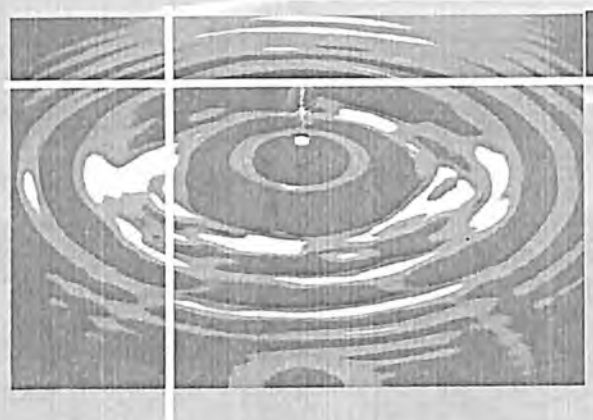
study higher levels of both IFN-γ and TNF-α by cHSPs in infertility group may suggest their involvement in the immunopathological condition associated with the infertility.

In this study, IL-4 was undetectable and IL-13 levels were not significantly different in any group in contrast to previous report in which cHSP60 specific IL-13 in PBMCs has been reported to be associated with the protective response (Cohen et al., 2005). It may suggest that cHSP specific Th2 cytokines (IL-4 and IL-13) does not play any role in pathogenesis related to chlamydial infection.

The higher levels of IL-10 may not suggest a Th2 response as studies showed that it is secreted by Th1 and Th2 cells as well as other cells (Trinchieri, 2007; Yssel et al., 1992). Further, it has been shown that in many chronic infections in human and experimental animals, $CD4^+$ T cells can produce high levels of both IL-10 and IFN-γ (Trinchieri, 2001). Hence the production of high levels of both the cytokines, IL-10 and IFN-γ in the absence of significant levels of other Th2 cytokines, suggests that the cells secreting IL-10 are not Th2 cells but other cells. Previous studies, suggested that immune sensitization to HSPs probably require prolonged exposure of them at elevated concentrations (Witkin et al., 1998a). As for cHSP60, there have been reports that during repeated and severe *C. trachomatis* infection there is enhanced recognition of cHSP60 by circulating lymphocytes (Witkin et al., 1993; Witkin et al., 1994) and it has been shown that PBMCs from women with tubal factor infertility responded more frequently to cHSP60 antigen (Kinnunen et al., 2003).

Hence the differential responses to cHSPs in *Chlamydia*-infected fertile and infertile women would be due to prolonged exposure to cHSPs in infertile cases. The correlation analysis of different cytokines produced upon stimulation with cHSP60 and cHSP10 and was observed a positive correlation for IL-10 and IFN-γ levels in infertility group suggesting

similar role of cHSP10 in pathology associated with the infertility as cHSP60. There are no studies to date on cHSP10 specific CMI responses and these data suggests that the cHSP10 specific immune responses may have crucial role in the immunopathological condition associated with the infertility. Reports on the immunogenicity of HSP10 antigens from other microbial pathogens suggest that the HSP10 family of proteins are capable of eliciting chronic inflammation and delayed hypersensitivity. In particular, HSP10 homologues of *Mycobacterium leprae* and *Mycobacterium tuberculosis* have been shown to stimulate T-cell responses (Launois et al., 1995; Mehra et al., 1992). Overall these results suggest that exposure to the cHSPs could significantly affect mucosal immune function by modifying the release of cytokines leading to severe immunopathological conditions related to infertility.



Chapter 8
Role of Chlamydial Heat Shock Proteins 60 and 10 in the apoptosis of cervical epithelial cells

Role of Chlamydial Heat Shock Proteins 60 and 10 in the Apoptosis of Cervical Epithelial Cells

Introduction

Chlamydiae are strict intracellular pathogens with a biphasic life cycle; the extracellular infectious form is the EB, which is metabolically inert. EBs infect susceptible host cells and transform into the RBs, which are the vegetative form of the organism, capable of metabolic activities, replicate intracellular within a membrane-enclosed vacuole called an inclusion and differentiate back to EBs (Moulder, 1991). Host-cell death observed at the end of the infection cycle is involved in release of EBs from the host-cell and contributes to the inflammatory response of the host (Hogquist et al., 1991). Apoptosis, or programmed cell death, is the mechanism for cellular self-destruction that functions to eliminate cells during immune selection, tissue development, and tissue regeneration (Wyllie AH 1980). Activation of human host cell apoptosis has been observed as a response to infection by a wide range of intracellular and extracellular pathogens (Grassme et al., 2001; Weinrauch and Zychlinsky, 1999). It has also been demonstrated that apoptosis induction in some bacterial infections can limit the spread of intracellular infection by provoking inflammatory responses or by delivering the intracellular pathogen to professional phagocytes (Fratazzi et al., 1997; Gao and Kwaik, 2000; Hacker and Fischer, 2002).

Chlamydiae have been reported to elicit the induction of host cell death under some circumstances and also actively inhibit apoptosis under others (Byrne and Ojcius, 2004). Several strains of *C. trachomatis* protect infected cells against apoptosis due to external ligands, including TNF-α, antibodies against Fas, and the kinase inhibitor staurosporine (Dean and Powers, 2001; Fan et al., 1998). However, epithelial cells that are preferential

target of *Chlamydia* display features of apoptosis during infection. Morphological changes associated with apoptosis such as chromatin condensation, blebbing and DNA fragmentation have been observed in infected cells confirming the apoptotic nature of host cell death (Ojcius et al., 1998).

Although, an inflammatory response is required for the resolution of primary *C. trachomatis* infection, on the other hand chronic inflammation is also responsible for the scarring process in trachoma and female genital tract. Thus, a number of inflammatory mediators are involved during infection, including IL-1β and TNF-α. TNF-α and other inflammatory cytokines which may aid in eradicating *Chlamydia* infection and also promote long-term tissue damage (Darville et al., 1997). It has been reported that human monocytes infected with *Chlamydia* secrete IL-1β. Caspase-1 plays a role in the maturation of IL-1β and IL-18, and has been shown to have no effect on *Chlamydia*-induced cell death, suggesting that caspase-1 is activated during infection (Los et al., 1999).

Chlamydial factor, cHSP60 has been shown to induce apoptosis, in vitro, in primary human trophoblasts, placental fibroblasts, and in JEG3 trophoblast cell line (Equils et al., 2006). Chlamydial HSPs are expressed constitutively throughout the life cycle and can be found in the outer membrane complexes of elementary and reticulate bodies. Chlamydial HSP60 has been reported to be responsible for proinflammatory pathologic manifestations of human chlamydial disease in the reproductive tract (LaVerda et al., 1999; Neuer et al., 1997; Neuer et al., 2000). Recently, cHSP60 has been identified as an immunodominant antigen being strong target in both humoral and cell mediated immunity whereas cHSP10 has been identified as human T-cell target (Follmann et al., 2008). In addition, serum and follicular fluid antibodies to cHSP60 has been associated with the development of immunopathology in

chlamydial infection, leading to blinding trachoma, PID, ectopic pregnancy, and tubal infertility following ocular and genital *C. trachomatis* infections (Arno et al., 1995; Ault et al., 1998; Brunham et al., 1985; Brunham et al., 1992; Claman et al., 1997; Cortinas et al., 2004; den Hartog et al., 2005; Eckert et al., 1997; Freidank et al., 1995; Kimani et al., 1996; Peeling et al., 1997; Toye et al., 1993). Serum antibodies to cHSP10 has also been associated with severity of human genital tract disease (Betsou et al., 2003; Betsou et al., 1999; LaVerda et al., 2000) and immune reactivity to cHSP10 has been significantly associated with tubal infertility (LaVerda et al., 1999).

There are number of studies that have been devoted on cell-mediated and humoral immune responses to cHSP60 and cHSP10, however, no study has been dedicated to their potential role in apoptosis of primary cervical epithelial cells that are privilege target for chlamydial infection. The fact that higher levels of cHSP60 and cHSP10 mRNA and proteins were detected in cervical epithelial cells of *C. trachomatis* infected infertile women than in fertile women might be related to different metabolic state of *Chlamydia* between infertile and fertile women and to different levels of apoptosis of endocervical epithelial cells (Jha et al., 2009). Therefore to assess their effect in apoptosis of primary cervical epithelial cells recombinant cHSP60 and cHSP10 were used. Quantitative measurement of apoptosis was performed by cytofluorometry. An apoptosis pathway-specific cDNA microarray analysis was used to examine the expression of epithelial cell genes that might be affected by cHSP60 and cHSP10. The upregulation of genes of interest was confirmed in real-time RT-PCR analysis.

Materials

Agarose, Bovine Serum Albumin, Glycine, Nonidet P-40, NaCl, SDS, Na_3VO_4, NaF, Glutamine, HEPES, Tris base, RPMI-1640, Penicillin, Streptomycin, Ponceau S, Phenyl methyl sulfonyl fluoride (PMSF), Diaminobenzamide, Coomassie, and other fine chemicals were purchased from Sigma Chemicals, USA. Trizol, Taq polymerase, dNTPs, $MgCl_2$, DNA ladder was from Invitrogen, USA. Primers for IL-18, IL-1β, TLR2, TLR4 and β-actin were synthesized by MWG-Biotech, Germany. Radiolabelled [α-^{33}P] dATP was procured from Board of Radiation and Isotope Technology (BRIT), Mumbai, India.

Experimental Methods

Culture of primary epithelial cells

Epithelial cells were obtained, after informed consent, from 15 healthy women attending the gynecology OPD of Safdarjung Hospital (New Delhi, India) who were referred for diagnostic laparoscopy undergoing routine checkup for reasons unrelated to this study. The Hospital's ethics review committee approved the study. At recruitment, a detailed clinical questionnaire was administered to each patient to collect information on reasons for referral and obstetric, medical and gynecological history (including menstruation and symptoms of genital and urinary tract infection). An exclusion criterion consisted of: - (i) occurrence of sexually transmitted disease during the last year and (ii) history of pelvic inflammatory disease. The cervical canal was wiped clean with cotton tipped swab and a cytobrush was placed within the endocervical canal and rotated one 360° turn so that cells from the endocervical region and the zone between the endocervical and ectocervical regions (transformation zone) could be obtained. The cytobrush was then placed in a sterile centrifuge tube containing PBS with penicillin (100 U/ml), streptomycin (100 µg/ml) and

glutamine (100 μg/ml). All cytobrush samples had negative results for blood contamination. No cervical specimens were taken from menstruating patients, or if blood was visible in the cervical area or if the epithelium appeared disrupted. Diagnosis of *C. trachomatis* and other STD pathogens were done as mentioned in Chapter 4.

