Contribution of *Chlamydia trachomatis* Inclusion Membrane Proteins B and C in Pathogenesis of Genital Chlamydial Infection in Humans

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By

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Under the Supervision of

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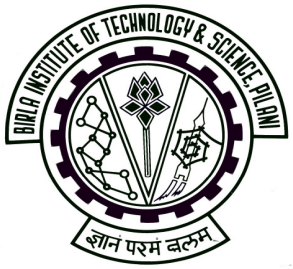

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CERTIFICATE

This is to certify that the thesis entitled **"Contribution of *Chlamydia trachomatis* Inclusion Membrane Proteins B and C in Pathogenesis of Genital Chlamydial Infection in Humans"** and submitted by **Rishein Gupta** ID No. 2004PHXF428P for award of Ph.D. Degree of the Institute, embodies original work done by him under my supervision.

Signature in full of the Supervisor

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New Delhi, India

Abstract

With an increase in the number of putative inclusion membrane proteins (Incs) in all chlamydial genomes, there is a need for understanding their contribution in host-pathogen interactions. Incs of *Chlamydia trachomatis* (CT) have been reported to play pivotal roles in the molecular and cellular interactions between pathogen and host cells. In this study, to detect *incB* and *incC* genes, full length gene specific PCRs were used. These genes encode for CT inclusion membrane protein B and C (IncB and IncC respectively) which are two early infection phase proteins expected to be involved in the establishment of infection within host cells. Using full length fusion proteins for CT IncB and IncC, we studied humoral and cell mediated immune responses in *C. trachomatis* infected women. Using ELISA and Western blot assay we detected IncB and IncC IgA and IgG in cervical washes and sera from CT-positive fertile and infertile women. Antibody titres to IncB and IncC were compared with antibodies to CT major outer membrane protein (MOMP), a highly antigenic, surface spanning, multi-epitope protein present in all CT serovars. In CT-positive sera, a positive correlation was found between antibody titres to IncB and IncC and CT MOMP antibodies. In addition we detected higher IncB and IncC IgG_2 titres in comparison to those of respective IgG_1, IgG_3 and IgG_4 in CT-positive patients. Using MTT assay, significantly high proliferative responses were observed in *in vitro* Inc-stimulated cervical cells and peripheral blood mononuclear cells (PBMCs) from CT-positive fertile women compared to CT-positive infertile women and controls. Lactate dehydrogenase (LDH)-cytotoxicity assay showed that there was no cellular damage in Inc- stimulated cervical cells or PBMCs from CT-

positive patients and controls. Secreted cytokines (IL-1ß, IL-4, IL-5, IL-6, IL-10, IFN- γ, IL-12, TNF-α and GM-CSF) in cervical cells and PBMCs on *in vitro* stimulation with IncB and IncC were assayed by real-time reverse-transcriptase PCR and ELISA. IFN- γ, IL-12 and GM-CSF was found to be elevated in Inc-stimulated cervical cells and PBMCs obtained from CT-positive fertile women compared to CT-positive infertile women and controls. In contrast, IL-1ß, IL-4, IL-5, IL-6 and IL-10 levels were found to be significantly higher in Inc-stimulated cells of CT-positive infertile women in comparison to CT-positive fertile women and controls. Overall our data suggests that CT incs namely, IncB and IncC modulate host immune responses and might be involved in immunoprotection/ pathogenesis of *C. trachomatis* infection.

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Introduction

Chlamydiae are intracellular bacterial pathogens that cause a spectrum of clinically important diseases in humans. *Chlamydia trachomatis* is a major cause of sexually transmitted diseases and trachoma, the leading cause of preventable blindness in humans [Schachter and Caldwell 1980; Jones, 1995; Pannekoek *et al.,* 2000]. *C. trachomatis* can cause cervicitis, endometritis and salpingitis, and is one of the most common pathogens associated with pelvic inflammatory disease (PID). Common sequelae of the infection include infertility, ectopic pregnancy and chronic pelvic pain in women, epididymitis and proctitis in men and arthritis in both men and women [Tavakoli *et al.,* 2002]. *C. trachomatis* may also cause pregnancy complications and implantation failure after *in vitro* fertilization [Toth *et al.*, 1993; Sulak, 2003; Romero *et al.,* 2004]. Moreover, chlamydial genital tract infections are an important risk factor for Human immunodeficiency virus (HIV) transmission [Laga *et al.,* 1993; Ho *et al.,* 1995]. *Chlamydia psittaci*, which predominantly infects animals and causes a variety of respiratory, gastrointestinal, and urogenital diseases, can also cause pneumonia in humans [Storz, 1998]. *C. pneumoniae* has been established as an important pathogen that causes infections of upper and lower respiratory tract [Grayston, 1992; Hammerschlag, 2000] and also plays a role in atherosclerosis [Saikku *et al.,* 1988].

Chlamydial infection is initiated by the extracellular elementary body (EB) which attaches to and invades epithelial cells at mucosal surfaces [AbdelRahman and Belland, 2005]. Within 8 to 12 hours (h) after invasion, EBs develop into metabolically active reticulate bodies (RBs). RBs divide by binary fission inside a host-derived endosome that eventually occupies much of the host cell cytoplasm (then termed the chlamydial

inclusion). RBs reorganize into EBs 18–30 h after invasion of host cells, and infected cells rupture and release infectious EBs at 48–72 h post-infection.

C. trachomatis infects epithelial cells and early stages of urogenital infection results in mucopurulent discharge. The infection is resolved in most infected women but in those with persistent or repeated infections, the infection can spread upwards from the endocervix to the fallopian tubes. Persistent infections lead to scarring of the fallopian tubes and result in infertility or ectopic pregnancy [Mardh *et al.,* 2004]. *C. trachomatis* infection usually remains confined to mucosal surfaces, however lymphogranuloma venereum (LGV) strains, which cause systemic illness are known to infect regional lymph nodes [Schachter*,* 1999].

Worldwide around 92 million new cases of chlamydial infections have been reported to be occurring every year with highest prevalence of *Chlamydia* in adults in South-East Asia (45 million) and Sub-Saharan Africa (15 million) [WHO, 2001]. In India, a high prevalence of chlamydial infection in symptomatic women (upto 40% in cervicitis and 36% in infertility) has been reported [Mittal *et al.,* 1993]. Further, a high percentage of chlamydial positivity has been reported in urban slum dwellers [Singh *et al.,* 2002]. Cervical *C. trachomatis* infection has also 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 [Mittal *et al.,* 1993; Malik *et al.,* 2006; Malik *et al.,* 2009]. Furthermore, in India, the occurrence of *C. trachomatis* serological variant or serovar D (48%), serovar E (34%), serovar F (12%) and serovar I (6%) in infected urogenital samples has been reported [Singh *et al.,* 2003].

With growing concerns over the increasing incidence of *C. trachomatis* infections globally and their deleterious effects on human health, there is an immediate

need for improved and comprehensive understanding of all the elements of the host immune system which contribute towards protection against establishment of infection. Unfortunately our understanding of chlamydial pathogenesis has been hampered by the fact that till date, there are no techniques to manipulate the organism genetically. Nevertheless, genome sequencing of several *C. trachomatis* strains has led to significant advances. The genome of *C. trachomatis* serovar D has been sequenced and reveals that at a size of 1.04 Mega bases it encodes only 894 proteins. The genome contains genes encoding a large group of proteins termed Inc proteins, that have been shown to be located in the inclusion membrane and probably regulate traffic between the inclusion and chlamydial cells. It is believed that *Chlamydia*—host cell interactions are mediated through their own unique Incs [Rockey *et al.,* 2002]. Inc proteins appreciably differ from each other by the primary amino acid sequence, but have a characteristic hydrophobic domain consisting of about 50 amino acids, this presumably determining their location in the inclusion membrane [Bannantine *et al.,* 2000]. The functions of Inc proteins are unknown, but the fact that expression of genes encoding some Inc proteins starts during the first half-hour after cell culture infection suggests that they can act as intermediaries in interactions between *Chlamydia* and eukaryotic cell.

Although *C. trachomatis* has evolved to survive intracellularly within the host cell, the host has in turn has evolved an intricate system to detect as well as to control infection. Host immune responses to *C. trachomatis* are well timed and coordinated where innate immune cells, B cells, and T cells act in concert and have distinct roles in recognizing different stages of the infection. These immune effectors affect clearance of *Chlamydia* organisms but may also contribute to the tissue pathology associated with *Chlamydia* infection. Although several Inc proteins have been identified in different

chlamydial species, their role in chlamydial biology, especially in the aspect of generating host immune responses is not well understood. There is a need to understand the role of these Inc proteins in immunopathogenesis of chlamydial infection which is important for controlling chlamydial growth and facilitating pathogen clearance.

The proposed study was undertaken with the aim of studying the role of two inclusion membrane proteins of *C. trachomatis*, IncB and IncC in generating immune mediated responses in host cells. These two proteins are products of CT 232 and CT 233 genes respectively, annoted in the *Chlamydia trachomatis* D/UW-3/CX (serovar D) genome database [Stephens *et al*., 1998; Carlson *et al*., 2005]. These genes are early-expression genes and are activated within an hour of the infection cycle. These gene products are thus very critical as they are believed to be involved in modification of the inclusion membrane- a crucial requirement for chlamydial intracellular survival. Therefore to study the role of these Inc proteins, full length recombinant fusion proteins were used and immune parameters, both humoral as well as cell mediated immune responses were assessed in *C. trachomatis* infected women. Seroprevalence of IncB and IncC antibodies in infected women was studied. Further, *in vitro* cell mediated immune responses to IncB and IncC stimulation were evaluated. This study will provide insights into functions of chlamydial inclusion proteins and their probable role in pathogenesis of chlamydial infection. This study could provide crucial lead for further research to delineate specific effector mechanisms of these chlamydial inclusion proteins in host-pathogen cross-talk.

Review of Literature

Taxonomy

Chlamydiae are obligate intracellular gram-negative eubacteria with a unique biphasic life cycle [Grayston and Wang, 1975]. The original taxonomic categorization was Order, *Chlamydiales*, with a single family, *Chlamydiaceae*, and one genus, *Chlamydia* [Moulder *et al.*, 1984]. The genus included four species, differing in host cell tropism but shared biological properties for their intracellular existence. These species were *C. trachomatis*, *C. psittaci* [Moulder *et al.*, 1984], *C. pneumoniae* [Grayston *et al.*, 1989] and *C. pecorum* [Fukushi and Hirai, 1992]. A revolutionary change in the taxonomy was proposed in 1999 on the basis of molecular markers. According to the proposed taxonomy, the family *Chlamydiaceae* was divided into two genera, *Chlamydia* and *Chlamydophila* (Fig.1.1). Non-*Chlamydiaceae* such as *ParaChlamydiaceae*, *Waddliaceae* and *Simkaniaceae* were also included in this family [Everett *et al.*, 1999]. This change in the taxonomic nomenclature for the *Chlamydiaceae* family proved to be controversial and was not universally accepted in the field [Schachter *et al.*, 2001]. In this thesis, the terminology pertaining to a single genus *Chlamydia* is used.

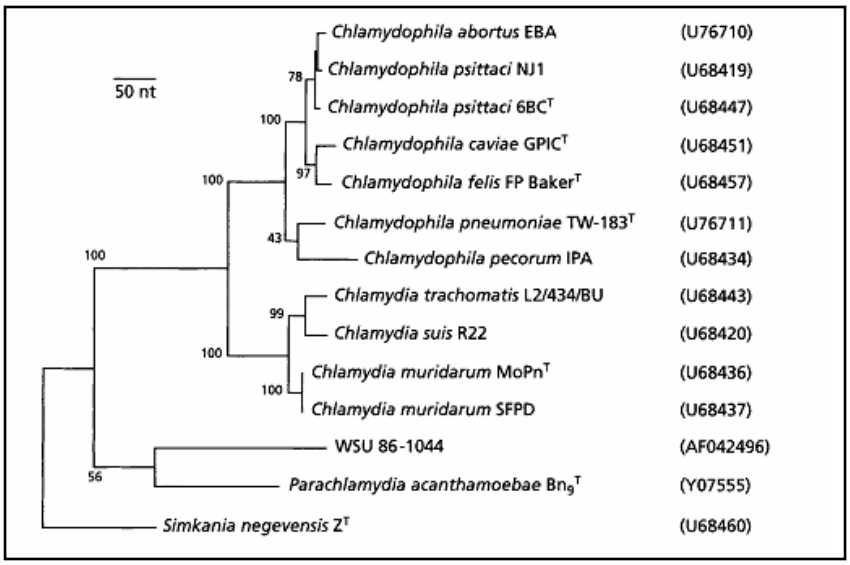


Fig.1.1. Phylogeny of 14 members of the order *Chlamydiales.*
Phylogenetic tree constructed using full-length 23S rRNA genes. Branch lengths are measured in nucleotide substitutions. Numbers show the percentage of times each branch was found in 1000 bootstrap replicates. Branches without bootstrap values occurred in less than 50 % of the trees. Values in parentheses are the GenBank sequence accession numbers. [Everett *et al.*, 1999]

C. trachomatis and *C. pneumoniae* are the two chlamydial species which are pathogenic to humans, whereas the other species occur mainly in animals. *C. trachomatis* has been isolated comprises of two human biovars, trachoma and lymphogranuloma venereum [LGV]. Based of genetic differences in the *omp1* gene encoding for the major outer membrane protein (MOMP) biovars have 21 sero- or genonotypes (including subtypes): A, B, Ba, C, D, Da, E, F, G, Ga, H, I, Ia, J, Ja, Jv, K, L1, L2, L2a, and L3 [Wang *et al.*, 1985, Frost *et al.*, 1991, Wang and Grayston 1991, Morré *et al.*, 1998, Dean *et al.*, 2000] . *C. pneumoniae* has only one serovar, with almost 100% DNA homology between the strains but less than 10% homology with the other *Chlamydiae* [Kuo *et al.*, 1995]. The number of serovars of *C. psittaci* is not comprehensively known [Everett *et al.*, 1999].

Life Cycle

The chlamydial developmental cycle was first described by Bedson and Bland in 1932 using used light microscopy [Bedson *et al.,* 1932]. Till early 1960's chlamydial infection was considered as viral. This idea was changed when the presence of DNA and RNA, a cell wall, ribosomes, and sensitivity to antibiotics such as tetracycline within the infectious form led to the conclusion that the organisms belonged to bacteria.

In vivo, the intracellular, biphasic developmental cycle of the gram-negative bacterium *C. trachomatis* is reliant on host cell ATP and nutrients for its existence. In this unique cycle, the bacterium is normally found in two highly specialized morphologic forms — the extracellular, metabolically inactive, and infectious **elementary body** (EBs; 200–300nm in diameter) and the metabolically active, intracellular form known as the **reticulate body** (RBs; 1000–1500nm in diameter) that divides by binary fission within the inclusion [AbdelRahman *et al.,* 2005]. The infectious EB enters the mucosal host cells in a process now known to be independent of host cell-surface heparin sulfate glycosaminoglycans [Stephens *et al.,* 2006], and following binding to a number of proposed ligands on *Chlamydia* [Hackstadt *et al.,* 1999]. Once inside, the epithelial cell-surface antigens of the EB appear to prevent fusion of the endosome with lysosomes [van Ooij *et al.,* 1997], allowing the EB to subsequently reorganize into the larger, replicative form of the RB. RBs successfully divide by binary fission, filling the endosome that has now become a chlamydial cytoplasmic inclusion. Multiplication then ceases after 48 – 72 h and nucleoid condensation occurs, enabling the RBs to transform into infectious EBs. The EBs are released from the cell and target new host cells for progression of infection [Wyrick *et al.,* 2000].

The life cycle of *Chlamydiae* is, thus a transition from EB into RB, RB into RB, and finally, RB into EB (Fig.1.2). Chromosomal material in EBs is condensed in the nucleoid by bacterial histone-like proteins HctA and HctB [Barry *et al.,* 1992; Brickman *et al.,* 1993]. Chlamydial EBs do not have a detectable peptidoglycan layer in the cell wall and are supported by a crosslinked outer-membrane complex (OMC), composed of cysteine rich proteins such as OmpA, OmcB and OmcA. Stability of EBs depends on disulfide-linked outer membrane proteins [Hatch *et al.,* 1981; Newhall *et al.,* 1983; Bavoil *et al.,* 1984; Hackstadt *et al.,* 1985].

Unique structural appendages termed "projections" extending approximately 30 nm from the surface of EBs, are shown by electron microscopy to penetrate the inclusion membrane and extend into the host cell cytoplasm. These projections are suggested to be involved in interactions between RBs and the host cell [Matsumoto *et al.,* 1982; Nichols *et al.,* 1985] and are randomly redistributed when EBs transforms to RBs. These structures have resemblance to flagella, a thought further supported by findings of flagella gene orthologues in *C. trachomatis* serovar D genome sequencing [Stephens *et al.*, 1998]. Chlamydial projections may serve as the type III secretion machinery in *Chlamydiae* [Bavoil *et al.,* 1984; Hsia *et al.*, 1997] and contribute to the parasitic relationship between intracellular *Chlamydiae* and host cells. Type III secretion system possessing pathogens like *Salmonella sp*., *Yersiniae sp*., *Shigella sp*. and *Pseudomonas sp.,* have also been reported to have similar membrane spanning structures [Ginocchio *et al.*, 1994; Michiels *et al.*, 1990; Parsot *et al.*, 1995; Roine *et al.*, 1997]. Upon infection, EBs became internalized in inclusions. There is very little information about the physiological processes involved in the transition from infectious EB to non-infectious

but metabolically active RB. Molecular signals triggering these changes are not yet defined.

Entry

Entry within host mucosal epithelial cells is an essential requirement for obligate intracellular parasites like *Chlamydiae*. There *are* several modes of entry for *Chlamydiae* including receptor mediated endocytosis in polarized epithelial cells [Wyrick *et al.*, 1989], pinocytosis [Prain and Pierce, 1989] and phagocytosis [Bryne and Moulder, 1978] for entry into cells. Involvement of adhesins for receptor mediated endocytosis have also been reported [Bavoil *et al.*, 1996; Hackstadt *et al.*, 1999]. There is no evidence of macropinocytosis [Finlay and Cossart, 1997] or upheaval of apical membrane through microvillus effacement in *Chlamydiae* [Jerse *et al.*, 1990].

Early intracellular events

There are series of rapid events which take place once the infectious EB comes in contact with the epithelial cell surface. The changes are essential to make the host niche ready for a productive infectious cycle. First, the EB endosome drops to pH 6.2 and then stabilizes at 6.6 due to retention of Na^+/ K^+/ ATPase [Schramm *et al.*, 1996]. There is no fusion with or maturation of EB-containing endosomes to lysosomes at this stage. High resolution transmission electron microscopy has shown that tubular endosomes containing transferrin-conjugated horseradish peroxidase adjacent to and not fused to EB-containing vesicles. Two hours post infection, the vesicles lacks transferrin receptor, mannose-6-phosphate, LAMP1, cathepsin D and vascular H^+ ATPase, markers of early and late endosomes and lysosomes respectively.

Next there is a rapid accumulation of F-actin and clathrin [Majeed and Kihlstrom, 1991] to scaffold the redistribution of EB endosomes to the perinuclear space, an event triggered by tyrosine phosphorylation of epithelial proteins [Birkelund *et al.* 1994, Fawaz *et al.* 1997] by EB receptor signaling. Further, homeostatic levels of intracellular calcium ($(Ca^{2+})_i$) make annexins III, IV and V bind specific endosomal membrane phospholipids for regulating homotypic fusion of EB-containing vesicles with each other and not with lysosomes. However, homotypic vesicle fusion does occur in all chlamydial species. In *C. trachomatis*, homotypic fusion results in a single vacuole containing EBs, and is called a **nascent inclusion**. This helps in genetic exchange as double infection of host cells with two *C. trachomatis* serovars, namely E and F, eventually resulted in a single inclusion with both serovars [Ridderhof and Barnes, 1989; Suchland *et al.*, 2000]. However, in *C. psittaci* and *C. pneumoniae* multiple inclusion formation takes place. In host cells doubly infected with *C. trachomatis* and *C. psittaci*, the *C. trachomatis* endosomes fused with one another but not with any of the multiple *C. psittaci* inclusions in the same infected host cell. Finally, concomitant early gene expression results in vacuole modification which ensures that the EB is not diverted from the endocytic pathway to the exocytic pathway [Hackstadt *et al.*, 1997].

Host cell responses are modified by several pathogenic gram-negative bacteria by injecting effector molecules within host cytoplasm by a type III or contact-dependent secretion system. *Yersinia sp., Salmonella sp.*, and *Shigella sp.* are known to directly modify host responses [Hueck *et al*., 1998; Ramamurthi and Schneewind, 2002]. A complex protein secretion system called type III secretion (TTS) system is reported for *Chlamydia* which functions as a 'molecular syringe' to deliver virulence 'effector' proteins into the host cell cytosol [Hsia *et al*., 1997; Fields *et al*., 2003]. TTS

injectisomes and surface projections protrude from chlamydial particle surfaces at all development stages [Bavoil and Hsia, 1998]. It has been observed that (i) in 'free' chlamydiae, the projections are clustered asymmetrically at one pole, in a regularly spaced hexagonal array and are not uniformly distributed at the surface (ii) on 'connected' chlamydiae, the cluster of projections was exclusively associated with the inclusion membrane, that is, the smooth, projection-free surface of the bacterium did not appear to make contact with the inclusion membrane [Matsumoto, 1981]. All chlamydial genomes have been shown to contain TTS genes [Stephens *et al*., 1998; Kalman *et al*., 1999; Read *et al.*, 2000, 2003; Shirai *et al.*, 2000; Horn *et al*., 2004].

Contact mediated chlamydial replication depends on TTS injectisomes [Hackstadt *et al*., 1997; Bavoil and Hsia, 1998; Bavoil *et al*., 2000]. Pre-loaded EB projections attach to receptors on the inclusion membrane and release their load [Fields and Hackstadt, 2000; Fields *et al.*, 2003; Hackstadt *et al*., 1997]. This causes a signal transduction cascade which prevents phago-lysosome fusion and allows the EB to differentiate into the RB. The inclusion is a tight fitting structure into which the EB enters and as a result of this, there is a contact established between the pathogen and the membrane from the initiation of the life cycle. This in turn up-regulates TTS pathways and provides a necessary molecular switch for the binary fission of RBs. The inclusion membrane expands within the space available in the host cell. RBs attached to the inclusion membrane replicate and also may be squeezed out of contact. These events coincides with the expression of Cop N protein, a TTS-secreted protein, whose paralog in *Yersinia sp.* which down-regulates TTS [Hsia *et al.*, 1997]. Differentiation of RB to EB involves TTS inactivation by physical detachment from the surface or Cop N-

mediated inactivation [Hackstadt *et al*., 1997; Rockey and Matsumoto, 1999; Fields and Hackstadt, 2000].

Development of the inclusion

Immediately after reductase activity at the epithelial surface and internalization of EB, a complex, lengthy reorganization begins and is fecilitated by ATP stores and its own protein synthesis [Hatch *et al*., 1996; Hatch *et al*., 1999]. Little is known about the kinetics and biochemistry of EB-RB transition. Depending on the chlamydial species, logarithmic growth starts at 6 hours post infection and carries on till 24-40 hours with a generation time of about 2.5 hours. The increase in chlamydia progeny is attributed to binary fission with dumbbell shaped RBs being formed. With the growth in the chlamydial microcolony, the enveloping inclusion membrane also expands. Along with expansion of the inclusion membrane, lipid synthesis occurs as inclusion development is seen to proceed normally in host cells exposed to cyclohexamide-an eukaryotic protein synthesis inhibitor. It is also reported that chlamydial inclusion can intervene vesicles released from the trans-Golgi [Hackstadt *et al*., 1997]. *Chlamydiae* also acquire phosphatidylinositol and phosphatidylcholine produced in the endoplasmic reticulum and cardiolipin that is contained in mitochondria and cholesterol [Wylie *et al*., 1997]. *Chlamydiae* also produce a number of specific proteins termed Inc proteins that also incorporate into the inclusion membrane [Bannantine *et al*., 2000; Rockey *et al*., 1995; Rockey *et al*., 1997; Scidmore-Carlson *et al*., 1999; Rockey *et al*., 2002].

The differentiation of EB to a metabolically active RB is one of the least-understood stages of the chlamydial developmental cycle. For example, the addition of antibiotics that block transcription or translation does prevents conversion of EB to RB when EBs are known to be metabolically inert with DNA in EBs binding to histone-like

proteins HctA and HctB [Parsot *et al*., 1995]. Further, a report that showed histone-DNA interactions in *Chlamydia sp.*, are degraded by the time of germination has not been able to throw light on this dilemma [Grieshaber *et al*., 2004]. Disruption of DNA-histones interactions depend on a metabolite, encoded by chlamydial gene CT804, a homolog of ispE, acts as an antagonist of HctA, in non-mevalonate pathway (MEP) of isoprenoid biosynthesis. It has been shown that the lethal effect of expression of HctA in *E.coli* is prevented by co-expression of CT804 (ispE). This study revealed that ispE is an intermediate enzyme of the MEP pathway. However, the *E. coli* orthologue of ispE did not rescue *E. coli* from the lethal effect of HctA expression. The mechanism that makes chlamydial IspE specific against HctA is not well elucidated. *Chlamydiae* also encode another histone-like protein HctB, which is prevented from functioning during the differentiation from EBs to RBs [Grieshaber *et al*., 2004]. In EBs there is also presence of a large amount of mRNA ("carryover mRNA") and ribosomes. It is very surprising owing to the fact that EBs are metabolically inactive. It could be explained by the fact that pathogens have a transcriptionally active genome immediately after the chromosome decondensation.

The significant fraction of this "carryover mRNA" is represented by mRNAs encoding late gene products as these mRNA are packed into EBs during the final stages of RB to EB differentiation [Belland *et al*., 2003]. However, the presence of these late stage mRNAs does not lead to their transcriptional expression at the early stages of a new infection process. *Chlamydia sp.,* are hypothesized to mechanistically differentiate between carryover mRNA and newly transcribed mRNAs. This is probably carried out by two possible mechanisms. (i) RNA binding protein(s) prevent carryover mRNAs from interaction with ribosomes and (ii) by the processing of carryover mRNAs in such a way

that translational start signals on 5' end becomes degraded. However, during further development of infection, carryover mRNA rapidly became degraded [Belland *et al*., 2003]. Active gene expression starts almost immediately after internalization of *Chlamydia* [Plaunt *et al*., 1988; Crenshaw *et al*., 1990; Wichlan *et al*., 1993].

Chlamydial histone-like proteins Hc1 and Hc2 down-regulate gene expression during differentiation of RB back into EB [Grieshaber *et al*., 2006]. It has been shown that co-expression of small RNA with *hctA*, the gene that encodes Hc1, in *E. coli* inhibited Hc1 translation, but was not able to affect *hctA* mRNA stability or transcription. It has also been demonstrated that IhtA, which inhibits translation of *hctA* mRNA, is present only in RB, while Hc1 was detectable in EB. During infection, IhtA was downregulated while Hc1 was upregulated upon differentiation of RB into EB. This has led to the suggestion that IthA could regulate transition of RB to EB during the chlamydial developmental cycle.

Escape from the host cell

Escape into the host cell cytoplasm is the last step of the chlamydial life cycle. Although the exiting process is simple, the real mechanisms that direct the end of the developmental cycle and trigger transition of RB back to infectious EB are unknown and may involve multiple events. A decrease in host nutrients may signal detachment of RB from the inclusion membrane and activation of histone proteins. Histone proteins can regulate stage-specific expression of genes and initiate DNA condensation [Tao *et al*., 1991, Barry *et al*., 1992].

Different chlamydial species exit host cells in different ways and this supports the idea of complexity of this final stage of the developmental cycle. Some *Chlamydiae sp*., lyse the host cell when exiting while others exit in a less aggressive manner. For

example, *C. trachomatis* serovar D (UW3 isolate) exit cells by exocytosis. The inclusion moves along the exocytic pathway to the epithelial surface for fusion with the plasma membrane. During this process the inclusion exvaginates at the plasma membrane and the host cell remains viable [Todd *et al*., 1985]. Other serovars of *C. trachomatis*, such as LGV strains, destroy the host cell when exiting. It was reported that *Chlamydiae sp.* encode a cytotoxin involved in the process of releasing bacteria from the cell [Belland *et al*., 2001].

Table 1. 1. Changes in *Chlamydia sp.* morphology during development cycle

Time (h)	**Changes in *Chlamydia sp.* Lifecycle**
0	EBs attach to surface of epithelial cells via receptors
8	Engulfment and conversion of EBs to RBs
18	RBs multiply by binary fission
24	DNA condenses and RBs convert to EBs
40	Cell rupture and release of infective EBs

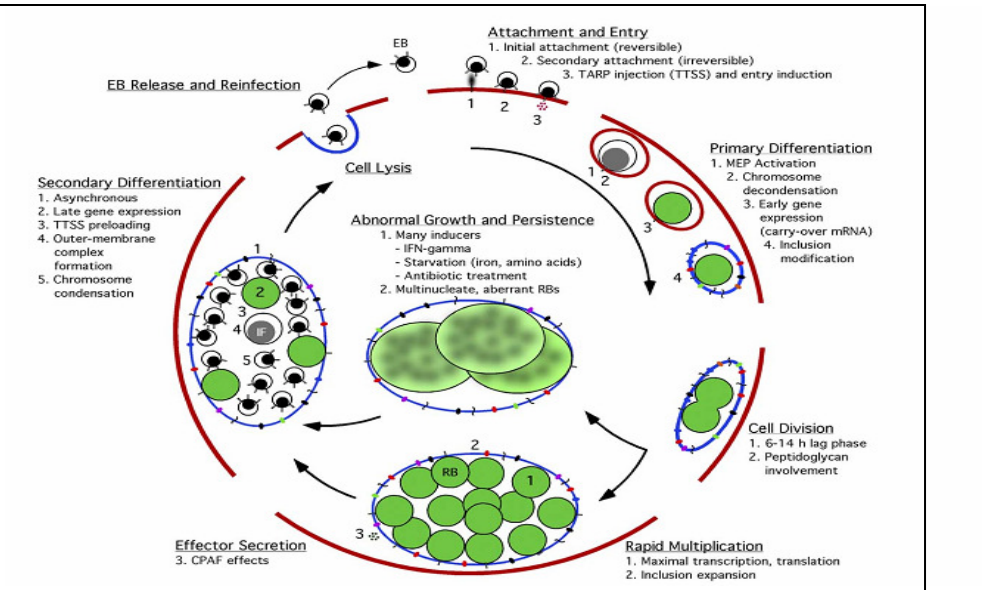


Fig. 1.2. Diagram of chlamydial developmental cycle.
The host cell cytoplasmic membrane (red line) is shown to depict the interactions of Chlamydial EBs and the origin of the inclusion membrane [Abdelrahman and Belland, 2005]

Infections caused by *Chlamydia trachomatis*

Trachoma

Trachoma is the world's leading cause of preventable blindness. The manifestations of ocular trachoma infections range from mild conjunctival lesions (follicular conjunctivitis) to severe forms that eventually lead to scarring and blindness. Severe forms develop through repeated or persistent infections by the *C. trachomatis* serovars A, B, Ba and C [Grayston and Wang, 1975; Grayston *et al.,* 1985; Abu el-Asrar *et al.*, 1989]. Therefore, trachoma is considered the prototype of chronic chlamydial infection. It has been estimated that about 500 million people have had the disease. In the developing countries, about 7 to 9 million people are estimated to be blind because of *C. trachomatis* infection [WHO, 2001]. Trachoma is endemic mainly in tropical and

subtropical countries. The main reservoir of the organism is the eye of an infected person, usually a child, and transmission may be potentiated by flies that carry infected secretion from person to person [Miller *et al.*, 2004].