Processing of Samples

Cervical samples were centrifuged at 200 *g* for 10 min at 4° C, with the resulting pellet yielding cervical epithelial cells. The epithelial cells were washed twice with PBS. To check the viability of the cells Trypan-blue staining was performed on a hemocytometer. The final pellet was resuspended in RPMI medium containing 10% (v/v) FCS, 1 mM glutamine and 1 mM pyruvate. The cells were seeded into 24-well tissue culture plates and incubated at 37°C in an atmosphere of 5% (v/v) CO_2 in air to allow the rapid attachment of any contaminating fibroblasts. After incubation for 1 h, the medium containing non-attached cells was removed and seeded into another plate. These epithelial cells (5 x 10^6 cells) were incubated for 3 days to reach 80-90% confluence in order to avoid contact inhibition of the cells. The epithelial cells were confirmed by staining with a cytokeratin-specific antibody (ICN pharmaceuticals, Costa Mesa, Calif., USA) and the absence of leucocytes was verified by the lack of staining with an antibody specific for the leukocyte common antigen, CD45 (Dako Corp., Carpinteria, Calif., USA). Further epithelial cells were stimulated with cHSP60 (5 μg/ml) and cHSP10 (5 μg/ml) along with unstimulated control cells. Cells were harvested after 4 h for RNA isolation and cDNA preparation. Approximately 0.5 x 10^6 cells were fixed in methanol for flow cytometric analysis and the rest of the cells in equal numbers were processed for nucleic acid preparation and western blot assay.

Cloning, Expression and Purification of cHSP60 and cHSP10

Cloning and expression of cHSP60 and cHSP10 have been previously described in Chapter 6.

Quantitative assessment of cell death and apoptosis using cytofluorometry

Cervical cells were analyzed for apoptotic effect of cHSP60 and cHSP10 stimulation. Cells were stained using Propidium Iodide (PI) which stains dead cells (necrotic cells) and FITC-labeled annexin V (Roche Molecular Biochemicals, Germany), which stains apoptotic cells. Cells were incubated with medium and 5 μg/ml of cHSP60 or cHSP10 for 4 h. Labeling procedures followed were those suggested as per manufacturer's manual. Briefly, cells were resuspended in annexin labeling solution containing 10 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM $CaCl_2$, and FITC-conjugated annexin V for 15 min. After washing twice with PBS, cell pellets were resuspended in PI (2 μg/ml) containing PBS and analyzed by flow cytometry. At least 10,000 events were analyzed, and apoptosis was presented as mean percentage positive cells stained with annexin V.

Complementary DNA Microarray

Atlas human apoptosis array (Clontech, Palo Alto, Calif.) containing 205 immobilized human cDNAs, housekeeping and negative control cDNAs, in duplicate dots on nylon membranes, were used to detect apoptosis genes. Labeled cDNA probes were prepared with reagents provided with the Atlas Human Array kit (Clontech Laboratories, Palo Alto, CA, USA). For each specimen, 2.5 μg of total RNA was incubated with Human Apoptosis-Specific Primers (R&D Systems, Minneapolis, MN, USA) for 5 min at 65°C and then at 42°C. A mixture of reaction buffer, dNTP mix, 50 U of Moloney murine leukemia virus reverse transcriptase and [α-^{33}P] dATP (>2,500 Ci/mmol, 10 μCi/μl) (BRIT, Mumbai, India)

was added to each sample, which was then incubated at 42°C for 60 min. The labeled cDNA probes were purified with column chromatography to remove unincorporated isotope using spin columns provided with Atlas pure total RNA labeling system (Clontech). C_0t-1 DNA was added to the labeled probe to reduce background hybridization to repetitive DNA sequences prior to the addition of the probe to the hybridization solution. A nylon membrane containing bound cDNA clones corresponding to 205 different human genes was prehybridized with a solution of hybridization buffer (ExpressHyb; Clontech) and 100 μg of sheared salmon testes DNA/ml (previously boiled for 5 min and chilled on ice prior to addition to the prehybridization solution) at 68°C. Each labeled cDNA probe was mixed into prehybridization buffer and incubated overnight at 68°C with the membrane. After hybridization, the membrane was washed with wash solution 1 (2×standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS)) and wash solution 2 (0.1×SSC, 0.1% SDS) at 68°C followed by a final wash of 2×SSC at room temperature. The washed membrane was wrapped in plastic wrap and exposed to a phosphor imaging screen. Imaging screens were scanned by use of a PhosphorImager (Typhoon 9700, Amersham, Uppasala, Sweden) and analyzed with ImageQuant software (GE Healthcare). The signals on each array were corrected for background with an average for blank columns and standardized with a housekeeping gene present on the same membrane (glyceraldehyde phosphate dehydrogenase). The duplicated intensity signals for each gene were summed for data analysis. Each experiment was performed in triplicate and the ratio of gene expression levels was determined by dividing the signal intensity on the control array. Differential gene expression was considered significant when the signal ratio was greater than 2:1. The

complete gene list for the Atlas human apoptosis cDNA expression array may be viewed at www.bdbiosciences.com/clontech/atlas/genelists/index.shtml.

Preparation of total RNA and preparation of cDNA probes

Total RNA was extracted from epithelial cells using Trizol reagent (Life Technologies Corp., California, USA). All RNA preparation was confirmed to be DNA-negative by PCR targeting the host actin gene in the absence of reverse transcription. The integrity of each RNA preparation was checked by analysis on ethidium bromide-stained formaldehyde-agarose gels (Gerard et al., 2002; Gerard et al., 2001). The concentration of the extracted total RNA was assessed using spectrophotometry. Next, the total RNA was treated with DNase Free™ reagent (Ambion, Austin, TX, USA) for 60 min, and then reverse-transcribed with Superscript II™ (Invitrogen, Carlsbad, CA, USA) at 37°C for 60 min using Random Primer™ (TaKaRa, Tokyo, Japan). The resultant cDNA mixture was stored in small aliquots at –20°C until further use.

Real-Time reverse transcriptase PCR

The SYBR green real-time RT-PCR was performed in RT^2 Profiler PCR Array System (SABiosciences Corporation, Frederick, USA) that contains a panel of 96-primer sets for thoroughly researched set of 84 apoptosis related genes, five housekeeping genes and three RNA and PCR quality control. Briefly, 2.5 μg of RNA was used to convert cDNA using first strand synthesis kit (Fermenta International Inc., Canada) followed by mixed with 2X SABiosciences RT^2 qPCR Mastermix provided with kit and 25 μl of mixture was added in each well. RT-PCR was performed on Eppendorf Mastercycler ep realplex using program consisted of 10 min of denaturation at 95°C followed by 15 sec at 95°C and 1 min at 60°C for 40 cycles and final extension of 5 min. at 72° C. Threshold cycle (Ct) for each well was

calculated using instrument software. Data was analyzed using PCR array data analysis web portal from the address: http://www.SABiosciences.com/pcrarraydataanalysis.php. In brief, the expression level of housekeeping genes was chosen for normalization in each plate. ΔCt of each gene of interest was calculated by subtracting average of Ct value of housekeeping genes using formula: $\Delta Ct=Ct^{GOI}-Ct^{AVG\ HKG}$. Further, ΔΔCt was calculated using the formula: ΔΔCt=ΔCt (group II) – ΔCt (group I), where group I is the control and group II is experimental. Finally, fold change ($2^{-\Delta\Delta Ct}$) in gene expression was calculated by dividing normalized expression of the gene of interest in the experimental sample to the normalized expression of same gene of interest in the control sample. The result was reported as mean fold changes in gene expression compared to housekeeping gene if the fold change is greater than 2.

Immunoblotting of proteins

Cells stimulated with cHSP60 and cHSP10 were washed with PBS and subsequently treated with lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 0.1% SDS, 50 mM NaF, 1 mM Na_3VO_4, and 1 mM phenyl methyl sulfonyl fluoride) containing the complete protease-inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined by the Bradford protein assay (BioRad Laboratories, USA) with BSA as standard. Extracted proteins (50 μg) were electrophoresed on 8–12% SDS-polyacrylamide gels and transferred to polyvinyl difluoride membranes (BioRad, USA); the membranes were reversibly stained with Ponceau S (Sigma Aldrich, St Louis, USA) to confirm complete transfer. Membranes were blocked with 5% nonfat dry milk in PBS-Tween-20 and incubated with mouse anti-IgGs against Nuclear Factor- kappaB (NF-κB) (p65), IκB (inhibitor of kappa B) (Alexis Biochemicals) and rabbit anti-IgG against caspase-3, Caspase-8, Caspase-9 (Santa

Cruz Biotechnology, USA). They were further incubated with the respective secondary IgG conjugated with horseradish peroxidase. Subsequently, blots were developed using diaminobenzamide as the detection agent and analyzed using the Image J software (http://rsbweb.nih.gov/ij/index.html).

Analysis of mRNA expression using semiquantitative RT-PCR

Total RNA and cDNA prepared for microarray and real-time RT-PCR were used for amplification of IL-1β (443 bp), IL-18 (279 bp) and β-actin (235 bp) gene. Primer sequences for RT-PCR were as follows: IL-1β (forward) 5'- GCT GAT GGC CCT AAA CAG ATG-3', IL-1β (reverse) 5'- ACG AAT CTC CGA CCA CCA CTA-3' (de Gruijl et al., 1999); IL-18 (forward) 5'-GCT TGA ATC TAA ATT ATC AGT C-3' and IL-18 (reverse) 5'-GAA GAT TCA AAT TGC ATC TTA T-3' (Pizarro et al., 1999); TLR-2 (forward) 5'-AGG CTG CAT TCC CAA GAC ACT-3' and TLR-2 (reverse) 5'-AGC CAG GCC CAC ATC ATT TT-3', TLR-4 (forward) 5'-TGG TGT CCC AGC ACT TCA TCC-3' and TLR-4 (reverse) 5'-TTC CTG CCA ATT GCA TCC TGT A-3' (Sasu et al., 2001); β-actin (forward) 5'-CCA ACC GCG AGA AGA TGA CC-3', and β-actin (reverse) 5'-GAT CTT CAT GAG GTA GTC AGT-3' (Jobin et al., 1997). Sequences for PCR primers specific genes of interest were synthesized by (MWG-Biotech AG, Ebersberg, Germany); 2 µl of cDNA was used as the template for individual PCRs with pairs of gene-specific primers. Each PCR mixture on contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 0.5 mM each primer, and 1.5 U of DNA polymerase (Invitrogen). Thermal cycling programs consisted of 10 min of denaturation at 95°C, followed by 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C for 23 cycles and a final extension of 5 min at 72°C. PCR products were analyzed by electrophoresis through 2%

agarose gels and visualized by ethidium bromide staining. RT-PCR data were analyzed by scanning densitometry of gel bands and normalized to β-actin signals obtained from the same time point. The normalized data were expressed as relative changes in mRNA levels between cHSP60 and cHSP10 stimulated cells and unstimulated controls. The numerical data were analyzed using a two-tailed Student *t* test. A p value of, 0.05 was considered significant.