C. trachomatis infections in women

The genital chlamydial agent was first isolated from the cervix of a mother whose baby had neonatal inclusion conjunctivitis [Jones *et al.,* 1959]. However, knowledge of the diseases caused by *C. trachomatis* in genital tract only began to be gained in the 1970's, after the development of cell culture techniques enabling isolation of the organism from urogenital samples [Grayston and Wang, 1975]. According to the World Health Organization, 92 million new cases occur each year worldwide [WHO, 2001]. The highest incidence of positive chlamydial culture is in the age group of less than 25 years [Hiltunen-Back *et al.*, 2003]. The incidence gradually decreases with increasing age [Grun *et al.*, 1997; Fenton *et al.*, 2001; Hiltunen-Back *et al.,* 2001]. Re-infections are also more common in women under 25 years of age than in older women [Xu *et al.*, 2000; Burstein *et al.*, 2001]. Risk factors related to sexual behavior include an increased number of sexual partners and a failure to use contraceptives, such as condoms [Grun *et al.*, 1997; Burstein *et al.*, 2001; Fenton *et al.*, 2001].

The clinical manifestations of *C. trachomatis* infection in women include acute urethral syndrome, urethritis, bartholinitis, cervicitis, upper genital tract infection (endometritis, salpingo-oophoritis, or PID), perihepatitis (Fitz-Hugh-Curtis syndrome) and reactive arthritis. Genital *C. trachomatis* infection is asymptomatic in up to 80% of women [Stamm, 1999].

The predominant *C. trachomatis* serovars in urogenital infections are the serovars D, E and F [Wang *et al.*, 1985; Saikku and Wang, 1987; van Duynhoven *et al.,*

1998]. Symptomatic genital tract infections in women have been suggested to be related to the chlamydial serovar G [Lan *et al.*, 1995], whereas asymptomatic infections are often caused by the chlamydial serovars D and F [Workowski *et al.,* 1994; Lan *et al.,* 1995]. Serovar E has been found in both symptomatic and asymptomatic women. Furthermore, it has also been shown that almost all patients with repeated chronic or recurrent *C. trachomatis* infections are infected with uncommon complex C serovars [Dean *et al.*, 2000]. In India, the occurrence of *C. trachomatis* serovar D, E, F, G and I in infected women has been reported [Mittal *et al.,* 1998; Singh *et al.,* 2003]. The relationship between different serovars and syndromes is not well elucidated and is of research interest.

C. trachomatis infection leads to mucopurulent cervicitis [Brunham *et al.*, 1984]. Infected patients show symptoms of vaginal discharge, post-coital vaginal bleeding and mild abdominal pain. Although the initial site of infection is the cervix, the urethra and rectum may also be involved [Stamm *et al.*, 1980; Cates and Wasserheit, 1991]. Culture studies have shown that 50 to 60% of the women infected with *C. trachomatis* have cervical and urethral infections, 30% have only cervical infections and 5 to 30% have only urethral infections [Paavonen, 1979; Paavonen *et al.*, 1982; Phillips *et al.*, 1987; Morris *et al.*, 1993]. Most women with chlamydial cervicitis or urethritis are completely asymptomatic or show mild symptoms [Paavonen, 1979; Stamm *et al.*, 1980; Cates and Wasserheit, 1991]. Local signs of infection such as endocervical bleeding, mucopurulent (green or yellow) endocervical discharge and edema within the area of ectopy are seen [Wolner-Hanssen *et al.*, 1988]. *C. trachomatis* has also been associated with endometritis (inflammation of the endometrium) both in the presence and in the absence of salpingitis [Eckert *et al.*, 2002]. Infection of the upper genital tract [e.g.

endometritis, salpingitis] may present irregular vaginal bleeding and abdominal or pelvic discomfort [Greenwood and Moran, 1981; Mårdh *et al.*, 1981; Dieterle *et al.*, 1998]. A physical examination may reveal uterine tenderness or cervical motion tenderness, but a marked proportion of women have completely normal physical findings [Cadena *et al.*, 1973]. *C. trachomatis* infection may persist sub-clinically in the endometrium for a long time [Paavonen *et al.*, 1985] and produce a chronic subclinical infection. It has been shown using endometrial biopsies from women with suspected pelvic inflammatory disease that severe plasma cell endometritis and lymphoid follicles with transformed lymphocytes were significantly more common in a *C. trachomatis* culture-positive group than in a culture-negative group [Paavonen *et al.*, 1985]. The presence of plasma cells in the endometrial stroma (*i.e.* plasma cell endometritis, PCE) seems to be characteristic of chronic endometritis [Greenwood and Moran, 1981]. The frequency of *C. trachomatis* as a causative agent in PCE has been reported to be between 18 to 52% [Kiviat *et al.*, 1986; Paavonen *et al.*, 1987; Paukku *et al.*, 1999] Severe PCE with lymphoid follicles has been shown to be significantly more common in endometritis caused by *C. trachomatis* than in non-chlamydial endometritis [Paavonen *et al.*, 1985]. The natural history and long-term prognosis of endometritis is unknown, but it may occasionally progress to PID with consequent sequelae and subsequent infertility [Munday *et al.*, 2000]. PID has been defined as a syndrome associated with the spreading of microbes from the vagina and cervix to the endometrium, salpingeal tubes and adjacent structures [Weström *et al.*, 1980]. Long-term complications of PID include ectopic pregnancy, TFI and chronic pelvic pain. The majority of PID cases are caused by *C. trachomatis*, *Neisseria gonorrhoeae* or both and anaerobic bacteria. Studies on the prevalence of *C. trachomatis* infection in patients with proven PID have shown that more than half of PID cases are

caused by *C. trachomatis* [Paavonen and Lehtinen, 1996]. Seroepidemiological studies have indicated that chlamydial infections account for a large proportion of these asymptomatic cases by demonstrating a strong link between the presence of serum antibodies to *C. trachomatis* and the presence of tubal pathology [Punnonen *et al.*, 1979]. *C. trachomatis* particularly invades columnar epithelium and the densely ciliated ampullary segment of the fallopian tube is most susceptible to chlamydial infection. In this way, chlamydial PID may cause distal tubal occlusion and subsequent infertility or partial distal occlusion with an increased risk for ectopic pregnancy. Women with PID have a 7- to 10-fold risk for ectopic pregnancy, and 43% of ectopic pregnancies may be due to chlamydial infections. Evidence suggests that ectopic pregnancy may be an acute or long-term complication of *C. trachomatis* infection [Egger *et al.*, 1998]. Chronic pelvic pain occurs in more than 15% of women with previous episodes of PID, and the incidence increases from 11% after one episode to 66% after three or more episodes. It appears to correlate with the presence of peritoneal adhesions. Epidemiological data has suggested that *C. trachomatis* infection is an independent risk factor for the development of cervical carcinoma [Zenilman *et al.*, 2001]. Additionally, *C. trachomatis* infections have been linked to the risk of cervical cancer [Gopalkrishna *et al.,* 2000].

C. trachomatis infections during pregnancy

C. trachomatis is the sexually transmitted bacterial pathogen to be found in an obstetric population, with 2–20% of pregnant women infected [McGregor and French, 1991]. Prevalence of *C. trachomatis* in 21.4% infected pregnant women has been reported from India [Rastogi *et al.,* 1999]. Untreated chlamydial infection during pregnancy is suggested to be associated with a number of adverse outcomes, including preterm labor, premature rupture of membranes, low birth weight, neonatal death and

postpartum endometritis and increased immunocompetency [Martin *et al.*, 1982; Gravett *et al.*, 1986; Martius *et al.*, 1988; Gencay *et al.*, 2000; Rastogi *et al.*, 2002]. *C. trachomatis* may persist in the upper genital tract for months or even years [Shepard and Jones, 1989], and the persistent infection in the endometrium may cause repeated adverse pregnancy outcomes. Early or recurrent pregnancy loss may be induced by asymptomatic *C. trachomatis* infection [Quinn *et al.*, 1987, Witkin and Ledger 1992, Witkin 1999], although not all studies have revealed such a link [Paukku *et al.*, 1999]. *C. psittaci* ovine abortion strain infection in humans is also suggested to cause abortion by inducing an acute inflammatory response in the placenta [Johnson *et al.*, 1985, Flanagan *et al.*, 1996].

C. trachomatis infections in men

The clinical significance of *C. trachomatis* in non-gonococcal urethritis (NGU) and accessory sexual gland infection in men has been established during the past two decades [Paavonen and Eggert-Kruse, 1999]. NGU is the most common clinical genital syndrome seen in males, and *C. trachomatis* is the most important etiological agent in it. Urethral *C trachomatis* infection has been found in 18- 24.7 % symptomatic male NGU patients in India [Bhujwala *et al.*, 1982; George *et al.*, 2003; Vats *et al.*, 2004]. Symptoms of NGU may develop after an incubation period of 7 to 21 days and include dysuria, mild-to-moderate whitish or clear urethral discharge or urethral pruritus. Up to 50% of infected men do not experience symptoms. Other clinical syndromes in men include acute epididymitis, acute proctitis, acute proctocolitis, conjunctivitis, and Reiter's syndrome. In young men, 'idiopathic' epididymitis is often caused by *C. trachomatis*. Unilateral scrotal pain is the primary symptom, and the common clinical signs of this infection include scrotal swelling, tenderness and fever. Chlamydial

proctitis may occur in homosexual men [Stamm 1999]. The prevalence of male chlamydial infection is dependent on age, number of sex partners and socioeconomic factors.

Lymphogranuloma venereum

Lymphogranuloma venereum (LGV) is a sexually transmitted systemic infection caused by the *C. trachomatis* serovars L1, L2 and L3. LGV is uncommon in industrialized countries but highly prevalent in parts of Africa, Asia and South America, and it occurs in both men and women. The LGV serovars of *C. trachomatis* are more invasive than the other genital serovars [Black, 1997]. It predominantly infects lymphatic tissue but may also occur as an acute symptomatic infection without apparent lymph node involvement or tissue reaction at the point of infection. Until 2003, LGV was considered a rare disease outside poor countries. Since then, it has emerged as a significant problem among men who have sex with men in Europe. In 2003, an outbreak of LGV was recognized in the Netherlands. Since that report, similar outbreaks have been seen in France. Cases have also been reported from Sweden and, from the United States and Canada. All the reported cases have been caused by the L2 serovar, 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].

Table 1. 2. *Chlamydia trachomatis* serovars and their associated human diseases [Brunham and

Rey-Ladino, 2005]

Serovars	Human disease	Pathology caused
A, B, Ba and C	Ocular Trachoma	Conjunctivitis, conjunctival and corneal scarring
D, Da, E,F, G, H, I, Ia, J, Ja, K	Oculogenital disease	Cervicitis,urethritis, endometritis,pelvic inflammatory disease, tubal factor infertility, ectopic pregnancy, neonatal conjunctivitis and infant pneumonia
L1, L2 and L3	Lymphogranululoma venereum	Submucosa and lymph-node invasion, with necrotizing granulomas and fibrosis

Diagnostic Tests for *C. trachomatis* Infections

There are three types of specific screening tests: (a) cell culture; (b) "direct" antigen or nucleic acid detection assays; and (c) DNA/ RNA amplification. Nonspecific tests of inflammation, such as leukocyte esterase dipstick, may also be used to screen for infection. Tests vary in the type of specimens, the level of skill required for sampling and transportation, the level of skill in testing laboratory, and the efficiency and accuracy of results. In women, specimens may be obtained from the endocervix, using a swab or from the urethra and vagina using a swab and first-catch urine. In men, specimens can be obtained by swabbing the anterior urethra as well as through first-catch urine. Chlamydial culture from endocervical or urethral swabs was long the "gold standard," but it has been largely supplanted as a screening test because it requires invasive and time-consuming specimen collection, cold transportation of specimens and a high level of laboratory technical expertise, slow processing time (3 to 7 days) and insensitive

relative to newer tests. Non culture assays include enzyme immunoassay (EIA) (e.g. Chlamydiazyme); DNA hybridization probe (e.g. PACE 2); and direct fluorescent assay (e.g. DFA). Rapid "point-of-care" tests are self-contained EIA kits that can be completed in 30 minutes in the clinician's office and have also been recommended [Pate *et al.*, 1998; Lauderdale *et al.*, 1999; Chernesky *et al.*, 1999]. These tests are designed to require very little expertise, but in practice they have significantly lower sensitivity than laboratory-based EIA testing. The newest generation of chlamydia-detection tests utilizes nucleic acid amplification methods. Several studies have shown that nucleic acid amplification tests are far superior to tests for detection of *Chlamydia sp.* than culture and non-culture based enzyme immunoassays and serological tests [Schachter et al., 2002]. Polymerase chain reaction (PCR) has been established as the standard assay for rapid diagnosis of genital chlamydial infection [Bobo *et al.,* 1991; Chen *et al.,* 2002]. National screening programs have shown that by using sensitive and cost-effective PCR-based methods to identify infected women [Miller *et al.,* 2004; Keise *et al.,* 2005], high rates of morbidity associated with this infection can be reduced. Detection of chlamydial species using real-time PCRs and microarrays has also been recommended [Sachse *et al.,* 2005; Jalal *et al.,* 2007].

Genome Organization

Chlamydia sp., are among the oldest described human pathogens, and have long fascinated researchers because they are obligate intracellular bacteria that have a unique developmental cycle and can infect virtually all cell types. Because no genetic tools or cell-free methods of culture are available to manipulate the chlamydial genome, genomics has provided important insights into chlamydial biology. Complete genome sequences are now available for the *C. trachomatis* pathotypes: ***Chlamydia trachomatis***

A/HAR-13 (serovar A), a trachoma strain; ***Chlamydia trachomatis*** **D/UW-3/CX** (serovar D), an STI strain; ***Chlamydia trachomatis*** **434/Bu (serovar L2)**, a classical LGV strain; and ***Chlamydia trachomatis*** **L2b/UCH-1/proctitis** (serovar L2b), a proctitis LGV strain [Stephens *et al*., 1998; Carlson *et al*., 2005; Thomson *et al*., 2008]. Whole-genome analysis has shown that the size of these 4 genomes varies by a maximum of 5,000 bp, with all 4 genomes comprised of ~1.04 Mbp. The four serovars also share a high level of synteny and genome-sequence conservation. This conservation is best illustrated by comparing the coding capacities of the three genomes. A comparison of the gene sets of single trachoma, STI and LGV strains revealed that 846 coding sequences (CDS) were common to all 3 genomes (the total CDS count for these genomes range from 889–920). Of the remaining genes, most differences that were noted represented differences in *in silico* gene prediction rather than real differences in gene content. Further, it is believed that horizontal gene transfer has probably not had a significant impact on the changes in disease pathology that are caused by this group of organisms [Thomson *et al*., 2008]. The plasticity zone varies in size between the different *C. trachomatis* genomes from approximately 20 to 25 Kbp [Stephens *et al*., 1998; Carlson *et al*., 2005; Thomson *et al*., 2008].

Inclusion Membrane Proteins of *Chlamydia*

Sequencing of the whole genome of *C. trachomatis* serovar D and usage of the RT-PCR technique has helped to identify multiply genes that are expressed in different stages during the developmental cycle. Temporal stages of gene expression based on different times post infection (p.i.) were classified as early (1- 2 h p.i.), mid-cycle (3-18 h p.i.), and late (19-48 h p.i.). Usage of genome-wide micro-arrays revealed various stage specific genes [Belland *et al*., 2003]. Genes encoding many inclusion membrane proteins

(Inc) have been identified as early expressed genes [Shaw *et al*., 2000]. Despite the fact that functions of most of inclusion membrane proteins have not yet been established, these proteins are considered as promising candidates for the role of interactors with the host cell [Scidmore *et al*., 2001; Alzhanov *et al*., 2004]. It is but logical that all proteins produced at the early stage of chlamydial life cycle serve two major purposes. The first is the effective establishment of nutrient delivery to developing bacteria and the second is the protection of the inclusion from fusion with lysosomes.

Incs were originally identified in *C. psittaci* [Bannantine *et al.*, 1998, Rockey *et al.*, 1995, Rockey and Rosquist, 1994] and subsequently found in *C. trachomatis* [Scidmore-Carlson *et al.*, 1999]. All Inc share a singular common feature of a predicted hydrophobic domain of 40 or more amino acid residues [Rockey *et al.*, 1995; Bannantine *et al.*, 1998; Scidmore-Carlson *et al.*, 1999]. However, there is little or no primary sequence similarity even within the predicted hydrophobic domains [Stephens *et al.*, 1998; Kalman *et al.*, 1999, Read *et al.*, 2000]. More than 40 proteins bearing this characteristic feature have been identified *in C. trachomatis* with almost half of them being localized to the inclusion membrane [Bannantine *et al.*, 2000, Shaw *et al.*, 2000]. Microinjection of specific antibodies has demonstrated the exposure of *C. psittaci* IncA [Rockey *et al.*, 1997] and five *C. trachomatis* Incs on the cytoplasmic face of the inclusion: Inc A, F, G [Hackstadt *et al.*, 1999] and CT226 and CT229 [Alzhanov *et al*., 2009]. Four of the five antibodies against *C. trachomatis* were prepared against the C-terminal hydrophilic domain. Only one (anti-CT226) was prepared against the N-terminal hydrophilic domain.

Portions of IncA [Hackstadt *et al.*, 1999, Rockey *et al.*, 1997] and IncG [Hackstadt *et al.,* 1999] are exposed to the host cell cytosol where they are free to

interact with host cell proteins. *C. psittaci* IncA is phosphorylated by an unidentified host kinase [Rockey *et al.*, 1997], although there is no evidence that *C. trachomatis* IncA is similarly phosphorylated. Cytoplasmic protein 14-3-3ß interacts with IncG resulting in its phosphorylation at a predicted C-terminal phosphorylation site. [Scidmore and Hackstadt, 2001].

Shaw *et al.,* in an RT-PCR-based survey of 39 confirmed and putative inclusion membrane proteins revealed that 29 of those were expressed early (2 h post-infection) in the developmental cycle [2000]. A thorough screen of temporal gene expression throughout the *C. trachomatis* L2 developmental cycle including >8% of the open reading frames in the chlamydial genome was conducted. The genes considered early, detected at 2 h post-infection, were those involved in DNA, RNA, or protein synthesis. Interestingly, structural genes or those involved in intermediary metabolism were not transcribed until between 6 and 12 h post-infection and were termed mid-cycle which correlates with ultra structural evidence of binary fission of RBs by 12h post-infection. For comparison, late genes, defined as those transcribed during the terminal differentiation of RBs to EBs, were not detected until 20 h post-infection [Shaw *et al*., 2000]. Collectively, the results suggest that modification of the inclusion membrane is a critical requirement for chlamydial intracellular survival and that only after that requirement is fulfilled do the organisms begin to multiply.

There is not much data on the functions of the Inc proteins. No detectable similarity to eukaryotic proteins implicated in vesicular trafficking has been observed, and no substantial clues as to their function based on homology searches have been found. There are no established genetic systems for chlamydiae and the lack of experimental approaches has led to the lacunae in defining functions of Inc proteins.

Despite this limitation, some progress is being made. Microinjection of anti-*C. trachomatis* IncA antibodies into *C. trachomatis*-infected cells blocks the homotypic fusion of inclusions but does not stop chlamydial multiplication [Hackstadt *et al.,* 1999] that is characteristic of *C. trachomatis* in multiply-infected cells [Blyth and Taverne, 1972; Ridderhof and Barnes, 1989]. The resulting inclusion morphology is indistinguishable from *C. psittaci* GPIC inclusions, which are not only non-fusogenic but appear to partition with the dividing RBs to create a multi-lobed inclusion structure composed of multiple membrane bound vesicles [Rockey *et al.*, 1996]. An unrelated but complementary approach to the study of *C. trachomatis* IncA supports this conclusion; clinical isolates of *C. trachomatis* that do not express IncA also develop a multi-lobed inclusion structure [Suchland *et al*., 2000]. IncA expression is also first detected at approximately 10h post-infection with *C. trachomatis* L2, a time corresponding to the initiation of fusion of inclusion [Hackstadt *et al.*, 1999]. In addition, *C. trachomatis* IncA interacts directly with *C. trachomatis* IncA in yeast two-hybrid systems, but *C. psittaci* IncA interacts neither with itself nor with *C. trachomatis* IncA [Hackstadt *et al.*, 1999]. The temperature dependence of fusion of *C. trachomatis* inclusions [van Ooij *et al.*, 1998] has also been correlated with exposure of IncA on the inclusion membrane [Fields *et al.,* 2002].

Screening for HeLa cell proteins interacting with *C. trachomatis* IncG in a yeast two-hybrid system led to the identification of 14-3-3ß [Scidmore and Hackstadt, 2001]. The association of 14-3-3ß with the inclusion membrane was confirmed by immunofluorescence localization with specific antibodies and expression of GFP-14-3-3ß in infected cells. 14-3-3ß is a member of a highly conserved family of dimeric phosphoserine-binding proteins [Fu *et al.*, 2000]. This family of proteins has been

implicated in a number of signal transduction pathways by virtue of their ability to alter the intracellular distribution of bound ligands. Many different proteins are bound by 14-3-3ß and, because they are dimeric, may function as bridging molecules between different ligands [Jones *et al.*, 1995; Liu *et al.*, 1995]. In addition, a possible role in regulation of vesicular transport is suggested by the observation that 14-3-3ß complement clathrin heavy chain mutants in yeast [Gelperin *et al.,* 1995]. The function of 14-3-3ß interaction with IncG in chlamydiae remains unknown. All serovars of *C. trachomatis* express IncG and show association of 14-3-3ß with the inclusion membrane. Surprisingly, *C. psittaci* and *C. pneumoniae* neither express IncG nor show association with 14-3-3ß [Scidmore and Hackstadt, 2001]. This suggests that the interaction of the inclusion with 14-3-3 ß is not required for all chlamydial species and that its function is not involved in the acquisition of properties shared by all chlamydiae.

Although one might expect that chlamydial proteins located at the interface of the inclusion with the cytoplasm would likely participate in defining interactions with the host cell, they would therefore be among the most conserved. Surprisingly, the Inc proteins are not at all well conserved between species [Kalman *et al.,* 1999; Read *et al.,* 2000; Stephens *et al.,* 1998]. *C. trachomatis* IncA, for example, shares only 21% identity with *C. psittaci* IncA and is approximately 26% smaller. IncA is not represented at all in *C. pneumoniae*. Many of the *C. trachomatis* Incs are not represented in *C. pneumoniae*, although *C. pneumoniae* possesses several unique genes encoding proteins that do have the predicted hydrophobic domain. Other *C. pneumoniae* proteins considered homologous to *C. trachomatis* Incs lack the expected hydrophobic domain. Such diversity in the chlamydial Inc proteins has not yet been explained. Proteins exposed on the cytoplasmic face of the inclusion would be exposed to cytosolic proteases and

possible processing through antigen presentation compartments. It is likely that the variation in these proteins serves to keep the collective concentration high but the copy number of individual protein antigens low. It may be that the hydrophobic domains play more of a structural role than regulating specific interactions.

There are reports of other proteins which are involved in modifying the inclusion membrane. These represent the inclusion membrane-associated proteins. These chlamydial proteins do not bear the characteristic hydrophobic domain but associate with the inclusion membrane. This is shown by the distribution of Cop N, a component of the chlamydial type III secretion system that is localized to the inclusion membrane [Fields and Hackstadt, 2000]. Cap1, a 31-kDa *C. trachomatis* protein identified by virtue of its recognition by protective $CD8^+$ T cells, also appears to be localized to the inclusion membrane [Fling *et al.,* 2001]. The extreme fragility of the *C. trachomatis* inclusion membrane has so far prohibited more standard biochemical fractionations and proteomic analysis of inclusion membrane proteins. It is likely, however, that other possibly peripherally associated chlamydial proteins will be found on the inclusion membrane.

Immunology of Chlamydial Infections

C. trachomatis is a powerful immunogen which stimulates the host's immunological processes. Inspite of *C. trachomatis* undergoing evolution to be alive within host cells, the host triggers off its own immunological defense mechanisms to combat infection. All components of host immunity including innate immune cells, B cells, and T cells act harmoniously to recognize different stages of the infection. These immune effectors bring about effective clearance of *Chlamydia* organisms, but do also contribute to the tissue pathology associated with *Chlamydia* infection.

Innate Immunity

The genital mucosa is the first line of host defense in genital *C. trachomatis* infection. The entry of chlamydial organisms at this surface is influenced by the timing of the estrus cycle. Shedding of the endometrial epithelium during certain stages of this cycle limits a vigorous *C. trachomatis* infection [Tuffrey *et al.*, 1986; Ramsey *et al.*, 1999]. Defensins inhibit *C. trachomatis* infection *in vitro* [Porter *et al*., 2005]. Innate immune effectors provide protection when chlamydial organisms are able to enter the mucosal lining. The role of epithelial cells are to this end are known as they are capable of initiating innate immune responses [Quayle, 2002]. Pro-inflammatory cytokines such as interleukin (IL) -1, IL-6, tumour necrosis factor-α (TNF-α) and Granulocyte-macrophage colony-stimulating factor (GM-CSF) are produced in *C. trachomatis* infection of both human and murine epithelial cells [Rasmussen *et al.*, 1997; Johnson, 2004]. In addition, chemokines such as IL-8 are secreted by infected epithelial cells and they recruit classical innate immune cells [Buchholz and Stephens, 2006] including natural killer cells and phagocytes such as neutrophils, macrophages and dendritic cells [DCs], which are abundant in the genital mucosa. These in turn produce more inflammatory cytokines such as TNF-α, which can lead to the restriction of *C. trachomatis* growth within the infected epithelial cells [Dessus-Babus *et al*., 2002]. These inflammatory cytokines also contribute to the pathology associated with *Chlamydia* infection. 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]. Interferon gamma (IFN-γ), a major inflammatory cytokine produced by innate cells can control chlamydial replication by upregulating the phagocytic potential

of macrophages, thereby promoting the engulfment and destruction of extracellular EBs [Zhong and de la Maza, 1988]

Adaptive Immunity

B cell responses to *C. trachomatis*

Components of the adaptive immune system are necessary to limit chlamydial infection and provide protection for subsequent infections. It has been shown that in severe combined immunodeficiency mice, which lack an adaptive immune system, the susceptibility to chlamydial infection is very high [Magee *et al.*, 1993]. B cells are able to recognize soluble antigens via B cell receptors. B cells and the antibodies produced by them can mediate immunity to *C. trachomatis* has been documented very early in literature when it was observed that the presence of *Chlamydia*-specific antibodies provided protective immunity against *C. trachomatis* infection in humans [Jawetz *et al.*, 1965; Barenfanger and MacDonald, 1974]. Monoclonal antibodies against chlamydial MOMP can neutralize infection *in vitro* [Peeling *et al.*, 1984; Peterson *et al.*, 1991] and in mice [Cotter *et al.*, 1995]. There is also evidence that Fc receptor mediated activities of antibodies also play an important role in combating infection [Moore *et al.*, 2002; Moore *et al.*, 2003]. B cells have not been found to be critically involved in controlling primary chlamydial infection. It has been reported that B cell-deficient mice control primary genital infection as efficiently as wild-type mice [Su *et al*., 1997]. However, clearance following secondary infection is slightly delayed in the absence of B cells [Su *et al*., 1997; Williams *et al*., 1997], suggesting the role of B cells in generating memory response to *C. trachomatis.* This protective effect is chlamydial serovars specific [Johansson and Lycke, 2001]. It is also reported 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]. These studies point clearly that there $CD4^+$ T cells and B cells are involved in providing immunity to *C. trachomatis* infection.

T-cell responses to *C. trachomatis*

T cell responses are highly essential for anti-chlamydial immunity as they can recognize established infection in affected cells and accordingly mount a immune response for clearance of infection. The role of both $CD4^+$ and $CD8^+$ T cells is documented. Nude mice, which lack T cells, have been reported to establish chronic chlamydial infection whereas wild-type mice clear infection [Rank *et al.*, 1985]. Transfer of polyclonal *Chlamydia*-specific T cells into *Chlamydia*-infected T cell-deficient mice also facilitates bacterial clearance [Ramsey and Rank, 1991]. $CD4^+$ T cells contribute majorly to controlling *Chlamydia* infection, although the role of $CD8^+$ T cells have been demonstrated [Landers *et al.*, 1991; Magee *et al*., 1995; Morrison *et al*., 1995; Williams *et al*., 1997]. *Chlamydia*-specific $CD4^+$ and $CD8^+$ T cell clones can protect against chlamydial infection on adoptive transfer into nude mice [Igietseme *et al*., 1993; Igietseme *et al*., 1994] or into immunocompetent mice [Starnbach *et al*., 1994; Su and Caldwell, 1995; Roan and Starnbach, 2006; Roan *et al*., 2006].

Differential antigen recognition by $CD4^+$ and $CD8^+$ T cells

$CD4^+$ and $CD8^+$ T cells recognize antigens that are processed through different pathways. $CD4^+$ T cells typically recognize antigens that are engulfed by professional antigen presenting cells (APCs). These antigens are processed by proteases within the lysosomal compartments of APCs, and the resulting peptides are presented by major histocompatibility complex (MHC) class II molecules to $CD4^+$ T cells [Trombetta and Mellman, 2005]. Chlamydial EBs in the extracellular space within tissues can be phagocytosed by professional APCs and thereby serve as a source of $CD4^+$ T-cell antigens. RB antigens can also be presented to $CD4^+$ T cells by professional APCs that have engulfed infected cells harboring RB organisms. $CD4^+$ T cells may therefore recognize antigens from multiple stages of chlamydial infection. In contrast, $CD8^+$ T cells typically recognize antigens that have access to the cytosol of infected cells. These cytosolic antigens are processed by the proteasome into peptide fragments, which are eventually shuttled to the cell surface in complex with MHC class I to activate CD8 T cells [Cresswell *et al*., 2005]. Although chlamydial bodies are confined to the inclusion during the intracellular stages of the developmental cycle, a number of chlamydial proteins have access to the host cytosol. These include proteins that are secreted into the cytoplasm [Zhong *et al.*, 2001; Clifton *et al.*, 2004] as well as inclusion membrane proteins that have domains which extend into the cytosol of the host cell [Bannantine *et al.*, 2000; Fling *et al.*, 2001]. Chlamydial proteins that have access to the cytosol have been demonstrated to serve as $CD8^+$ T-cell antigens [Fling *et al*., 2001; Starnbach *et al*., 2003].