Results

Apoptotic effect of cHSP60 and cHSP10 stimulation of endocervical epithelial cells

We first examined whether cHSP60 and cHSP10 elicited an apoptotic effect on endocervical epithelial cells. Cells were incubated with medium alone (control) or with 5 μg/ml of cHSP60 and cHSP10 for 4 h. Cell death was assessed by staining with PI and FITC-labelled annexin V to detect necrotic and apoptotic cells respectively, using flow cytometry. Treatment with cHSP60 significantly increased the mean percentage of apoptotic cells (57.4 ± 5.9 % vs 9.3 ± 1.2 % in control cells, $p < 0.05$). Similarly, treatment with cHSP10 significantly increased the mean percentage of apoptotic cells (47.8 ± 4.8 % vs 9.3 ± 1.2 % in control cells, $P < 0.05$) **(Figure 8.1a)**. No significant difference in necrotic cell death (PI positive cells) was observed in cHSPs stimulated cells as compared to control cells **(Figure 8.1b).**

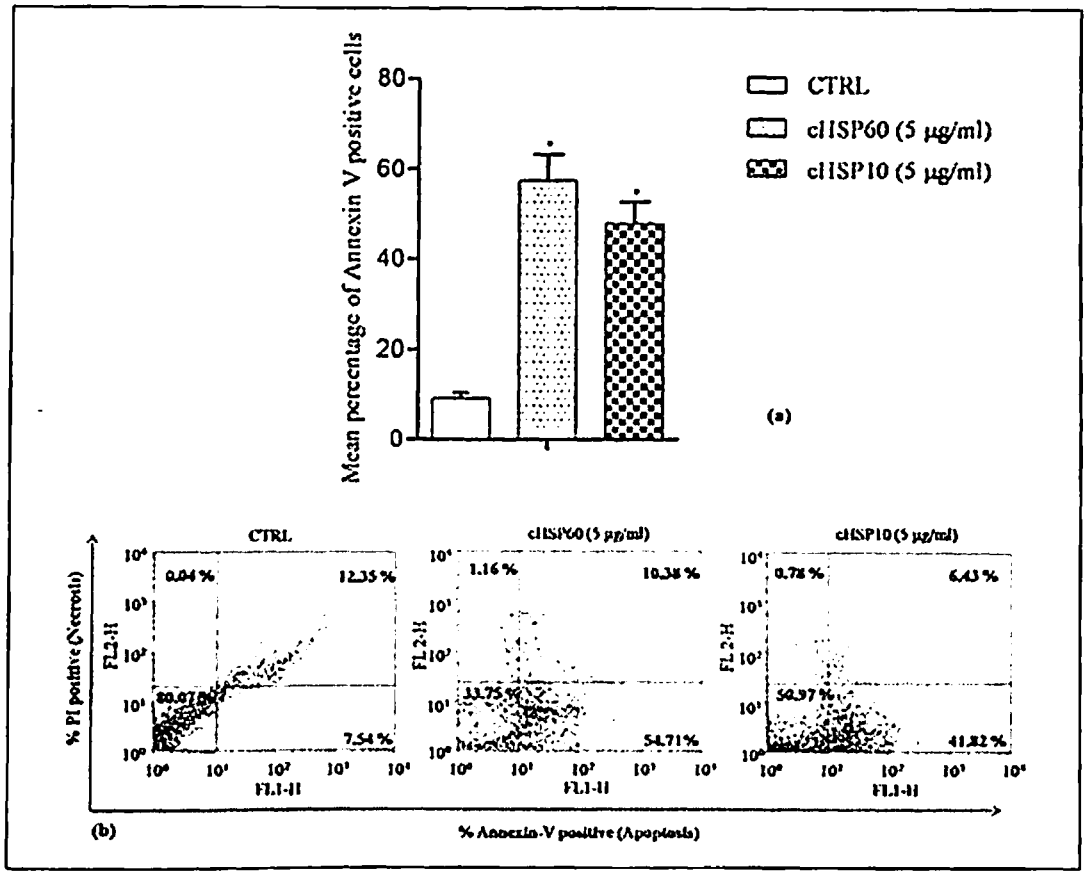


Figure 8.1: Detection of apoptosis by flow cytometry in *in vitro* stimulated epithelial cells. (a) Apoptosis was detected by PI and FITC-labeled Annexin V staining assay in 4 h cultures of primary epithelial cells in the presence of 5 µg/ml cHSP60 or cHSP10. Each column denotes the mean percentage and SEM (error bar) from experiments carried out in triplicate with cervix epithelial cells derived from 3 donors. * $P < 0.05$; Kruskal-Wallis test. (b) Figures (dot plot) shown are representative of three independent experiments.

Effect of cHSP60 and cHSP10 stimulation on mRNA expression (cDNA microarray)

To study the changes in mRNA expression of genes related to apoptosis in epithelial cells, cDNA microarray approach was done using the Clontech atlas human apoptosis expression array (205 different human genes related to apoptosis). In cHSP60 stimulated cells, 10 genes were significantly ($P < 0.05$) upregulated and 6 genes were significantly ($P <$

0.05) downregulated. Similarly, in cHSP10 stimulated cells, 11 genes were significantly ($P < 0.05$) upregulated and 4 genes were significantly ($P < 0.05$) downregulated. The fold change expression of genes and their accession numbers are listed in **Table 8.1**.

Table 8.1: Modulation of gene expression in epithelial cells after stimulation with cHSP60 and cHSP10 for 4 h.

S. No.	Gene Description	Accession No.	cHSP60	cHSP10
1	Cyclin dependent kinse 4	M14505	(-)8.3±0.32	(-)5.2±0.46
2	Cyclin-B1	M25753	(-)17.1±0.19	(-)4.6±0.24
3	Phospholipase D1	U38545	3.8±0.62	9.2±0.57
4	ERK-1	X60188	4.6±0.44	5.4±0.19
5	MAPK Kinase kinase 3	U78876	9.3±1.2	3.2±0.41
6	PCNA	M15796	(-)2.4±0.22	Unchanged
7	E2F transcription factor	M96577	(-)3.9±0.61	Unchanged
8	P53 induced protein	AF010315	4.1±0.2	Unchanged
9	Bcl-2 antagonist of cell death	U66879	Unchanged	3.1±0.12
10	IL-1 beta convertase	U13699	11.8±1.1	8.3±1.3
11	Caspase-3	U13737	4.5±0.64	5.9±0.22
12	Caspase-8	U60520	3.3±0.23	6.4±0.67
13	Caspase-9	U56390	7.1±0.19	4.6±0.32
14	TNF-receptor associated factor-6	U78798	3.1±0.91	5.7±0.81
15	Caspase-8 & FAAD-like apoptosis regulator	AF010127	2.2±0.33	9.7±0.41
16	TNF superfamily member 6	Z70519	Unchanged	1.8±0.11
17	TNF receptor family member 1B	M32315	(-)14.3± 1.3	(-)7.2± 0.82
18	TNF receptor family member 10B	AF016268	(-)2.8±0.41	(-)6.6± 0.36

Note: The expression of genes was analyzed using an apoptosis cDNA expression array from Clontech containing 205 immobilized human cDNAs. Values in columns cHSP60 and cHSP10 represent normalized fold change ± standard deviation after stimulation as compared to unstimulated control, (-) indicates fold down regulation and rest are fold upregulated genes.

Analysis of mRNA expression using real-time RT-PCR

To validate the data obtained using the human apoptotic cDNA microarray and to further characterize mRNA expression profile, few genes of interest were subsequently quantified by real-time RT-PCR. IL-1 β-convertase, caspase-3, -8 and -9 were upregulated in cDNA microarray were also upregulated in real-time RT-PCR upon stimulation with cHSP60 and cHSP10. The fold change expression of all genes and their accession numbers after stimulation of cHSP60 and cHSP10 are listed in **Table 8.2.**

Table 8.2: Modulation of gene expression in epithelial cells after stimulation with cHSP60 and cHSP10 for 4 h.

S. No.	Gene Description	Gene Bank ID	cHSP60	cHSP10
1	Apoptosis peptidase activating factor 1	NM_001160	13.6±0.75	6.2±0.86
2	BH3 interacting domain death antagonist	NM_001196	(-)5.5±1.16	Unchanged
3	NLR family apoptosis inhibitory protein	NM_004536	(-)7.1±0.38	(-)9.6±2.41
4	Interleukin β convertase	NM_033292	7.3±1.22	6.2±0.44
5	Caspase-3	NM_004346	9.1± 1.46	3.6±0.74
6	Caspase-8	NM_001228	4.7±0.54	10.2±1.41
7	Caspase-9	NM_001229	7.2±0.69	7.9±0.36
8	Fas-associated death domain	NM_003824	(-)4.5±1.44	(-)6.1±2.17
9	Fas-ligand	NM_000639	12.4±1.11	Unchanged
10	Bcl-2 interacting domain	NM_003806	5.6±1.91	5.9±0.74
11	TNFRSF1A associated death domain	NM_003789	Unchanged	4.6±1.22
12	TNF receptor family member 1B	NM_001065	(-)9.4±0.58	Unchanged

Note: The expression of mRNA was analyzed using real-time RT PCR. Values in columns cHSP60 and cHSP10 represent normalized fold change ± standard deviation after stimulation as compared to unstimulated control, (-) indicates fold down regulation and rest are fold upregulated genes.