Antigen specific T-cell responses to *C. trachomatis*

Both $CD4^+$ and $CD8^+$ T cells respond to *C. trachomatis* infection, but antigen specific T cell recognition has being investigated in the last decade. *C. trachomatis*-specific T-cell epitopes in mice have been enumerated and their role in generating immunity against a single antigen over the course of infection has been studied [Fling *et al*., 2001; Starnbach *et al*., 2003]. Furthermore, T-cell receptor (TCR) transgenic and retrogenic mice specific for defined *Chlamydia*-specific $CD4^+$ and $CD8^+$ T-cell antigens have allowed for examination of the early response of *Chlamydia*-specific T cells to cognate antigens [Roan and Starnbach, 2006; Roan *et al*., 2006]. Both TCR transgenic and retrogenic mice serve as an abundant source of antigen-specific T cells that can be adoptively transferred into wild-type recipient mice, thereby boosting the frequency of these cells so that they can be detected during the early phases of the infection before the T cells have clonally expanded. By adoptively transferring *Chlamydia*-specific TCR transgenic and retrogenic cells into recipient mice, it has been demonstrated that in response to genital infection with *C. trachomatis*, both *Chlamydia*-specific $CD4^+$ and $CD8^+$ T cells are activated and proliferate in the iliac lymph nodes that drain antigen from the genital tract.

Furthermore, both subsets of T cells develop the ability to produce IFN-γ before migrating to the infected genital mucosa [Roan and Starnbach, 2006; Roan *et al.*, 2006]. In these studies, proliferation of both Chlamydia-specific $CD4^+$ and $CD8^+$ T cells is not initiated until several days following genital infection with *C. trachomatis*. The period between chlamydial inoculation and activation of the antigen-specific T cells may define the amount of time required for chlamydial antigens to reach the draining lymph nodes

where they can be presented to naïve T cells. The delay in T-cell activation may reflect structural differences between the immune system in genital tissues and those found in other mucosal tissues such as the intestine. The intestinal mucosa contains Peyer's patches in which pathogen-derived antigens can be presented to T cells within hours after infection [McSorley *et al*., 2002]. In contrast, the genital mucosa lacks organized lymphoid elements, and the initiation of T-cell responses against genital pathogens must occur outside the genital mucosa in regional lymph nodes such as the iliac lymph nodes. The genital and intestinal mucosa also differ in cell surface adhesion molecules responsible for recruiting lymphocytes [Rott *et al*., 1996; Perry *et al*., 1998].

T cells recognizing antigens expressed early during the chlamydial developmental cycle may play a bigger role in controlling infection because they may recognize infected cells before non-infectious chlamydial RBs have differentiated back into infectious EBs. In contrast, T cells recognizing antigens expressed late during the developmental cycle may be ineffective at containing infection because recognition and destruction of chlamydia-infected cells late in the developmental cycle may simply result in the release of infectious EBs which can go on to infect new sites. T-cell responses may also be influenced by the subcellular localization of Chlamydia protein antigens. In particular, $CD8^+$ T cells typically recognize proteins that have access to the cytosol of non-professional APCs. However, $CD8^+$ T cells can be initially primed against not only cytosolic antigens but also non-cytosolic antigens. Priming of non-cytosolic antigens can occur via cross-presentation, a process whereby professional APCs phagocytose and present antigens from infected epithelial cells to T cells. Although $CD8^+$ T cells specific for non-cytosolic antigens can be primed, during the subsequent effector phase these T cells may never recognize epithelial cells infected with *C. trachomatis* as epithelial cells

are unable to cross-present non-cytosolic antigens. As a consequence, these $CD8^+$ T cells specific for non-cytosolic antigens may not recognize the typical cell type infected with *C. trachomatis*, and may therefore not contribute significantly towards containing the infection. The CrpA antigen has access to the cytosol [Bannantine *et al.*, 2000], and indeed T cells specific for this antigen are protective [Starnbach *et al.*, 2003; Roan and Starnbach, 2006].

Despite host immune cells detecting and responding to chlamydial infection, *Chlamydia* persists within the host. Chlamydial persistence both *in vitro* and *in vivo* has been documented. *In vitro*, an aberrant, dormant persistent form of chlamydiae can be induced by low levels of IFN-γ [Beatty *et al*., 1993]. Nutrient deprivation or antibiotics can also induce *Chlamydia* to differentiate into a phenotypically similar form [Coles *et al.*, 1993; Raulston, 1997; Hogan *et al.*, 2004]. However these dormant forms are reversible to metabolically active forms following removal of conditions. Chlamydial persistence *in vivo* is not directly established. Chlamydial antigens, DNA and RNA have been detected in culture-negative human patients [Rahman *et al*., 1992; Patton *et al.*, 1994; Gerard *et al*., 1998], suggesting the presence of unculturable chlamydial organisms that remain within hosts. In mice, the immune system itself may induce *Chlamydia* into an unculturable form by using immunosuppressant cortisone [Cotter *et al*., 1997]. Chlamydial persistence may cause pathological outcomes associated with infection. As the immune response to the *Chlamydia* wanes, persistent chlamydial may reactivate and replicate, and in turn restimulate inflammation and recruitment of immune effectors to the site of infection. The inflammatory effectors deployed by the immune system may then drive the organisms back into a persistent form. Repeated cycles of chlamydial

persistence alternating with reactivation may stimulate the chronic inflammation in *Chlamydia*-induced disease.

Chlamydial persistence can also be due to the fact that the pathogen has mechanisms to avoid recognition by the host immune components. *C. trachomatis* can directly degrade RFX-5 and USF-1, transcription factors that induce expression of MHC class I and MHC class II respectively within infected cells by CPAF, a chlamydial protease [Zhong *et al*., 1999; Zhong *et al*., 2000; Zhong *et al.*, 2001] Chlamydia-specific $CD4^+$ and $CD8^+$ T cells are then not able to recognize infected cells as MHC expression is affected. Deubiquitinases [DUBs] encoded by *C. trachomatis* also help the pathogen in evading T cell responses [Misaghi *et al.*, 2006]. Removal of ubiquitin from chlamydial proteins by the DUBs, interferes with the targeting of these proteins to the proteasome and further reduced MHC class I presentation of the chlamydial antigens to $CD8^+$ T cells. CPAF is also known to degrade pro-apoptotic BH3-only proteins [Pirbhai *et al*., 2006]. The ability of *Chlamydia sp.,* to degrade BH3-only proteins, sequester pro-apoptotic proteins and induce anti apoptotic factors explains why *Chlamydia*-infected cells are largely resistant to immune-mediated induction of apoptosis *in vitro* [Byrne and Ojcius, 2004; Miyairi and Byrne, 2006]. The influence of *Chlamydia sp.*, on host cell apoptosis is complex [Perfettini *et al*., 2000]. Apoptosis can be mediated through the expression of chlamydial proteins associated with death domain [Byrne and Ojcius, 2004; Miyairi and Byrne, 2006]. In addition, induction of TNF-α may promote apoptosis of both infected as well as bystander cells [Perfettini *et al*., 2000; Jendro *et al*., 2004]. Towards the end of the developmental cycle, it may be beneficial for the organism to induce apoptosis in order to avoid necrosis, which can stimulate inflammation and enhance *Chlamydia*-specific immune responses. Apoptosis of *Chlamydia*-infected cells

towards the end of the developmental cycle may also aid in the release of infectious EBs. There is *in vivo* indirect evidence for immune evasion by *C. trachomatis.* It has been shown that previous exposure of humans to *C. trachomatis* does not provide robust immunity against re-infection [Brunham and Rey-Ladino, 2005].

Gaps in Existing Research

Chlamydial Incs have been suggested to actively modify the inclusion membrane and are involved in development of an intracellular protective niche for survival of the pathogen. Incs are also known to provide contact with host cells but their importance in development of immunity remains to be elucidated. It is also important that the balance between protective and pathological host immune responses to infection be maintained as it finally determines the resulting outcome of chlamydial infection. The ineffective responses which are not able to eliminate chlamydial infection from the host can cause serious chronic inflammation leading to deleterious effect on host cell components and tissue pathology. The governing factors that are responsible for the immune response to chlamydial Incs in infected humans involve the nature of these proteins, the antigens presented during infection and the resultant effector responses generated by immune cells. A better understanding of all these contributory factors is very important for developing an effective vaccine capable of generating sterilizing immunity in *C. trachomatis* infected individuals.

Aims and Objectives

The aim of the study is to provide an insight into the probable roles of two inclusion membrane proteins of *Chlamydia trachomatis* in the host-pathogen cross-talk at the site of infection. The specific objectives of the present study have been defined as under.

1. Propagation of **Chlamydia trachomatis** obtained from clinical samples in cell culture system.

Patients attending the Gynaecology outpatient department of Safdarjang Hospital, New Delhi, India were enrolled after approval of the hospital's ethical committee. Inoculum was prepared from *C. trachomatis* positive endocervical samples and propagated in *in-vitro* cell culture systems.

2. Isolation of **C. trachomatis** inclusion proteins of interest

Full length gene-specific primers were designed for PCR detection and isolation of *C. trachomatis* CT 232 (*incB*) and CT 233 (*incC*) from *C. trachomatis* positive clinical samples.

3. Cloning and expression of inclusion proteins

incB and *incC* PCR products were cloned into pGEM-T vector, characterized by restriction digestion and colony PCR and then used for expression of recombinant IncB and IncC proteins respectively in protein expression vectors.

4. Functional analysis of Chlamydial inclusion membrane proteins B (IncB) and C (IncC)

Humoral and cell mediated immune responses to *C. trachomatis* IncB and IncC were evaluated in *C. trachomatis* infected women to determine the functional role of these proteins.

Introduction

Chlamydia trachomatis (CT) is known to have tremendous impact on human reproductive health and acute infection is a common cause of mucopurulent, often asymptomatic, cervicitis in women. Infection of the female upper genital tract with CT results in pelvic inflammatory disease (PID) and salpingitis. The long-term complications of chronic CT infection include secondary tubal infertility (infertility consequent on infectious disease and synechia development affecting women who could be fertile in the absence of this process) and ectopic pregnancies [Barlow *et al.,* 2001].

The chlamydial inclusion provides the ideal environment known to support chlamydial replication. Efforts have been made to understand the origins and functions of this unusual vesicle. All interactions with host cells are mediated via activities localized at the inclusion membrane. These include mechanisms for nutrient availability, evasion of lysosomal fusion, congenial conditions to support replication and signals for chlamydial development. In the inclusion membrane of chlamydiae-infected cells, no protein of host origin has been identified, but several chlamydial proteins termed inclusion membrane proteins (Incs) have been characterized in several species such as CT serovar L2 [Bannantine *et al.,* 1998; Scidmore-Carlson *et al.,* 1999; Bannantine *et al.,* 2000], *C. pneumoniae* strain TW183 [Shirai *et al.,* 2000], and *C. psittaci* [Rockey *et al.,* 1995; Bannantine *et al.,* 1998b]. The increasing number of new chlamydial Incs in chlamydial genomes has ascertained the importance of characterizing proteins of this family to gain a better understanding of their association in the pathogenesis of chlamydial infection. The absence of systems for genetic transformation of chlamydiae considerably impedes studies of the structure and functions of inclusion membrane

proteins. Moreover, expression of Inc genes in heterologous systems is complicated by insolubility of Incs in cytoplasm and their toxicity for host cell [Bannantine *et al.,* 1998; Bannantine *et al.,* 2000; Subtil *et al.,* 2001].

In this study we characterized two inclusion proteins of CT, IncB and IncC. Gene-specific PCRs were used for detection of *incB* and *incC* genes in CT-infected endocervical samples. IncB and IncC PCRs amplicons were cloned into pGEM®-T vector systems and further characterized by restriction digestion and colony PCR.

Materials

Agarose, Bovine Serum Albumin, Glycine, Glutamine, HEPES, Streptomycin, Gentamycin sulphate, Adenosine, Tris base, RPMI-1640, Kanamycin sulphate, Ponceau S, Diaminobenzamide, Coomassie, and other fine chemicals were purchased from Sigma Chemicals, USA. Terrific Broth, phenyl methyl sulfonyl fluoride, aprotinin, leupeptin and pepstatin were purchased from Amresco, USA. *Taq* polymerase, dNTPs, $MgCl_2$, were from Invitrogen, USA. Anti-GST and GST-purification system were purchased from Amersham, USA and pGEM®-T vector systems were purchased from Promega, USA.

Experimental Methods

1. Study population

A total of 124 women (aged 22 to 36 years) attending the outpatient department of Safdarjang Hospital, New Delhi, India, for gynaecological complaints namely discharge, lower abdominal pain, pelvic inflammatory disease (PID) and infertility were enrolled in the study. The study was approved by the hospital ethical committee and informed consent was obtained from each patient. Procedures followed for enrollment of

patients and sample collection were in accordance with the ethical standards for human experimentation established by the Declaration of Helsinki of 1975 (revised in 1983).

2. Collection of samples

The vulva and cervix were examined for any lesions if any. After cleaning the endocervix with a cotton swab, four cervical specimens were collected on separate cotton swabs and placed in sterile vials containing phosphate-buffered saline (PBS) and vial containing sucrose phosphate glutamate (SPG) transport media. Swabs collected from patients were stored at 4°C and transported to the laboratory within 1 h for diagnosis of CT and other STD pathogens.

3. Detection of CT 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 CT major outer membrane protein (MOMP) 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 labelled monoclonal antibodies directed against CT MOMP which is a common protein antigen located in the outer membrane of the cell wall in all CT serotypes. This FITC labelled antibody binds specifically to CT present in the methanol-fixed smears previously applied to a well on a microscope slide. When viewed under a fluorescent microscope, CT exhibits a bright apple-green fluorescence which are 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 elementary bodies (EBs) were detected by this direct

fluorescent assay (DFA). Samples were simultaneously confirmed for positivity by polymerase chain reaction (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 ^{o}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 *M. hominis* and *U. urealyticum* by diluting the cervical samples in arginine-containing and urea-containing liquid media, respectively, thereafter incubating the media at 37 ^{o}C.

4. Extraction of DNA

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 dissolved in appropriate volume of PBS. 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).The absorbance was measured and the sample DNA concentration was calculated using the formula- Concentration of DNA (µg/ml) = $[(A_1\text{-}A_{ref})f_1\text{–}(A_2\text{-}A_{ref})f_2]$ D where A_1- Absorbance at 260nm; A_2- Absorbance at 280nm; A_{ref} - Absorbance at 320nm; f_1 -

62.9; f_2- 36.0; D-(diluent volume + sample volume) / sample volume. This DNA was used as a template to screen for presence of CT and subsequent detection of *incB* and *incC* genes.

5. Polymerase Chain Reaction (PCR) for detection of CT

DNA from endocervical samples were tested for chlamydial positivity by PCR analysis using CT 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], 500mM KCl), 0.5 µl 10mM 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 (0.5 pg/ml) stained gels were visualized on Alpha Imager gel documentation system (AlphaInnotech, San Leandro, USA).

Samples found positive for CT by pCT PCR were further confirmed by real-time PCRs. For real-time PCR assays, the *RealArt™ C. trachomatis RG PCR Kit* (QIAGEN *Diagnostics* GmbH, Hamburg, Germany) was used for amplification of a 106 bp target region of the chlamydial o*mpA* gene. The *RealArt™ C. trachomatis Plus RG PCR Kit* (QIAGEN *Diagnostics* GmbH) was used for amplifying 111bp fragment of the pCT after removal of *ompA* PCR primers and probes. The analytical sensitivity of the *RealArt™ C. trachomatis Plus RG PCR Kit* was determined by setting up plasmid dilution series from

0.66 to 0.002 copy equivalents μl^{-1}. For the *ompA* based *RealArt™ C. trachomatis RG PCR Kit* dilution series were set from 10 to 0.078 copy equivalents µl -1 according to standard protocols. All real time PCRs were run on the Rotor-Gene™ 3000 instrument (Corbett Research, Australia).