Caspases-3, 8, 9 and NF-κB activation

To examine whether protein expression correlated with mRNA expression, western blot assay was performed to quantify caspase-3, -8, and -9 in the same experimental conditions. Expression of caspase-3 was significantly higher in both cHSP60 (7742 ± 1114.5 arbitrary units, $P < 0.01$) and cHSP10 (4662 ± 776.4 arbitrary units, $P < 0.05$) stimulated cells than in control cells (869 ± 66.7 arbitrary units). Furthermore, expression of both caspase-8 (cHSP60; 5877 ± 398.01 arbitrary units, $P < 0.01$) (cHSP10; 6915 ± 415.5 arbitrary units, $P < 0.01$) and -9 (cHSP60; 1300 ± 257.8 arbitrary units, $P < 0.05$) (cHSP10; 1392 ± 257.4 arbitrary units, $P < 0.05$) were also significantly higher in cHSP60 and cHSP10 stimulated cells than in control cells (caspase-8; 458 ± 169.7 arbitrary units) (caspase-9; 409 ± 182.7 arbitrary units) **(Figure 8.2)**.

Expression of NF-κB and IκB were also analyzed and it was upregulated in both cHSP60 (3940 ± 509.84 arbitrary units, $P < 0.05$) and cHSP10 (4924 ± 442.58 arbitrary units, $P < 0.05$) stimulated cells compared to control cells (1907 ± 719.85 arbitrary units). In contrast, expression of IκB was found to be downregulated in stimulated cells (cHSP60; 6576 ± 490.85 arbitrary units, $P < 0.05$) (cHSP10; 4629 ± 272.2 arbitrary units, $P < 0.05$) compared to unstimulated control cells (11817 ± 1464.08 arbitrary units) **(Figure 8.3).**

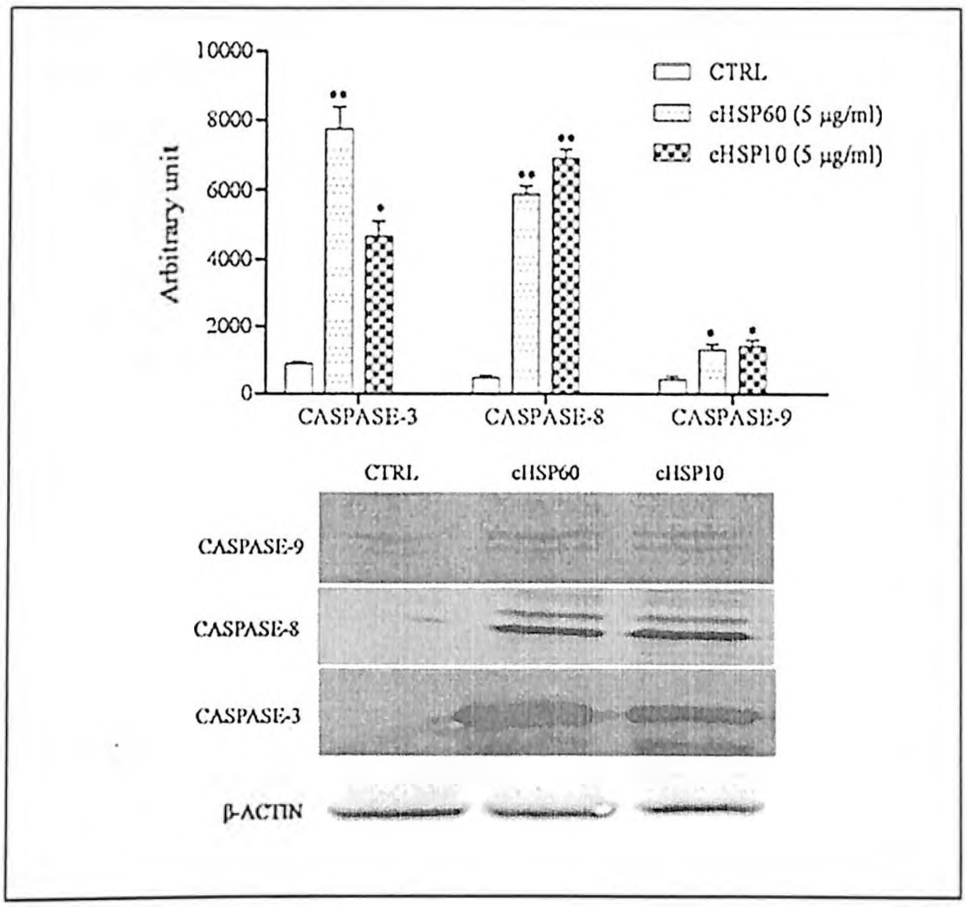


Figure 8.2: cHSP60 and cHSP10 induces increased expression of caspase-3, caspase-8 and caspase-9. Cervical epithelial cells from 3 healthy women were cultured in the presence of the cHSP60 (5 µg/ml) and cHSP10 (5 µg/ml). Total protein extracts (50 µg) were prepared from cultures and were electrophoresed on 8–12% SDS-PAGE gel, followed by Western blot analysis using anti-caspase-3, -8 and -9, with β-actin as loading control. The data presented are the mean ± SD of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ as compared with medium-treated cells.

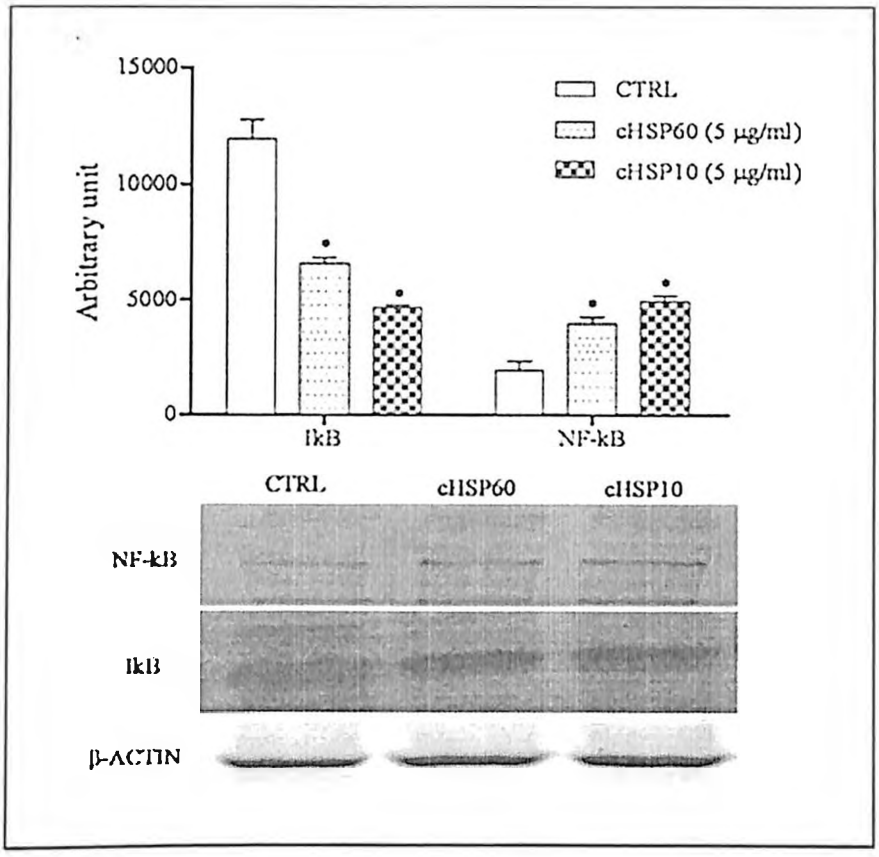


Figure 8.3: cHSP60 and cHSP10 induces NF-κB and downregulates IκB. Cervical epithelial cells from 3 healthy women were cultured in the presence cHSP60 (5 μg/ml) and cHSP10 (5 μg/ml). Total protein extracts (50 μg) were prepared from cultures and were electrophoresed on 8–12% SDS-PAGE gel, followed by Western blot analysis using anti-NF-κB and IκB, with β-actin as loading control. The data presented are the mean ± SD of three independent experiments. *$P < 0.05$ as compared with medium-treated cells.

Detection of IL-1β, IL-18, TLR-2 and TLR-4 using semiquantitative RT-PCR

Both IL-1β and IL-18 were quantified in supernatants of cells treated with cHSP60 and cHSP10. The levels of both cytokines were significantly higher in cHSP60- (IL-1β; 9310 ± 1095.5 arbitrary units, $P < 0.05$) (IL-18; 7633.6 ± 504.07 arbitrary units, $P < 0.05$) and

cHSP10- (IL-1β; 7534 ± 523.1 arbitrary units, $P < 0.05$) (IL-18; 5843 ± 250 arbitrary units, $P < 0.05$) stimulated than in control cells (IL-1β; 1153.6 ± 162.1 arbitrary units) (IL-18; 292.3 ± 184.7 arbitrary units) **(Figure 8.4)**.

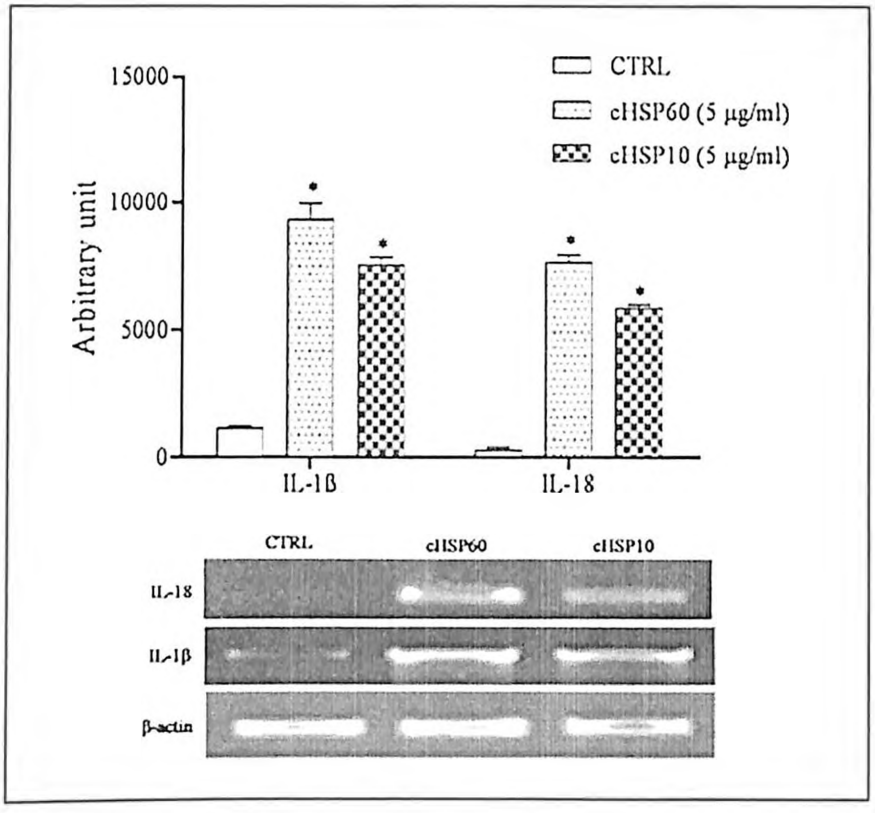


Figure 8.4: mRNA expression of IL-1β and IL-18 upon stimulation with cHSP60 and cHSP10. RT-PCR analysis of mRNA levels was done at 4 h after stimulation of epithelial cells of 3 healthy women cHSP60 (5 µg/ml) and cHSP10 (5 µg/ml). The levels of IL-18 and IL-1β were normalized against corresponding levels of β-actin. The graph shows mean levels of expression in triplicate represented by bars (mean ± SD). * $P < 0.05$ as compared with medium-treated cells.

Since, cHSPs has been identified as ligands for TLRs; mRNA expressions of TLR-2 and TLR-4 in cells treated with cHSPs were compared with control cells. The levels of both TLR-2 and TLR4 were significantly higher in cHSP60- (TLR-2; 4881 ± 319.5 arbitrary units,

P < 0.05) (TLR-4; 8318 ± 225.6 arbitrary units, *P < 0.01*) stimulated cells compared to controls (TLR-2; 1542 ± 555.4 arbitrary units) (TLR-4; 2558 ± 618.1 arbitrary units) **(Figure 8.5)**. There was no significant difference in expression levels of either TLR-2 or TLR-4 in cHSP10 stimulated cells compared to controls.

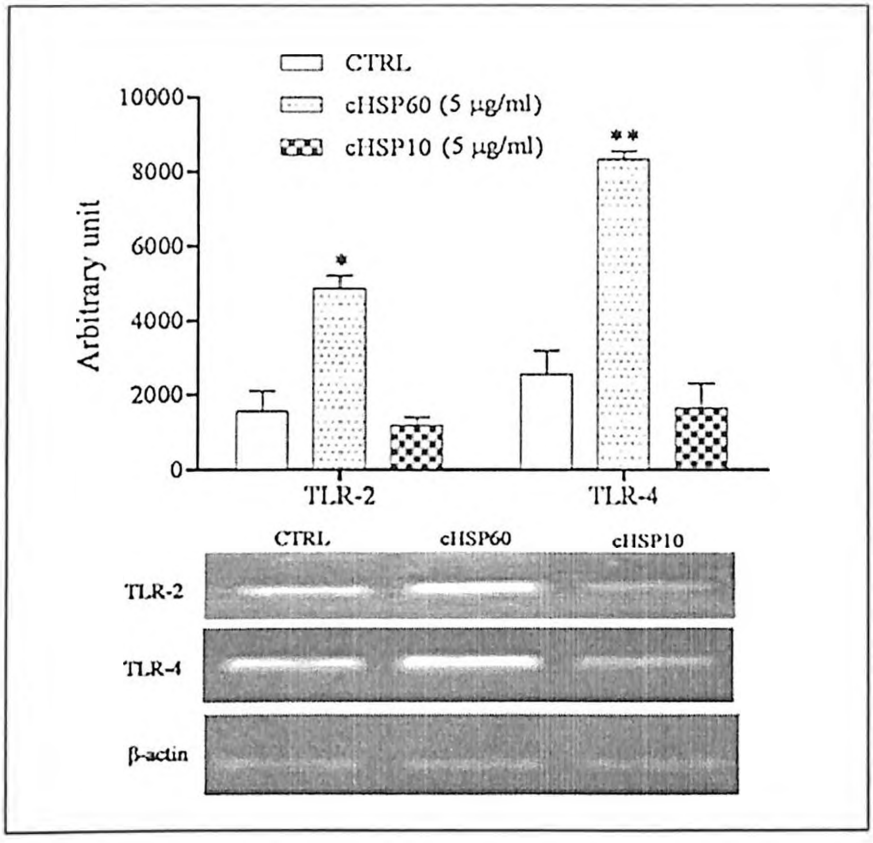


Figure 8.5: mRNA expression of TLR-2 and TLR-4 upon stimulation with cHSP60 and cHSP10. RT-PCR analysis of mRNA levels was done at 4 h after stimulation of epithelial cells of 3 healthy women with indicated concentrations of cHSP60 and cHSP10. The levels of TLR-2 and TLR-4 were normalized against corresponding levels of β-actin. The graph shows mean levels of expression in triplicate represented by bars (mean ± SD). * *P < 0.05* and ** *P < 0.01* as compared with medium-treated cells.

Discussion

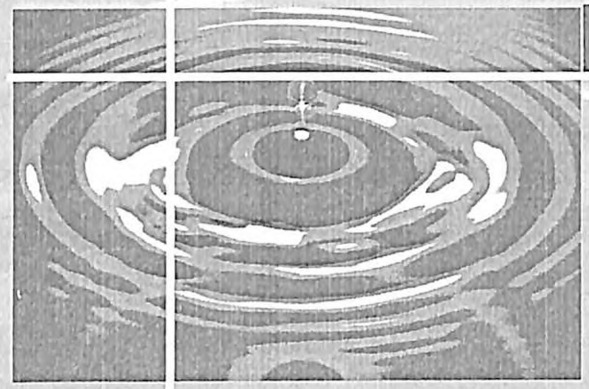
C. trachomatis is known to modulate host cell to escape immune response and prolong their persistence to cause fallopian tube damage, ectopic pregnancy and infertility in women. In chronic chlamydial infection, cHSPs are produced in large amounts and play a role in pathogenesis. In the present study, we investigated the ability of cHSP60 and cHSP10 to induce apoptosis of primary cervical epithelial cells. A significant increase in phosphatidylserine exposure which is known to be associated with apoptosis, has been observed in cHSP60 and cHSP10 stimulated cells as well as an upregulation of caspase-1, -3, -8 and -9. These results agree with an earlier *in vitro* study by Equils et. al., where in authors have shown that cHSP60 induces an apoptosis that was caspase-dependent in primary human trophoblasts and placental fibroblasts (Equils et al., 2006). In addition, the expression of several genes related to apoptosis was found to be increased. For example, the mRNA level of apoptosis peptidase activating factor-1 (Apaf-1) was upregulated by both cHSP60 and cHSP10 in real-time RT-PCR assay. This result agrees with the report of Samali et. al. showing that cytochrome c, procaspase-3, procaspase-9 and HSPs were all present in mitochondria and that the presence of Apaf-1 alone was able to initiate the apoptosis activation process (Samali et al., 1999). The mRNA level of Fas-ligand was also found to be upregulated by cHSP60 suggesting a role in Fas/CD95-mediated apoptosis requiring the activation of caspase-8 (Li et al., 1998). However, upon cHSP10 stimulation the level of Fas-ligand was unchanged which may be explained by the fact that cHSP10 plays an ancillary role in chaperone function with cHSP60.

Members of the transcription factor family, NF-κB, are key regulators of the expression of many genes involved in immune regulation. During chlamydial infection, activation of NF-κB has been reported in persistent infection of epithelial cells (Paland et al., 2006). In the present study, we have also observed the ability of cHSP60 and cHSP10 to activate the epithelial cells to trigger NF-κB activation and to down regulate IκB as well as to induce the release of IL-1β and IL-18. Similarly, Kol *et. al.* have shown that cHSP60 and human HSP60 activate vascular endothelium, smooth muscle and macrophages to produce proinflammatory cytokines mediated by NF-κB (Kol et al., 1999). In another study by Takenaka *et. al.,* it was reported that recombinant HSP60 of *Helicobacter pylori* induces inflammatory responses through TLR-mediated NF-κB in cultured human gastric epithelial cells (Takenaka et al., 2004). Our results also suggest the involvement of cHSPs in activation of NF-κB signaling pathway and production of proinflammatory cytokines.

In addition, cHSP60 has been known to be ligand for TLR2 and TLR4 and it subsequently activaties NF-κB leading to release of proinflammatory cytokines from immune cells (Bulut et al., 2002; Costa et al., 2002; Da Costa et al., 2004; Erridge et al., 2004; Prebeck et al., 2001). In the present study, higher expression of TLR-2 and TLR-4 mRNA in cHSP60 stimulated epithelial cells suggests a role of TLRs in recognition of cHSP60. This data agrees with an earlier study wherein Vabulas *et. al.* reported that cHSP60 are recognized by both TLR-2 and TLR-4 (Vabulas et al., 2001). We did not observe any significant difference in the mRNA levels of TLR-2 and TLR-4 in cHSP10 stimulated and unstimulated epithelial cells. This suggests that either cHSP10 is not recognized by TLRs or if recognized then it does not stimulate downstream signaling. However, further studies are required to elucidate this factor. The observed effects were attributable to recombinant cHSPs and not to

LPS contamination as our cHSP60 and cHSP10 preparations were treated with polymyxin B. Therefore, binding of cHSPs to TLR mediate both apoptosis and NF-κB activation. Indeed, TLR signals for apoptosis is through TLR adapter molecule, myeloid differentiation factor 88 (MyD88) via a pathway involving Fas-associated death domain protein and caspase-8, the initiator caspase associated with Fas-mediated apoptosis (Fan et al., 2005; Janssens and Beyaert, 2002). Through TLRs, cHSPs also induce the synthesis of the precursor of the pro-inflammatory cytokine interleukin-1β. Interestingly, binding of cHSPs to TLR also activates caspase-1 which contains active site cysteine necessary to cleave cytokine precursors pro-IL-β and pro-IL-18 (Cerretti et al., 1994; Ghayur et al., 1997), resulting in proteolysis and secretion of mature IL-1β (Aliprantis et al., 2000). Our findings support the earlier reports showing that *C. trachomatis* infection causes the activation of caspase-1 which had been correlated with the release of mature IL-18 from epithelial cells (Lu et al., 2000) and to IL-1β, involved in oviduct pathology (Cheng et al., 2008).