6. *Chlamydia trachomatis* culture and isolation of DNA

Detection of CT in cell culture was performed as described previously by Mittal *et al.,*[1993]. In brief, cervical specimens in SPG transport media were vortexed and 100µL sample was used as inoculum for cell culture. Initially McCoy cells were grown in 25 centimetre square flasks upto 90 % confluence, supplemented with Earle's Minimum Essential Media (EMEM), 10% Fetal Calf Serum having 20 µg/ml Gentamycin and 2 µg/ml Amphotericin B at 35 ^{o}C in a 5% CO_2 environment. Before infection, old media was decanted and cells were treated with DEAE –Dextran (prepared in PBS at 30:1 ratio) and incubated at 35 ^{o}C for 20 mins. The disintegrating monolayers were gently washed off using PBS and inoculum was introduced in flasks at multiplicity of infection (MOI) of 2. Infection was done by shaking tissue culture flasks at 100 rpm for 30 minutes followed by centrifugation at 2000 rpm (900g) for 30 mins. Post-infection, flasks were supplemented with Delbecco's Minimum Essential Media (DMEM), 10% FCS, 2 µg/ml Amphotericin B and 0.5 µg/ml Cyclohexamide and incubated at 35^{o}C in a 5% CO_2 environment. Cultures were harvested at 60 hours post infection (hpi). Detection of chlamydial inclusions was carried by DFA using monoclonal antibody against CT MOMP, in infected McCoy cell cultures on coverslips and visualized on a Zeiss epiflourescence microscope (Carl Zeiss, Germany). Chlamydial EBs were released from the cells by mechanical rupturing using glass beads and sonication. Culture tubes were centrifuged at 3000 rpm for 10 mins at 4 ^{o}C and

supernatants were transferred to 1.5 ml microfuge tubes and centrifuged at 16,000 rpm for 60 mins at 4 ^{o}C. Resulting cell pellets were dissolved in appropriate quantity of SPG (4X) and used for isolation of genomic DNA or subsequent culture.

Genomic DNA was isolated from cell pellets using Wizard Genomic DNA Purification Kit (Promega Corporation, U.S.A) according to manufacturer's instructions. In brief, cell pellets were resuspended in appropriate quantity of Nuclei Lysis Solution. RNase was then added to the solution and the samples were incubated at 37^{o}C for 20 mins to remove RNA contamination. Appropriate quantity of Protein Precipitation Solution was added to the RNase treated cell lysates and after snap chilling and brief centrifugation the samples were subjected to Isopropanol treatment. Samples were centrifuged at 16,000 rpm for 5 mins and 600 µl of 70% ethanol was added to resultant cell pellets. After air-drying for 15 minutes, 100 µl of DNA Rehydration Solution was added and DNA was rehydrated by incubating solution for 4 hours at 37^{o}C. DNA was quantified spectrophotometrically as mentioned above and used as template for cloning of CT *incB* and *incC* genes.

7. CT *incB* and *incC* PCRs

Two sets of primers, set 1(S1) and set 2(S2) were used for PCR amplification of both CT *incB* (CT 232) and *incC* (CT 233) genes in patients' samples. For *incB*-S1 and *incC*-S1 primer sets used were same as mentioned earlier [Kostrjukova *et al.,* 2005]. The sequences for primers were: for *incB* S1 forward primer 5'- CAT ATG GTT CAT TCT GTA TAC AAT TC-3' and reverse primer 5'- GAT CCC CTA TTC TTG AGG TTT TGT TTG -3'; for *incC* S1 forward primer 5'- CAT ATG ACG TAC TCT ATA TCC GAT -3' and reverse primer 5'- CTC GAG TTA GCT TAC ATA AAG TTT G -3'. Standardization of the optimum conditions for *inc* PCRs was performed initially.

Reaction cocktails with gradually increasing concentrations of 1.5mM -3.5mM $MgCl_2$ and annealing temperatures (50 °C -67 °C) were used to determine the best amplification conditions through gradient PCR. For PCRs, reaction volumes (total volume 25 µl) contained: 5 µl (750 ng/ ml) DNA, 2.5 µl 10X PCR buffer (200mM Tris-HCl (pH 8.4), 500mM KCl), 0.5 µl 10mM dNTP mixture, 0.75 µl 50mM $MgCl_2$, 3 µl of each primer (5 pM/µl), and 0.1 µl Taq DNA Polymerase (5 U/µl, Invitrogen, USA). PCR reaction tubes were subjected to cycling parameters for *incB*-S1 amplification at 94 °C / 1 min, 55 °C / 45 sec, 74 °C / 1 min for 34 cycles and *incC*-S1 amplification at PCR at 94 °C / 1 min, 54 °C / 45 sec, 74 °C / 2 min for 36 cycles. Further, in this study, unique primer pairs (S2) for both *incB* and *incC* genes were designed. Standard bioinformatics tools were used to determine the nucleotide sequence of *incB* and *incC*. The position the genes in the published CT genome database (available from http://chlamydia-www.berkeley.edu) have been identified using **BLAST** (http://www.ncbi.nlm.nih.gov). Unique primer pairs (S2) for both *incB* and *incC* genes were designed using Primer3 software (primer3_www.cgi v 0.1 beta 1a). Primer sequences for *incB* S2 were: forward primer 5'-GGA TCT ATG GTT CAT TCT GTA TAC AAT TC-3' and reverse primer 5'-GCA TGT CCT ATT CTT GAG GTT TTG TTT G-3'; for *incC* S2 forward primer 5'-CGG GGT ACC ATG ACG TAC TCT ATA TCC GAT-3' and reverse primer 5'-CCC AAG CTT TTA GCT TAC ATA TAA AGT TTG AG-3'. For S2 primer sets, all reaction tubes were subjected to cycling parameters for *incB*-S2 amplification at 94 °C / 1 min, 57 °C / 50 sec, 74 °C / 2 min for 35 cycles and *incC*-S2 amplification at PCR at 94 °C / 1 min, 61 °C / 45 sec, 74 °C / 2 min for 32 cycles. All PCR reactions were performed on DNA Eppendorf Mastercycler personal Thermal Cycler (Eppendorf GmbH, Hamburg, Germany).

Sensitivity limits of incB and incC PCR reactions were determined by using serial dilutions (1:10) of DNA template at 750ng/ ml as starting concentration for all PCR reactions. All amplified PCR products were electrophoresed on 1.2% (w/v) agarose gels and ethidium bromide (0.5 pg/ml) stained gels were visualized on Alpha Imager gel documentation system and the expression intensities were calculated using band densitometry analysis.

Specificity of all PCR products was confirmed by restriction digestion analysis. Restriction digestion maps using gene sequences of *incB* and *incC* available in published CT genome database (available from http://chlamydia-www.berkeley.edu) were generated using bioinformatics softwares (http://tools.neb.com/NEBcutter2/index.php). Ten microlitres of *incB* and *incC* PCR products were subjected for restriction endonuclease digestion using *SphI* (for *incB*) and *HincII* (for *incC*). A 20 µl cleavage reaction was performed using 5mM $MgCl_2$, and 10U of restriction enzyme for 10 hours at 37 °C. Reactions were terminated at 65 °C for 30 min and digested products were analysed by agarose gel electrophoresis as mentioned above.

8. Cloning of *incB* and *incC* PCR products

(i) pGEM®-T Vector

The pGEM®-T Vector System was used for cloning of PCR products. The vector was prepared by cutting pGEM®-5Zf(+)(b) and pGEM®-T Easy Vectors with EcoR V and adding a 3´ terminal thymidine to both ends. These single 3´-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases. These

polymerases often add a single deoxyadenosine, in a template-independent fashion, to the 3´-ends of the amplified fragments. The high copy number pGEM®-T vectors contain T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the a-peptide coding region of the enzyme ß-galactosidase. Insertional inactivation of the α-peptide allows recombinant clones to be directly identified by colour screening on indicator plates. The multiple cloning region of the vector includes restriction sites conveniently arranged for use for checking orientation of cloned inserts [Clark 1988; Mezei *et al.,* 1994; Robles *et al.,* 1998].

(ii) Extraction of DNA from agarose gel

The *incB* S1 and *incC* S1 PCR reaction mix was fractionated by agarose gel electrophoresis. DNA fragments of right size were excised from agarose gel and were eluted from the gel slice using gel extraction kit according to manufacturer's instructions. (Qiagen, CA, USA)

(iii) Ligation

The amplified DNA fragment resolved on agarose gel and extracted from gel was cloned in pGEM®-T Vector. Ligation was set up as follows.

pGEM®-T Vector DNA (50ng)	1.0 µl
inc DNA (75.5 ng)	3.0 µl
Ligation buffer (2X)	5.0 µl
T4 DNA ligase (1U)	1.0 µl

Total 10.0 µl

The ligation reaction mixture was incubated at 14ºC for 16 hrs.

Escherichia coli (E. coli) DH5α competent cells were prepared and the ligated product was transformed and subjected to Amp^r selection.

(iv) Competent cell preparation

The procedure used for the preparation of competent cells was a slight modification of the one described by Cohen *et al.,* [1972]. This protocol was frequently used to prepare batches of competent bacteria that yielded $5x10^6$ to $2x10^7$ transformed colonies per µg of supercoiled plasmid. The host cell culture was streaked on a Luria Bertini (LB) plate from the frozen glycerol stock. A single colony was inoculated into 5ml LB tube. 500µl of the overnight grown culture was further inoculated into 50ml (1:100) LB and allowed to grow for 2-3 hrs until A_{600} reached 0.4-0.5. The culture was chilled on ice, transferred to ice cold 50ml polypropylene tubes and centrifuged at 4000 rpm for 10 mins. The supernatant was decanted and the pellet was resuspended gently in 20ml of ice cold sterile 0.1M $CaCl_2$ and incubated on ice for 30mins. The cells were then centrifuged at 4,000 rpm for 10mins. The pellet was resuspended in 1ml of ice cold 0.1M $CaCl_2$. Chilled glycerol was added to the cells to a final concentration of 20%. About 200µl 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 hrs to enhance the competency of the cells and then stored in aliquots of 200µl at -70°C [Dagert and Ehrlich, 1979].

(v) Transformation of competent cells

Transformation of the competent *E. coli* cells was performed according to procedures described by Sambrook and Russel [2001]. Briefly a 100µl aliquot of competent cells was thawed on ice and 1µl of the *incB* or *incC* clones was added to it, mixed by tapping and incubated on ice for 30 mins. The cells were subjected to heat shock at 42°C for 90 secs in a water bath and were immediately chilled on ice for 1-2 min. 850µl of LB was added to the cells and the cells were incubated at 37°C for 1hr in a shaker incubator with constant shaking. The cells were pelleted and resuspended in 100µl of LB and plated on LB agar plates containing 100µg/ml of ampicillin, 10µl of 0.8 M IPTG and 50µl of 20mg/ml of X-gal and incubated at 37°C for 12hrs for selection of recombinant clones (white) over non-recombinant clones (blue).

(vi) 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 *NdeI* and *EcoRI* as described above and checked on agarose gel for the right size of insert of *incB* and *incC* genes respectively. The orientations of positive colonies were further determined by DNA sequencing on an automated DNA sequencer (ABI3770, Applied Biosystems, Fostercity, CA, USA) and by colony PCR and using T7 universal and *inc*-gene specific primers.

9. Expression of IncB and IncC proteins

Recombinant clones containing full length gene sequences of *incB* and *incC* cloned into pGEX expression vectors (Amersham Pharmacia Biotech Inc., NJ, USA) were obtained as a kind gift from Dr. Guangming Zhong at Department of Microbiology and Immunology, University of Texas Health Science Center at San Antonio, USA. These clones were further propagated in Terrific Broth (Amresco, Ohio, USA) and production of glutathione S-transferase (GST) fusion proteins was performed as follows.

(i) pGEX expression vectors

The Glutathione S-transferase (GST) Gene Fusion System used in this study is a versatile system for the expression, purification, and detection of fusion proteins produced in *E. coli*. The system is based on inducible, high-level expression of genes or gene fragments as fusions with *Schistosoma japonicum* GST. Expression in *E. coli* yields fusion proteins with the GST moiety at the amino terminus and the protein of interest at the carboxyl terminus. The protein accumulates within the cell's cytoplasm. This vector system allows the proteins of interest to be expressed as a fusion protein with glutathione *S*-transferase (GST) fused to the N terminus of the chlamydial protein. GST fusion proteins are purified from bacterial lysates by affinity chromatography using immobilized glutathione. GST fusion proteins are captured by the affinity medium, and impurities are removed by washing. Fusion proteins are eluted under mild, non-denaturing conditions using reduced glutathione. The purification process preserves protein antigenicity and function. If desired, cleavage of the protein from GST can be achieved using a site-specific protease whose recognition sequence is located immediately upstream from the multiple cloning site on the pGEX plasmids. Fusion proteins can be detected using colorimetric or immunological methods.

(ii) Expression of fusion proteins

Fusion protein expression was induced with isopropyl-β-D-thiogalactoside (IPTG; Invitrogen, Carlsbad, CA). To ensure that each fusion protein is produced with adequate quantities of full-length fusion proteins, induction of fusion protein expression was individually optimized using the following variables: IPTG concentration (0.1 to 5 mM), starting number of bacteria (optical density (OD), 0.5 to 1.5), incubation temperature (10°C to 37°C), and time (0.5 h to overnight). After protein induction, bacteria were harvested via centrifugation. The bacterial pellets were resuspended in a Triton lysis buffer (1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 75 IU/ml of aprotinin, 20 μM leupeptin, and 1.6 μM pepstatin in PBS, pH 7.5) and were lysed by short pulses of sonication on ice. After a high-speed centrifugation to remove debris, bacterial lysates were aliquoted and stored at −80°C.

(iii) Purification of fusion proteins

Expressed fusion proteins were assessed by purifying the fusion proteins from lysates using glutathione-conjugated agarose beads (Amersham). 10 mL GST binding buffer (25 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA) were added to cell pellets on ice. 10% Triton X-100 detergent was added to 0.5% final concentration to lyse cells and gently mixed for 5 min. Cell suspensions were frozen for 1h at –70 °C. Cells were thawed and sonicated thrice on ice for short pulses to shear DNA in order to lower viscosity. Cell debris was centrifuged at 16,000 rpm for 30 mins at 4 °C in Oakridge tubes and supernatants were transferred to new tubes. 500 □L of washed GST beads (prewashed in GST binding buffer and resuspended to about 50% slurry) was added to supernatants and tubes were rocked with beads at 4 °C for 5 h. Tubes were centrifuged at 10,000 rpm for 10 mins at 4 °C, supernatants discarded and beads were transferred to

1.5 ml microfuge tubes. 500 □L of elution buffer was added to bead pellets to elute GST proteins (elution buffer- 10mM reduced glutathione/ 50mM Tris-HCL pH 8.0/ 5% glycerol). After incubation at room temperature for 15 minutes, microfuge tubes were centrifuged and supernatants containing eluted proteins were used. Protein concentrations were determined using Bradford assay (Sigma-Aldrich, MO, USA). The non-specific effects of LPS contamination were controlled by treating recombinant proteins with polymyxin B (Sigma-Aldrich). The fusion proteins were checked on sodium dodecyl sulphate (SDS)-polyacrylamide gels stained with a Coomassie blue dye (Sigma-Aldrich) and specificity of fusion proteins were confirmed by western blot analysis using goat anti-GST antibody (1:500) (Amersham). Protein samples that showed a prominent band at the expected molecular weight position were separated into aliquots and frozen at -80°C to be used in further assays.

(iv) Polyacrylamide gel electrophoresis

A 12% separating gel 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 12,000g for 5 min at room temperature. Proteins were analysed by SDS-PAGE according to method of Laemmli [1970] at a constant voltage of 85V in a Bio-Rad mini gel apparatus. 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.

(v) Western Blot analysis

Western blot analysis was performed following the method described by Towbin *et al*., [1979]. 2µg of purified protein was resolved on SDS-PAGE and transferred to PVDF at a constant current of 100 ampere for 2hrs. Blocking was done by using 2% BSA in PBST for 3hrs. For probing the blot with antibody, the membrane was incubated for 2hrs at room temperature with anti- GST 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 and H_2O_2.

Results

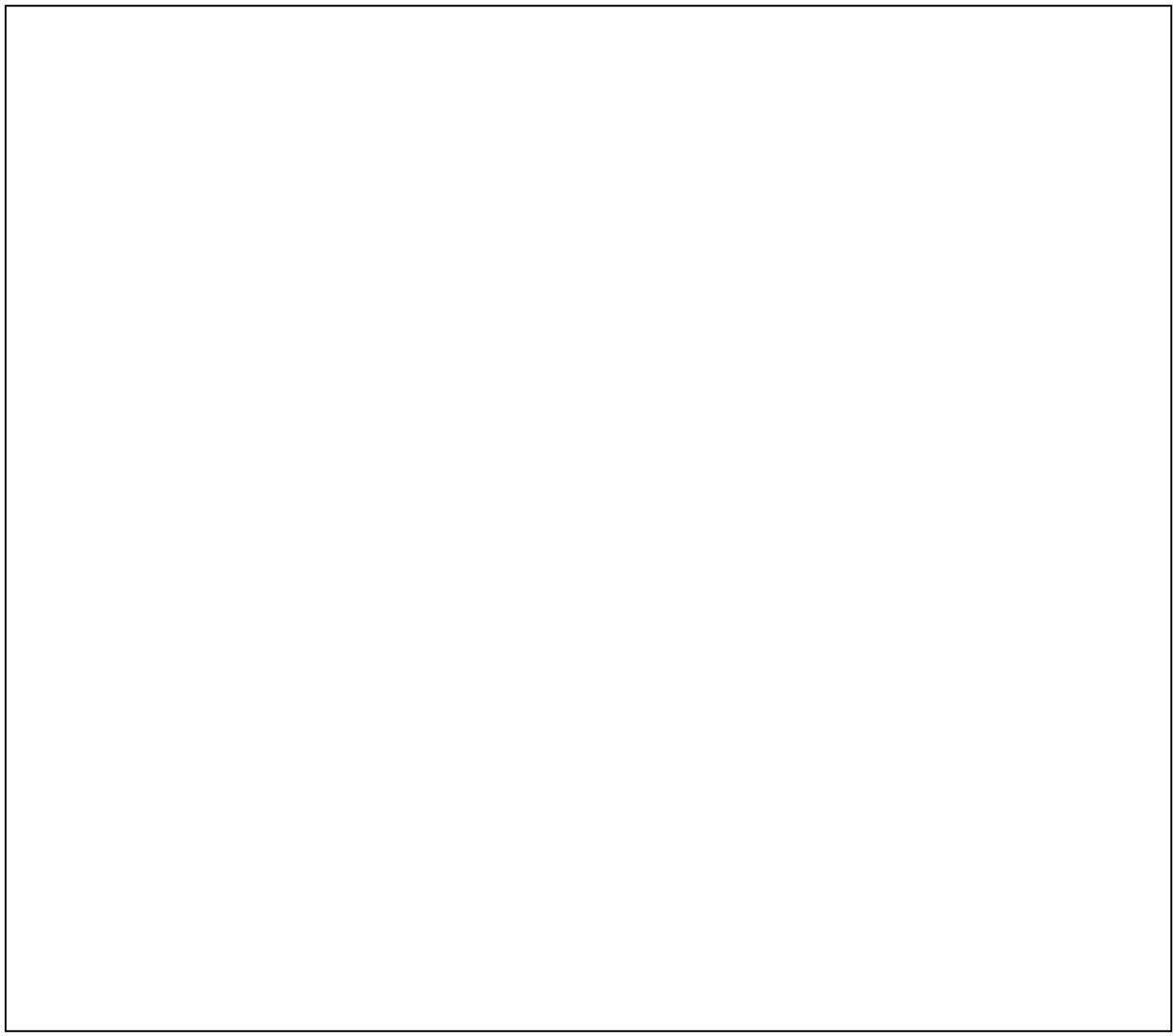
Diagnosis of CT

Cervical CT infection was diagnosed in 41/ 124 (31.8%) patients using pCT PCR which detected 200 bp amplicon of pCT (Table 2.1). All 41 samples were found to be positive for CT by both real-time PCRs targeting pCT and *ompA* gene. Positivity for CT infection was further confirmed by propagation in cell culture system (Fig. 2.1).

Table 2.1. CT positivity by PCR in women (n=124) enrolled for the study

Clinical conditions	(n)	Age [Range]	PCR No. positive (%)
Cervicitis	(64)	25 [2229]	24 (37.5)
PID	(47)	27 [2334]	13 (27.65)
Infertility	(13)	26 [2532]	4 (30.76)

Note. Table showing number of patients positive for CT by 200bp plasmid PCR; PID-Pelvic Inflammatory Disease; Values in parenthesis denote percentage positivity unless otherwise stated; [ ] denotes age range of enrolled patients with various clinical manifestations.



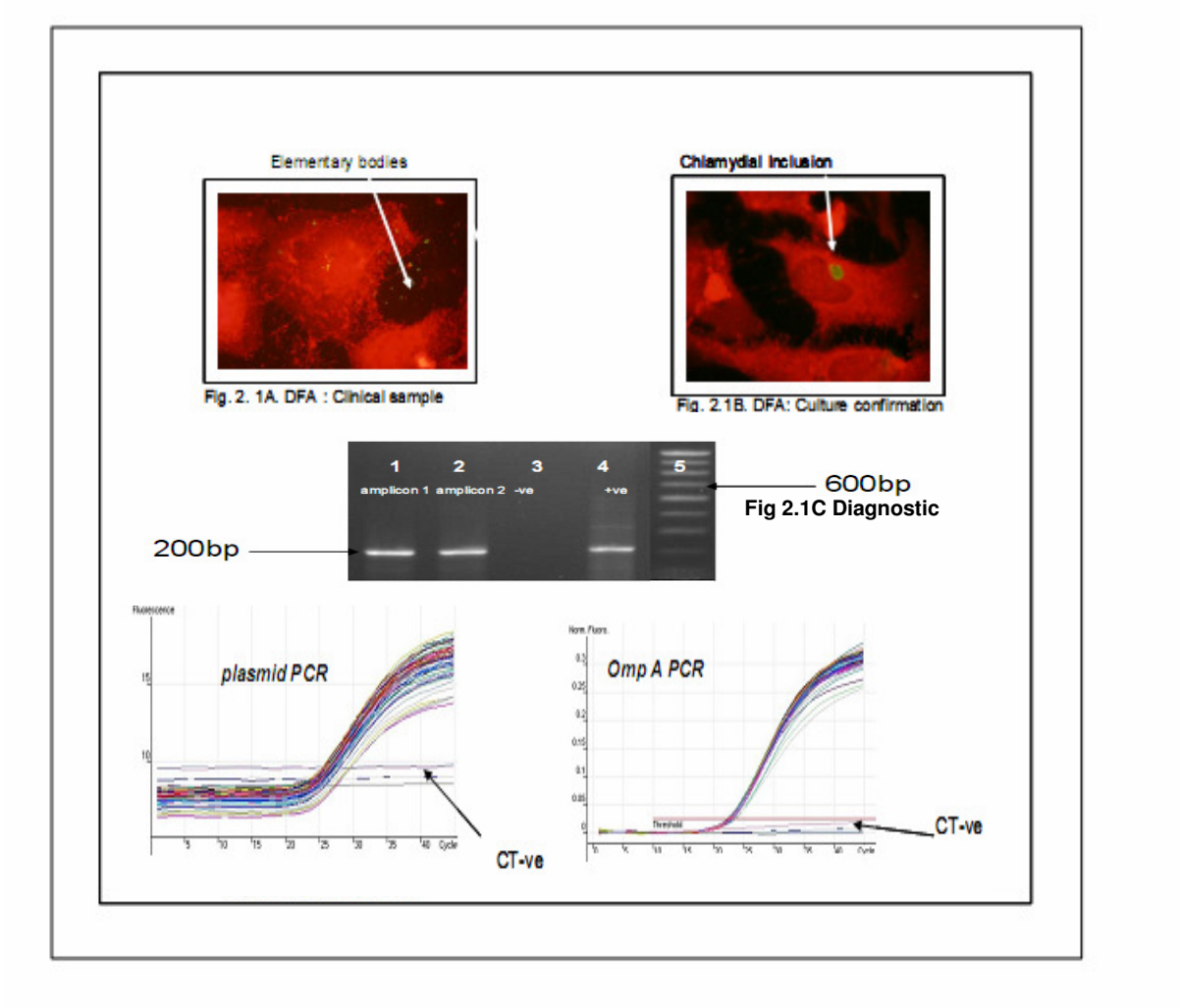


Fig. 2. 1A. Representative figure showing presence of chlamydial elementary bodies in clinical sample by Direct fluorescence assay. (100X magnification); **Fig. 2. 1B**. Representative figure showing presence of chlamydial inclusion at 66 hpi in McCoy infected with clinical isolate (100X magnification); **Fig. 2. 1C.** Representative gel showing PCR positivity for detection of CT in endocervical swabs collected from patients. 1% Agarose Gel electrophoresis of CT plasmid PCR 200 bp amplicons. Lane 1, 2 are CT detected in patient samples , Lane 3 is CT negative patient sample, Lane 4 is positive control, DNA of standard CT serovar D propagated in McCoy cells. Lane 5 is

100 bp Ladder (Invitrogen). Fig. **2. 1D.** Representative figure showing amplification curves for CT positive patient samples as detected by CT plasmid and *ompA* real-time PCRs.

Diagnosis of other STD pathogens

Among the CT-positive patients (n=35), 2 (5.71%) were infected with *Candida sp*., 3 (8.5%) had bacterial vaginosis, 3 (8.5 %) were infected with *M. hominis*, and 4 (11.4 %) with *U. urealyticum*. Amongst CT-negative patients (n=75), 5 (6.6%) were infected with *Candida sp*., 7 (9.3%) had bacterial vaginosis, 6 (8 %) were infected with *M. hominis*, and 29 (24.7%) with *U. urealyticum*. None of the patients had *N. gonorrhoeae or T. vaginalis*.

Detection of incB and incC genes

Visualization of 357 bp (S1) and 360 bp (S2) amplicons for *incB* gene and 545 bp (S1) and 554 bp (S2) amplicons for *incC* gene in a 1.2% (w/v) agarose gel was considered as a positive result for amplification of *inc* genes (Fig.2.2). 39/41 (95.12%) and 40/ 41 (97.56%) of CT-positive patients showed positivity for *incB* and *incC* genes respectively.

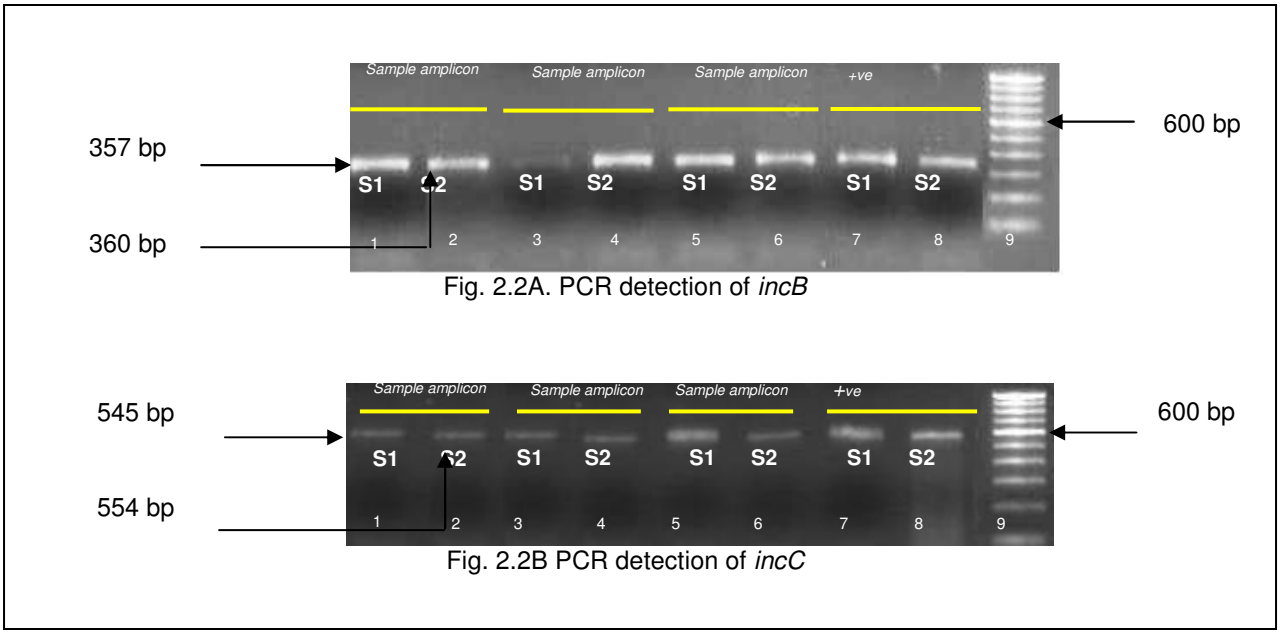


Fig 2.2. Representative agarose gels showing PCR detection of *incB* and *incC* genes in endocervical samples of CT-positive patients.

(A) Agarose gel showing PCR positivity for CT-*incB* gene.

Lanes 1, 3, 5- *incB* S1 PCR amplicons in patients 1, 2, 3 respectively; lane 7-positive control; lanes 2, 4, 6 - *incB* S2 PCR amplicons in patients 1, 2, 3 respectively; lane 8-positive control; lane 9 is 100 bp Ladder (Invitrogen).

(B) Agarose gel showing PCR positivity for CT-*incC* gene.

Lanes 1, 3, 5- *incC* S1 PCR amplicons in patients 1, 2, 3 respectively; lane 7-positive control; lanes 2, 4, 6 - *incC* S2 PCR amplicons in patients 1, 2, 3 respectively; lane 8-positive control; lane 9 is 100 bp Ladder (Invitrogen).

Sensitivity and Specificity of *inc* PCRs

The sensitivity, specificity, positive predictive value and negative predictive value of *incB* and *incC* S1 and S2 PCRs using 200bp pCT PCR as gold standard was

determined (Table 2.2). One sample was considered as false positive as it was found to be negative for pCT PCR but positive for *incB* and *incC* PCRs.

Table 2.2. Performance of *incB* and *incC* PCRs compared to diagnostic PCR for detection of CT

Assay	Sensitivity	Specificity	PPV*	NPV**
incB S1 PCR	97.56	100	100	98.80
incB S2 PCR	95.00	98.80	97.43	97.64
incC S1 PCR	97.50	98.8	97.5	98.8
incC S2 PCR	90.24	100	100	95.4

Note * Positive Predictive Value, **Negative Predictive Value

. The analytical sensitivity of *incB* and *incC* S1 and S2 PCR was determined by densitometric analysis of PCR amplicons. Amplicon intensities were measured in arbitrary units with a starting concentration of DNA at 750 ng/ ml. Densitometric analyses showed that *inc* gene PCRs showed positivity for detection at upto 1:1000 fold dilution (7.5 pg/ ml) of starting DNA concentration *i.e* 750 ng/ ml (Fig 2.3 A). Specificity of *incB* and *incC* PCR products was confirmed by restriction digestion analysis. Amplified PCR products had a restriction enzyme site for *SphI* in *incB* S1 PCR products and for *HincII* in *incC* S1 PCR products. Appearance of two fragments of

211bp and 146bp for *incB* S1 PCR amplicon and 408bp and 137bp for *incC* S1 PCR products was considered positive for confirmation of *inc* genes respectively (Fig. 2.3 B).