In conclusion, in our *in vitro* study it has been shown for the first time that cHSP60 and cHSP10 are involved in apoptosis of primary cervical epithelial cells with concomitant production of proinflammatory cytokines such as IL-1β which has been known to be involved in oviduct pathology. Also, it has been suggested that apoptosis induction in some intracellular bacterial pathogens provoke inflammatory responses by delivering the pathogen to professional phagocytes (Gao and Kwaik, 2000; Hacker and Fischer, 2002). In women with persistent *C. trachomatis* infection, the release of extracellular cHSP60 may therefore lead to cell apoptosis and to an inflammatory response resulting in fibrosis and scarring of female genital tract that may contribute to severe tubal pathologies including infertility.



Conclusions and Future Scope of Work

Conclusions and Future Scope of Work

Conclusions

In the present study seroprevalence of antibodies to cHSP60 and cHSP10 was evaluated in symptomatic women. Further role of cHSP60 and cHSP10 in immunopathogenesis of infertility was studied in *C. trachomatis* infected women. ELISA was performed to measure antibody responses using specific peptide sequences against cMOMP, cHSP60 and cHSP10 in patient's sera. Among *C. trachomatis*-positive women, the seroprevalence of cMOMP (66.7%) was higher followed by cHSP60 (64.0%) and cHSP10 (61.3 %) suggesting that cMOMP may be more immunogenic than cHSP60. High titers of antibodies to cHSP60 and cHSP10 were detected in women with PID/infertility than cervicitis. This implies that seropositivity of cHSP60 may be a useful marker for disease severity. The present investigation showed that women who were associated with *C. trachomatis* secondary infertility are more likely to have anti-cHSP60 antibodies than women having primary infertility. Considering IgG MOMP, PCR and DFA as test standard, cHSP60-ELISA showed high sensitivity and specificity in the secondary infertile women in comparison to patients having primary infertility, cervicitis and discharge. Also, cHSP60-ELISA had higher sensitivity and specificity in secondary infertile women than cHSP10-ELISA. Thus our study revealed that cHSP60 antibodies are more reliable serological marker for the sequelae resulting in infertility in subfertile patients. Further, different pattern of expression of cHSP60 and cHSP10 in cervical epithelial cells were observed using real-time RT-PCR and flow cytometry in infertile women compared to fertile women reflecting probable difference in the metabolic state of the *Chlamydia* with the presence of an abnormal cryptic form of *C. trachomatis* in infertile women.

In addition, full length cHSP60 gene (CT110) was amplified in *C. trachomatis* positive patient's samples. PCR amplicons were characterized by restriction digestion analysis and PCR products were subsequently cloned in pGEM-T vector. Further, specificity of positive clones was confirmed by restriction digestion and colony PCR. The recombinant clones of cHSP10 were provided as a gift from Dr. Sylvette Bas. Clones of both cHSP60 and cHSP10 were used for expression of recombinant proteins in protein expression vectors. Purified cHSP60 and cHSP10 were further used for stimulating cervical mononuclear cells from *C. trachomatis*-infected fertile and infertile women to study the specific cytokines (IFN-γ, IL-10, TNF-α, IL-4 and IL-13) released at the local site of infection. Levels of IFN-γ, IL-10 and TNF-α were higher when stimulated with both cHSP60 and cHSP10 in the infertile as compared to fertile and uninfected women. The enhanced levels of IL-10 detected in our study may be responsible in prolonging infection by exerting immunostimulatory effects and may contribute towards fibrosis and tubal infertility. IFN-γ delays the developmental cycle of *Chlamydia* so that chlamydial RBs persist longer, which might result in persistent unapparent infection and also, play a role in immunopathogenesis by promoting inflammatory damage and fibrosis. TNF-α which display anti-chlamydial properties, is known to play an important role in the initiation of inflammatory response. In the present study higher levels of both IFN-γ and TNF-α were detected upon stimulation with cHSPs in infertilite women which may suggest their involvement in the immunopathological condition associated with the infertility. IL-4 was undetectable and IL-13 levels were not significant and may suggest that cHSP specific Th2 cytokine does not play any role in pathogenesis related to chlamydial infection. Overall our results suggest that exposure to cHSPs could

significantly affect mucosal immune function by modifying the release of cytokines leading to severe immunopathological conditions such as infertility.

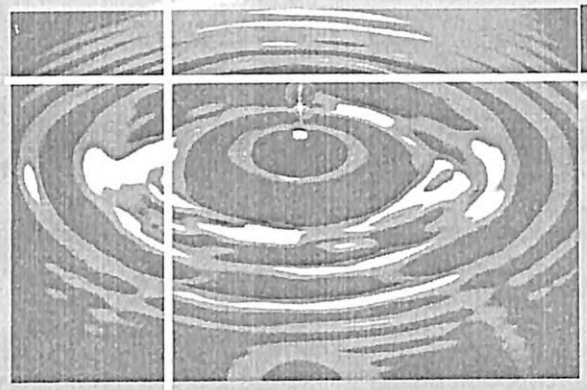
Furthermore, *in vitro*, study was also performed to assess the effect on apoptosis of cervical epithelial cells upon stimulation with recombinant cHSP60 and cHSP10. Our data has shown that the stimulation of epithelial cells with cHSP60 and cHSP10 resulted in significant increase in the number of apoptotic cells (Annexin V positive cells) compared to control cells. Using microarray analysis, caspases- 1, 3, 8 and 9 was found upregulated in both cHSP60 and cHSP10 stimulated cells. These results were further validated by real-time RT-PCR and western blotting. The proinflammatory role of cHSPs in stimulated cervical epithelial cells was also evident as upregulation of NF-kβ, IL-1β and IL-18 was detected in cHSPs stimulated cells as compared to control cells. In conclusion, it was shown for the first time *in vitro* that cHSP60 and cHSP10 are involved in apoptosis of primary cervical epithelial cells with concomitant production of proinflammatory cytokines such as IL-1β known to be involved in oviduct pathology. In women with persistent *C. trachomatis* infection, the release of extracellular cHSP60 may therefore lead to cell apoptosis and to an inflammatory response involved in fibrosis and scarring of female genital tract that may contribute to infertility.

Future Scope of Work

Reproductive morbidity associated with sexually transmitted *C. trachomatis* infection in India, is a cause of concern. This study suggests the importance of chlamydial infection in the pathogenesis of infertility in infected women. Therefore, a predictive diagnostic test based on cHSP60 for upper genital infection would be a desirable tool for various immunopathological sequelae such as infertility. The results presented in this study will help

in designing serological test based on cHSP60 for the diagnosis and prognosis of women at higher risk of developing sequelae to *C. trachomatis* infection. Also, this has a clinical relevance as it will help the gynecologist for timely therapy, thereby improving the reproductive health of women.

The intent of the study provides some perspective on understanding the role of cHSPs in immunopathogenesis of *C. trachomatis* infection in infertile women. However, further research is warranted to precisely define the potential contribution of cHSP60 and cHSP10 and other conserved chlamydial antigens in the immunopathological process in infertile women.



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Appendix

Appendix

Preparation of reagents

Stock solution of commonly used reagents

1M Tris

121.1 gm of Tris base was dissolved in 800 ml of double distilled water and pH set (6.8, 7.4, 8.0) with concentrated HCl. Volume was made up to 1 liter and autoclaved.

0.5M EDTA

186.1 gm of disodium EDTA-$2H_2O$ was added in 800 ml of double distilled water, stirred vigorously on a stirrer, pH set to 8.0 with NaOH (~20 gm of NaOH pellets) and volume made up to 1 liter and autoclaved.

3M sodium acetate

204.5 gm of $C_2H_3O_2Na$. $3H_2O$ was dissolved in 400 ml of ddH_2O, pH set to 5.3 with glacial acetic acid, volume made up to 500 ml and autoclaved.

10% SDS

10 gm of electrophoresis grade SDS was dissolved in 70 ml of ddH_2O, heated at 60°C to dissolve and the volume made up to 100 ml.

Ethidium Bromide (10 mg/ml)

10 mg of ethidium bromide was dissolved in 1 ml ddH_2O, stored in a opaque bottle.

Calcium Chloride (0.1 M)

1.47 gm of $CaCl_2.2H_2O$ was dissolved in 100 ml of ddH_2O and sterilized by autoclaving.

IPTG (1M)

238 mg of IPTG was dissolved in 1 ml of ddH_2O, filter sterilized and stored at -20°C in 50 ml aliquots.

DEPC water

0.1% diethylpyrocarbonate was added to 1 liter ddH_2O in a fume hood and mixed well. After incubating it for 1 hr at 37°C it was autoclaved.

Sodium Phosphate (1M)

Monobasic

138 gm of $NaH_2HPO_4.H_2O$ was dissolved in 800 ml of ddH2O and volume made up to 1liter.

Dibasic

268 gm of $Na_2HPO_4.7H_2O$ was dissolved in 700 ml of ddH2O and volume made up to 1liter.

Phosphate Buffer Saline (PBS)

8 gm of NaCl, 2 gm of KCl, 1.44 gm of Na_2HPO_4 and 0.2 gm of KH_2PO_4 were dissolved in 800 ml of dw. pH was set to 7.4 with HCl. Final volume was made up to 1 liter and sterilized by autoclaving at 15 lb/sq.in for 20 min. and stored at room temperature.

Ammonium persulfate (10%)

To 1 gm of ammonium persulfate, 10 ml of dw was added and the solution stored for several weeks at 4°C.

10 X TAE buffer (Tris acetate, EDTA)

4.84 gm of Tris base in 80 ml of dw was dissolved and 1.2 ml of glacial acetic acid and 2 ml of 0.5 EDTA pH 8.0 were added. Final volume was made up to 100 ml.

10X TBE buffers (Tris borate, EDTA)

8 gm of Tris base, 55 gm of boric acid and 9.3 gm $Na_2EDTA.$ H2O were dissolved in 700 ml dw and the final volume made up to 1 liter.

SDS-PAGE electrophoresis buffer

3 gm of Tris base, 14.4 gm of glycine and 1 gm of SDS were dissolved in 1 liter of dw.

Protein transfer buffer

5.8 gm of Tris base, 2.9 gm of glycine and 0.33 gm of SDS were dissolved in 500 ml of dw. 200 ml of ethanol was added and the final volume was made up to 1 liter.

2X SDS-PAGE sample buffer

(Tris-Cl (pH6.8) 100 mM, DTT 200 mM, SDS 4%, Bromophenol blue 0.2%, Glycerol 20%, β-mercaptoethanol 10%).

10X Ligation buffer

(Tris-Cl pH 7.8 500 mM, $MgCl_2$ 100 mM, DTT 100 mM, ATP 10 mM)

10 X Amplification buffer

(Tris-Cl (pH 8.3) 100 mM, $MgCl_2$ 15 mM, KCl 500 mM, Gelatin 0.1%)

DNA loading dye (6X)

0.2 gm bromophenol blue, 0.2 gm of xylene cyanol and 30 ml of glycerol were dissolved and volume set to 100 by autoclaved dw.

SDS-PAGE reagents

Composition of resolving gel (12%) 10 ml

(30% acrlyamide solution 4.0 ml, 1.5 M Tris-Cl (pH 8.8) 2.5 ml, dw 3.3 ml, 10% SDS 100 ml, 10% APS 100 ml, TEMED 10 ml)

Composition of stacking gel (5%) (5.0 ml)

(30% acrylamide solution 0.83 ml, 1.0 M Tris-Cl (pH 6.8) 0.68 ml, dw 3.4 ml, 10% SDS 50 ml, 10% APS 50 ml, TEMED 5 ml)

Staining solution

1 gm of coomassie blue was dissolved in 450 ml of methanol. 100 ml of glacial acetic acid was added and the volume made up to 1 liter by double distilled water, filtered through Whatman no.1 filter and stored at room temperature.

Destaining solution

Methanol: water: acetic Acid were mixed in the ratio of 45:45:10 and stored at room temperature.

DNA loading dye (6X)

0.2 gm bromophenol blue, 0.2 gm of xylene cyanol and 30 ml of glycerol were dissolved and volume set to 100 by autoclaved ddH2O.

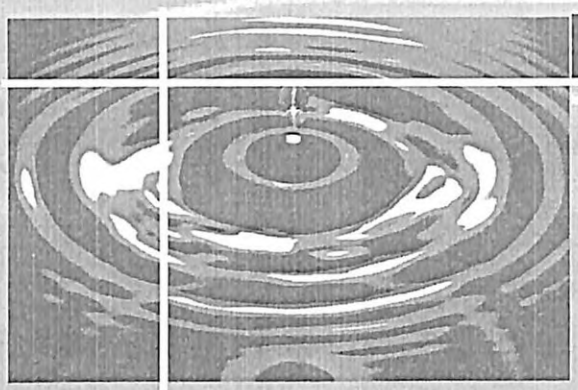
Preparation of bacterial culture media

LB medium (Luria Broth)

10 gm of Tryptone, 5 gm of Yeast, 5 gm of Sodium chloride (Hi Media) were dissolved in 1liter of double distilled water. Media were sterilized by autoclaving for 20 min at 15 lb/sq.in.

LB Agar

15 gm of agar powder, 10 gm of Tryptone, 5 gm of Yeast, 5 gm of Sodium chloride, (Hi media) were dissolved in double distill water. Media were sterilized by autoclaving for 20 min at 15 lb/sq.in. LB agar was allowed to cool to 45°C and poured (~30 ml per plate) in 90 mm disposable petri plates (Tarsons) along with appropriate antibiotics and allowed to solidify.



Publications and Biographies

Publications

RESEARCH PUBLICATION

1. **Jha R,** Vardhan H, Bas S, Salhan S and Mittal A. Cervical epithelial cells from *Chlamydia trachomatis* -infected sites coexpress higher levels of chlamydial heat shock proteins 60 and 10 in infertile women than in fertile women. **Gynecologic and Obstetric Investigation.** 2009, Jul. 29, 68(3):160-166.
   (This article and author's expert commentary was featured on MDLinx.com site, world's most up to date index of articles for physicians and other healthcare professionals, http://www.mdlinx.com/internalmdlinx/news-article.cfm/2821183).
2. **Jha R,** Jha H C, Biswas S and Mittal A. Prediction of three-dimensional structure of *Chlamydia trachomatis* heat shock protein 60: An immunodominant antigen. **Internet Journal of Genomics and Proteomics.** 2009, Vol. 4, No. 2.
3. **Jha R,** L. Neff, M. Vuillet, U. Spenato, B. Ninet, J. Schrenzel, C. Gabay, A. Mittal, S. Bas. Beta-Defensin 2 in *Chlamydia trachomatis* Infection. **(Submitted)**
4. **Jha R,** Srivastava, P, Salhan S, A. Mittal, S. Bas. Interleukin-17 and -22 in the cervix of women with primary and recurrent *Chlamydia trachomatis* Infection. **(Submitted)**
5. **Jha R,** Vardhan H, Bas S, Salhan S and Mittal A. *Chlamydia trachomatis* heat shock protein 60 and 10 induce apoptosis in endocervical epithelial cells. **(Submitted)**
6. Dutta R, **Jha R,** Gupta S, Gupta R, Salhan S and Mittal A. Seroprevalence of antibodies to conserved region of *Chlamydia trachomatis* heat shock proteins 60 and 10 in women attending Gynecology outpatient department. **British Journal of Biomedical Science.** 2007, 64(2):78-83.
7. Dutta, R. **Jha, R.** Salhan, S. Mittal, A. *Chlamydia trachomatis*-specific heat shock proteins 60 antibodies: A prognostic marker for sequalae in Chlamydia infected women. **Infection.** 2008;36:374-8. (DOI 10.1007/s15010-008-7129-9)
8. Srivastava, P. **Jha, R.** Bas, S. Salhan, S and Mittal, A. In infertile women, cells from *Chlamydia trachomatis* infected site release higher levels of Interferon-gamma, Interleukin-10 and Tumor Necrosis Factor-alpha upon heat shock protein stimulation than fertile women. **Reproductive Biology and Endocrinology.** 2008, 6:20. (DOI:10.1186/1477-7827-6-20)
9. Srivastava, P. Gupta, R. Jha, H C. Bhengraj, A R. **Jha, R.** Salhan, S and Mittal, A.Serovar specific immune responses to peptides of variable regions of *Chlamydia trachomatis* Major Outer Membrane Protein in serovar D infected women. 2008. **Clinical and Experimental Medicine.** 2008 Dec;8(4):207-15.
10. Gupta, R. **Jha, R.** Salhan, S. Eickhoff, M. Krupp, G and Mittal, A. Existence of plasmid less clinical isolate of *Chlamydia trachomatis* in India is a cause for concern and demand the use of real-time PCR assays. **Internet Journal of Microbiology.** 2008, Vol.5, No.2.
11. Vardhan H, Dutta R, Vats V, Gupta R, **Jha R,** Jha HC, Srivastava P, Bhengraj AR and Mittal A. Persistently elevated level of IL-8 in *Chlamydia trachomatis* infected HeLa 229

cells is dependent on intracellular available iron. **Mediators of Inflammation.** 2009: 417658. (doi: 10.1155/2009/417658)

12. Vardhan H, Bhengraj AR, **Jha R** and Mittal A. *Chlamydia trachomatis* alters Iron-regulatory protein-1 binding capacity and modulates cellular iron homeostasis in HeLa 229 cells. **Journal of Biomedicine and Biotechnology.** 2009:342032.
13. Vardhan H, Bhengraj AR, **Jha R** and Mittal A. Higher expression of ferritin protects *Chlamydia trachomatis* infected HeLa 229 cells from reactive oxygen species mediated cells death. **Biochemistry and Cells Biology.** 2010. **(In press)**
14. Vardhan H, Gupta R, **Jha R,** Bhengraj AR and Mittal A. Ferritin heavy chain mediated iron homeostasis regulates expression of Interleukin-10 in *Chlamydia trachomatis* infected HeLa cells. **(Submitted)**
15. Vardhan H, Bhengraj AR, **Jha R** and Mittal A. Thioredoxin-1 supports survival of Chlamydia trachomatis in HeLa 229 cells. **(Submitted)**
16. Srivastava P, Bhengraj AR, Vardhan H, Jha HC, **Jha R,** Singh LC, Salhan S and Mittal A. Differing effects of azithromycin and doxycycline on production of cytokine in cells from Chlamydia trachomatis infected women. **(Submitted).**

PATENT

- **R. Jha** and A. mittal. Development of Dot-blot assay for prognosis of sequelae to *Chlamydia trachomatis* infection in women using chlamydial heat shock protein 60. (Filed at ICMR/DRDO, file no. 186/DEL/2008)

CONFERENCE PRESENTATIONS

Oral presentations:

1. **Jha R** and Mittal A. Role of chlamydial heat shock proteins in the pathogenesis of genital chlamydiasis. National Conference on Emerging Trends in Life Sciences Research. **BITS-Pilani, Rajasthan,** 6-7th Mar. 2009.
2. **Jha, R.** Srivastava, P. Vardhan, H. Salhan, S and Mittal, A. Expression of Chlamydial heat shock proteins 60 and 10 in cervical cell of infertile women infected with *Chlamydia trachomatis* may contribute to immunopathogenesis. 2nd Mediterranean Clinical Immunology Meeting, **Antalya, Turkey,** 4-7 Oct. 2008.

Poster presentations:

3. **Jha, R.** Srivastava, P. Vardhan, H. Bas, S. Salhan, S and Mittal, A. Expression of chlamydial heat shock protein 60 and 10 in cervical cells modulates mucosal immune response in infertile women. International congress on Bio-immunoregulatory Mechanisms Associated with Reproductive Organs: Relevance in Fertility and in Sexually Transmitted Infections. **NII, New Delhi,** 9-13 Feb. 2009.
4. **Jha R,** Jha HC, Vardhan H, Gupta R, Srivastava P, Bhengraj AR and Mittal A. Three-dimensional structure of *Chlamydia trachomatis* heat shock protein 60 reveals cross presentation of epitopes in infected women causing infertility. ICSB 2007, Chinese University of Hong Kong, **Hong Kong,** 19-22 Nov. 2007.

5. **Jha, R.** Jha, H C. Gupta, R. Vardhan, H. Srivastava, P. Bhengraj, A R. and Mittal, A. Similarity in domains of HSPs in humans and *Chlamydia trachomatis* are suggestive of their role in causation of damaging sequalae in infertile women. 11th ADNAT Convention. CCMB, **Hyderabad,** 23-25 Feb. 2007.
6. Srivastava, P. **Jha, R.** Bas, S. Salhan, S and Mittal, A. higher Chlamydial heat shock protein specific levels of Interferon-gamma, Interleukin-10 and Tumor Necrosis Factor-alpha at the site of infection is associated with infertility of women. HUGO's 13th human genome meeting. 27-30 Sep. 2008, **Hyderabad,** India.
7. Gupta, R. **Jha, R.** and Mittal, A. Is Chlamydia trachomatis plasmid indispensable for its survival? In Proceeding of International conference on chemistry Biology Interface: Synergistic New frontiers, CBI SNF, **New Delhi,** 2004.
8. Dutta, R. **Jha, R.** Gupta, S. Gupta, R. Salhan, S. and Mittal A. Detection of anti-cHSP 60 & 10 in the serum of chlamydial infected women, a serological marker: An association with the severity of disease. In Proceeding of International conference on immunology. IIS, **Chandigarh,** 2005.
9. Jha, H C. Gupta, R. Srivastava, P. Vardhan, H. **Jha, R.** Bhengraj, A R. Prasad, J and Mittal, A. Chlamydia pneumoniae and associated risk factors in coronary artery disease patients. International symposium on recent advances in cardiovascular sciences (RACS). "Global conference on heart disease. DIPSAR, **New Delhi,** 14-15th Feb 2007.
10. Jha, H C. Srivastava, P. **Jha, R.** Gupta, R. Vardhan, H. Bhengraj, AR. Prasad, J and Mittal, A. Role of Chlamydia pneumoniae and its association with other risk factors in coronary artery disease. Biosparks'07, 5th Annual Research Festival, SLS, JNU, **New Delhi,** 23-24th Feb. 2007.
11. Gupta R, Srivastava P, Bhengraj AR, **Jha R,** Jha H C, Vardhan H, Salhan S and Mittal A. Chlamydia trachomatis heat shock protein 60-specific antibody and cell-mediated responses can predict tubal factor infertility in infected women. Sixth meeting of the European society of chlamydia research, **Aarhus, Denmark,** 1-4 July 2008.

Biography of Candidate

RAJNEESH JHA

Personal Information

Address: Lab no-204, Institute of Pathology (ICMR)

Safdarjung Hospital Campus,

Post box-4909, New Delhi-110029
Phone: 91-11-26198402-06 (Extn. 304)
E-mail: rjha.molbio@gmail.com
Date of Birth: 1st March, 1978; Gender: Male; Marital status: Single; Nationality: Indian;
Passport no. : F8602167

Educational Qualification:

M.Sc. (Microbiology) (1st Div.):	**76.90%**
SOS in Microbiology, Jiwaji University, Gwalior, India	
B.Sc. (Zoology) (1st Div.):	**62.50%**
C.M. Sc. College, Darbhanga, Bihar, India	
Higher Secondary (1st Div.):	**70.66%**
C. M. Sc. College, Darbhanga, Bihar, India	
High school (1st Div.):	**73.44%**
M.L. Academy, Darbhanga, Bihar, India	

CERTIFICATE COURSE

Successfully completed distance online certificate course on Intellectual Property in between 1st April to 15th **May 2009 from World Intellectual Property Organization (WIPO) Geneva, Switzerland.**

HONOURS AND AWARDS

- Qualified **GATE** (Graduate Aptitude Test in Engineering), **2004, conducted by IIT, Madras,** in Life Sciences discipline.
- **Junior Research Fellowship (JRF)** in a **Defence Research and Development Organization (DRDO)** project at IOP-ICMR, New Delhi, from 8th Oct 2004 to 7th Oct. 2006.
- **Senior Research Fellowship (SRF)** in a **DRDO** project at IOP-ICMR New Delhi, from 8th Oct 2006 to 19th Nov. 2007.
- Awarded **ICMR** independent **SRF** for the project entitled "Molecular characterization of

Chlamydial Heat Shock Proteins 60 (cHSP60) and its role in pathogenesis of genital Chlamydiasis. (Nov. 2007 to till-date)

- Awarded full Air-fare as **"Travel grant"** from **Department of Biotechnology (DBT),** for attending ICSB 2007 at Chinese University of Hong Kong, **Hong Kong,** Nov. 2007. (Grant No. BT/HRD/29/364/07 dated 28th Dec. 2007)
- Awarded Travel support from **Department of Science and Technology (DST)** for attending ICSB, 2007, Hong Kong. (Grant No. SR/PF/1293/2007-2008 dated 30th Oct 2007)
- Awarded HK $ 1000 cash as **"Appreciation Award"** from Secretary, ICSB for presenting poster at International Conference in Structural Biology 2007, Chinese University of Hong Kong, **Hong Kong,** Nov. 2007.
- Awarded 500 Euros as **"Travel Grant"** from European Federation of Immunological Society (EFIS) for attending 2nd Mediterranean Clinical Immunology Meeting at **Antalya, Turkey** from 4-7 Oct. 2008.
- Awarded full Air-fare as **"Travel Grant"** from Council for Scientific and Industrial Research (CSIR) for attending 2nd Mediterranean Clinical Immunology Meeting at **Antalya, Turkey** from 4-7 Oct. 2008. (Grant No. TG/3543/08-HRD dated 8th Oct. 2008)
- Awarded Travel support from Centre for **Cooperation in Science & Technology among Developing Societies (CCSTDS)** to attend Clinical Immunology Meeting, Antalya, Turkey. (Grant No. DO/Lr/TF-III/08 dated 26th Sep 2008)

SYMPOSIA ATTENDED

- 2nd Mediterranean Clinical Immunology Meeting, **Antalya, Turkey,** 4-7 Oct. 2008. Organized by European Federation of Immunological Society (EFIS), International Union of Immunological Society (IUIS) and Turkish Society of Immunology.
- International Conference in Structural Biology 2007, Chinese University of Hong Kong, **Hong Kong,** 19-22 Nov. 2007.
- 11th ADNAT Convention. Centre for Cellular and Molecular Biology (CCMB), **Hyderabad,** 23-25 Feb. 2007.
- 21st Annual Conference, Indian Association of Pathologists & Microbiologists. 16th April 2006, **Institute of Pathology (ICMR),** New Delhi.
- Seminar on "Infection to Immunity" organized by Society for young scientists held at All India Institute of Medical Science (AIIMS), New Delhi on 8th Oct. 2007.
- International congress on Bio-immunoregulatory Mechanisms Associated with Reproductive Organs: Relevance in Fertility and in Sexually Transmitted Infections. **National Institute of Immunology (NII), New Delhi,** 9-13 Feb. 2009.
- National Conference on Emerging Trends in Life Sciences Research. **BITS-Pilani, Rajasthan,** 6-7th Mar. 2009.
- Attended **International Symposium on Molecular Pathology and Applied Genomics** on 6-7th Nov. 2009 at India Habitat Centre, New Delhi, India.

RESEARCH INTEREST

- Immunology of intracellular pathogen
- Biomarker and vaccine research
- Pathogen induced stress biology
- Host-pathogen interaction

RESEARCH EXPERIENCE

During my PhD I have gained experience in the following techniques:

Molecular Biology: Genomic & Plasmid DNA Isolation; PCR; Gene Cloning; RNA Isolation; RT- PCR; Real-time PCR; Isotopic labelling of cDNA probes; Restriction mapping; DNA sequencing; Expression of eukaryotic and prokaryotic genes in bacteria; Purification of Expressed proteins; Western Blotting; EMSA, Nylon Microarray.

Immunology: ELISA; Immunoelectrophoresis; immunoprecipitation; Dot-blot assay; MTT assay for Cell proliferation; Nitric oxide estimation; Flow Cytometry (Surface and Intracellular staining); Immunocytostaining; Immunohistoschemistry.

Biochemistry: Protein purification (ion exchange, and affinity chromatography), protein estimation; Spectrophotometric enzyme assays; Polyacrylamide gel electrophoresis (SDS-PAGE).

Cell and tissue Culture: Culture and maintenance of cervical and peripheral blood mononuclear cells (PBMC); Mammalian adherent and nonadherent cell culture; Density gradient cell separation; Propagation of *C. trachomatis* in HeLa, McCoy and THP-1 cell-lines.

Microbiology: Bacterial cultures and staining methods (Giemsa and Gram staining); Characterization & identification of Bacteria; In-situ hybridization (rRNA probe); Direct fluorescent assay (DFA).

Computer and Biostatistics: MS Office; NCBI; BLAST; Clustal W; Just Bio; Scan Prosite; Active Pocket determination; CASTp; pyMOLE; Emboss software; FTP server; GCG server; SPSS version 12.1; Excel; Prism5; EPI Info; ANOVA.

Biography of Supervisor

Name in Full: **Dr. Aruna Singh nee Mittal**

Designation: **Scientist F, Institute of Pathology (ICMR)**

Educational Qualifications: **M.Sc., PhD**

Email: amittal_iop@yahoo.com

Area(s) of Research: **Immunology/Chlamydia**

Awards/Special recognitions:

- 'Shakuntala Amir Chand Prize for Young Scientists' by ICMR for miniaturizing radiometric assay for *M.Leprae* viability and drug resistance in 1984.
- ICMR Kshanika award for research work on Immunobiology of Chlamydia for the year 2003.
- ICMR Lala Ram Chand Kandhari award for research work for the year 2006
- Title of '*Chlamydia* Farmer' by Center for Disease Control, Atlanta, USA, 1989.
- Awarded 'Biotechnology Overseas *long-term* Associateship' by Department of Biotechnology, Ministry of Science & Technology and worked at Center for Disease Control, Atlanta, USA, 1988-89.
- Selected for taking advance training at Rockfeller University, New York, USA under 'Indo-US SSP Program', 1984.

Patent filed: 3

Technology Transfer: 1

Membership of National/International bodies:

i) **Member-** International Union against sexually transmitted infections(Asia-Pacific)

ii) **Fellow-**Indian college of Allergy & Applied Immunology

iii) **Life member-** Indian Immunology society

iv) **Member-** Indian Association of Pathologists and Microbiologists

Extramural Projects awarded:

National: 7

International: 2

Supervisor:

Ph. D students =10,

MSc students dissertation done/completed=11,

MD Thesis=1

Joint supervisor for Ph. D student= 1

Complete list of books, monographs etc. published. (Chapters):

1. Nath I and Mittal A. Subpopulation of lymphocytes. In manual of symposium-workshop 'New Directions in foetal liver transplantation', 20: 1986.
2. Mittal A. Rapid radiometric in vitro assay for evolution of M. leprae viability, drug screening and drug resistance: In Handbook of Practical Immunology. ed. G.P. Talwar, 288, 1992.
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