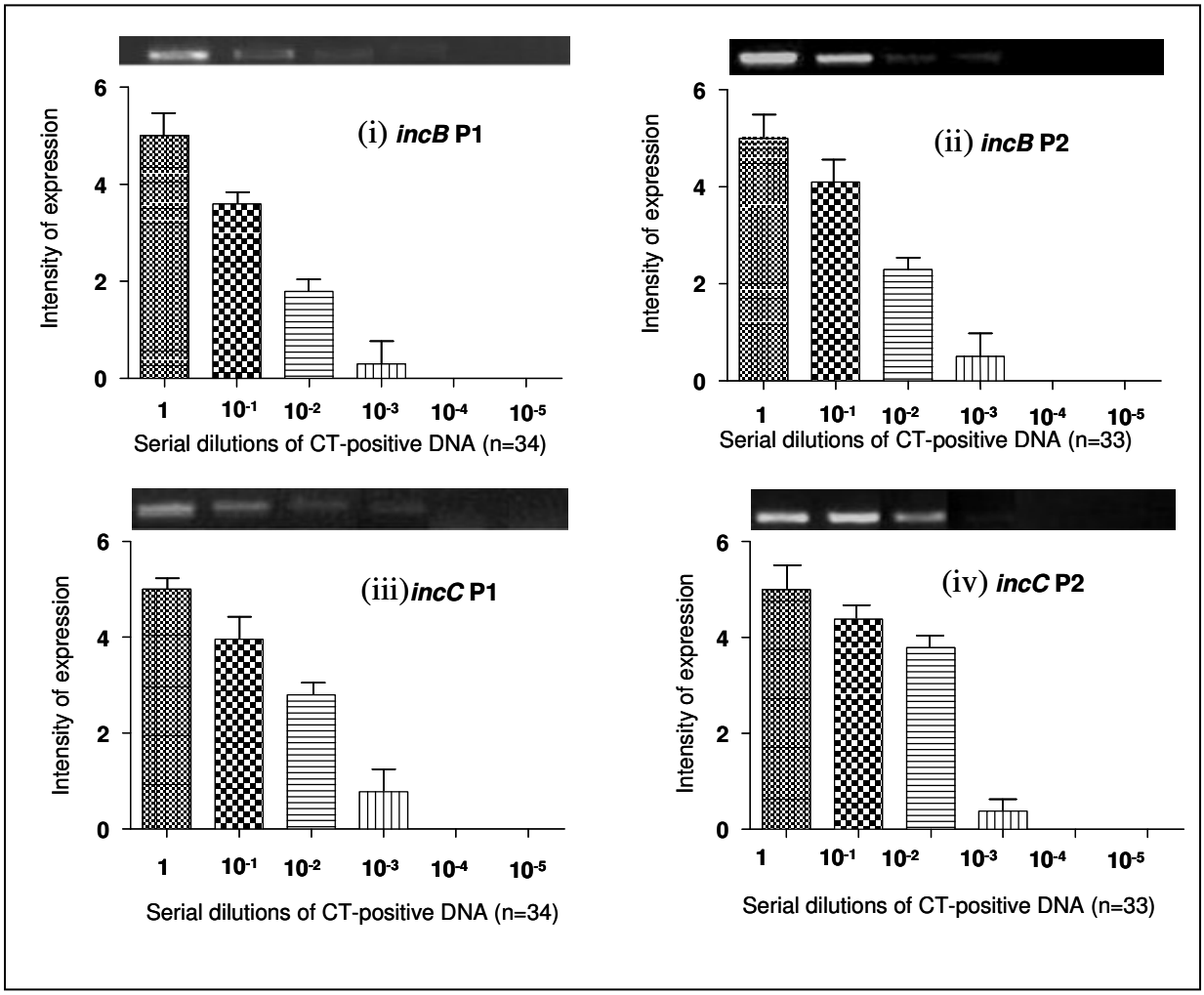


Fig. 2. 3A. Sensitivity of *incB* and *incC* PCRs.

incB and *incC* PCR products were analysed by band densitometry and the intensities of expression of amplicons was compared in arbitrary units. The minimum quantity of CT-positive DNA for which *inc*-PCR band could be visualized was determined. The highest concentration of starting DNA template was 750ng/ ml and was serially diluted to determine analytical sensitivity.

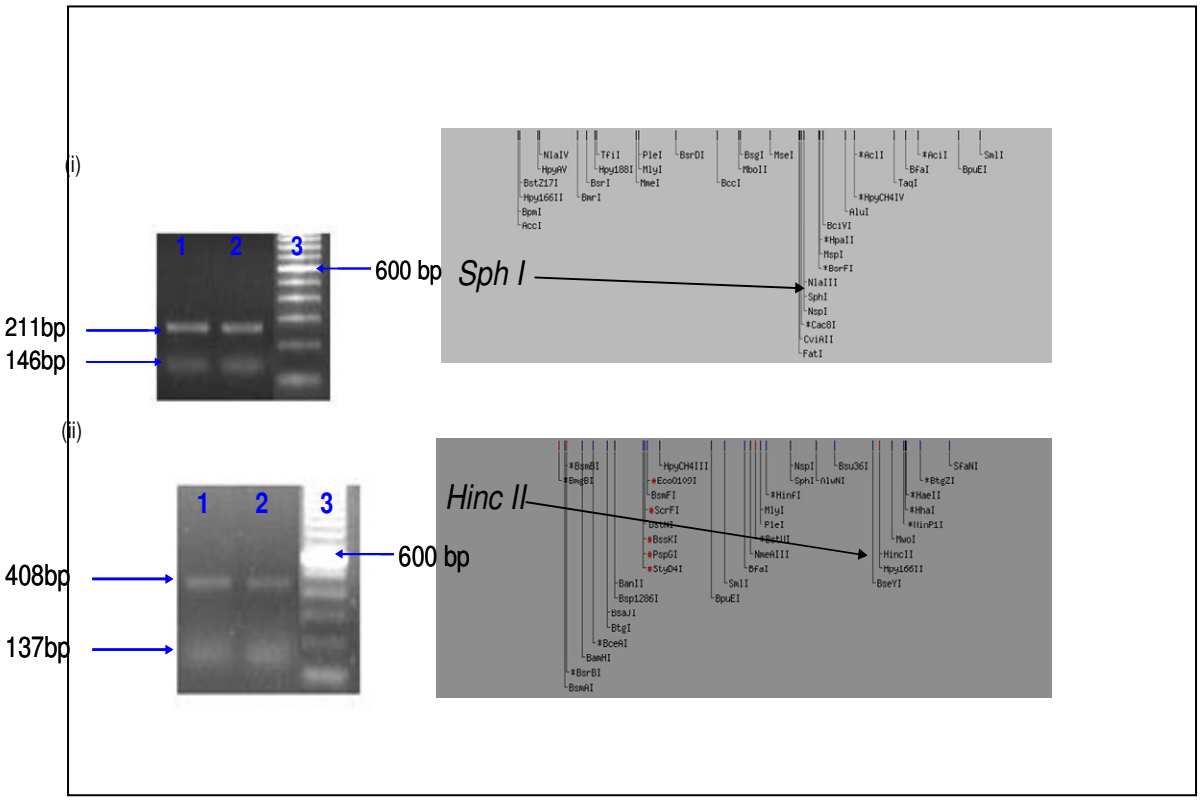


Fig. 2. 3 B. Specificity of *incB* and *incC* PCR products by restriction digestion analysis.

(i) *incB* S1 PCR products (lanes 1, 2) were digested using *SphI* to yield two fragments of 211bp and 146bp. Lane 3 was 100bp ladder (Invitrogen).

(ii) *incC* S1 PCR products (lanes 1, 2) were digested using *HincII* to yield two fragments of 408bp and 137bp. Lane 3 was 100bp ladder (Invitrogen)

Cloning of *incB* and *incC* PCR products

Using universal T7 and *inc* gene specific primers the positive clones containing full-length gene sequences of *incB* and *incC* cloned into pGEM-T vector was determined. Clones with correct gene-orientation were confirmed by the visualization of 403 bp and 615 bp amplicons of *incB* and *incC* colony PCR products (Fig. 2.4).

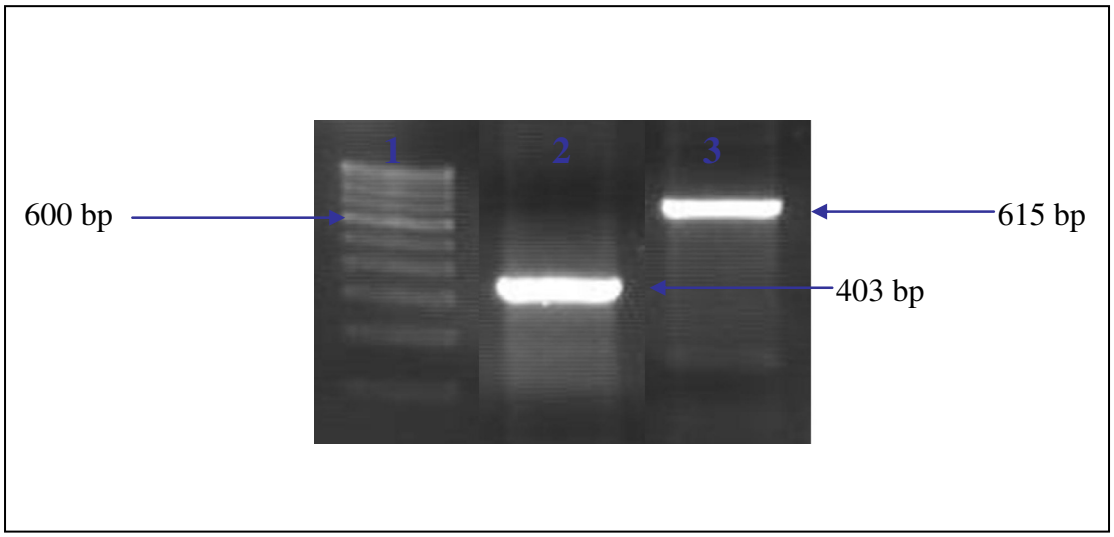
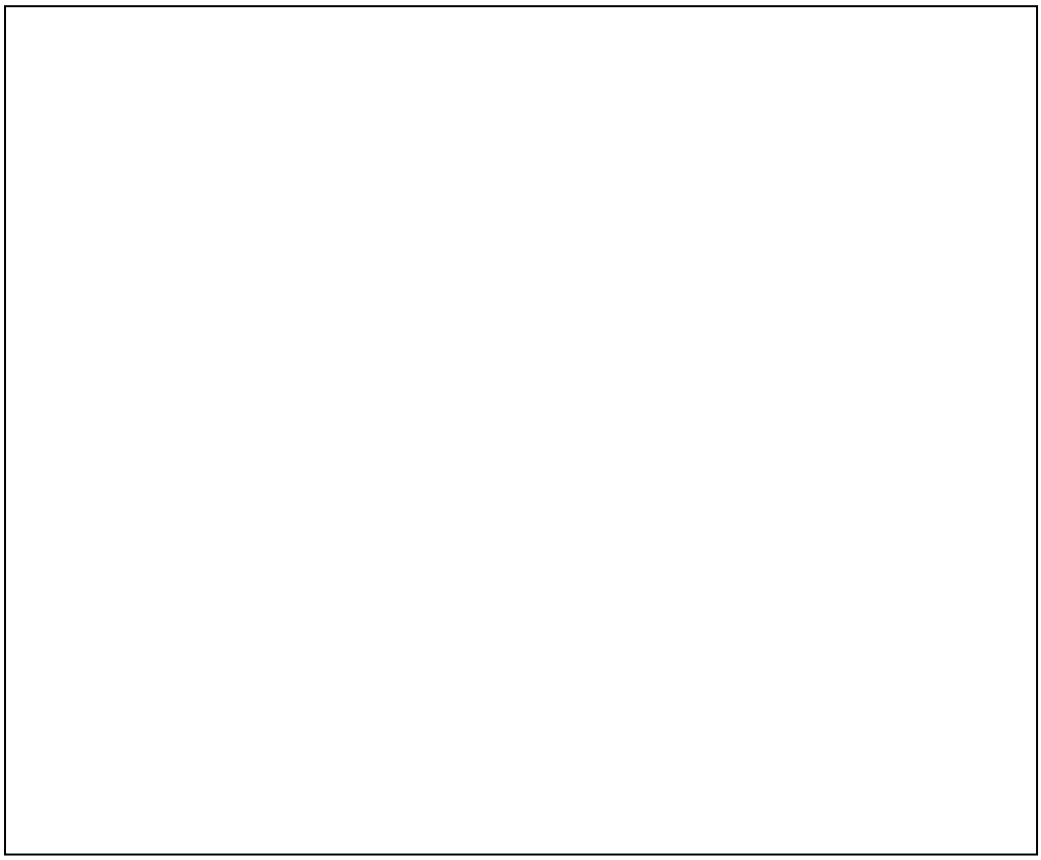


Fig. 2. 4 Colony PCR to determine orientation of positive clones in pGEM-T vector.

Lane 1- 100 base pair ladder (Invitrogen, USA)
Lane 2- Colony with forward orientation of *incB* gene using T7/ *incB* reverse primer (*incB* S1).
Lane 3- Colony with forward orientation of *incC* gene using T7/ *incC* reverse primer (*incC* S1).

Positive clones were further confirmed by restriction digestion analysis using suitable restriction enzymes whose sites were present in regions flanking the multiple cloning site for insertion of *inc* PCR amplicons. Restriction digestion with *Nde I* and *Eco RI* yielded positive clones with fragments of 390 bp and 551 bp for *incB* and *incC* genes respectively (Fig. 2.5)



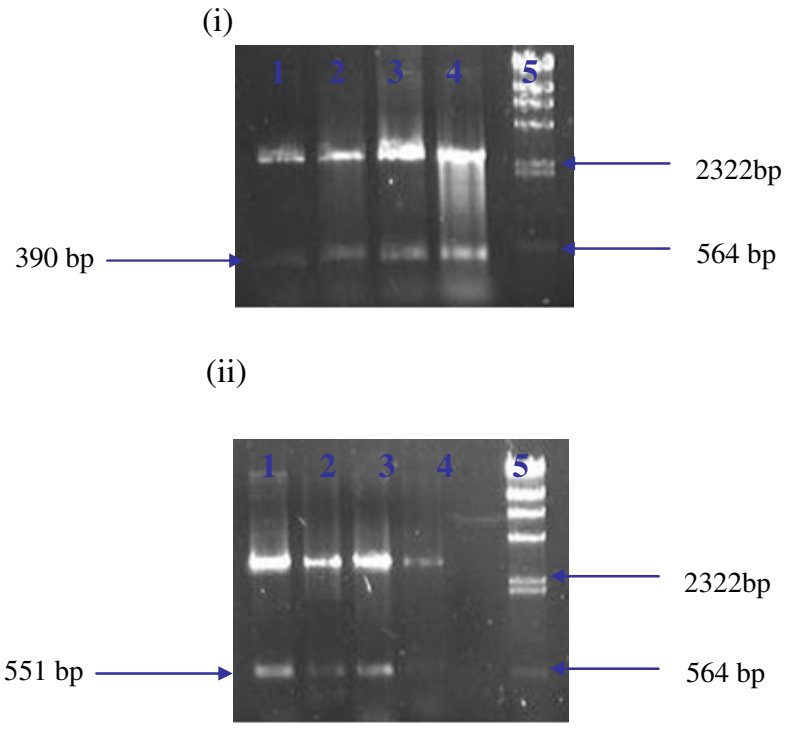


Fig. 2. 5. Specificity of *incB* and *incC* pGEM-T clones by restriction digestion analysis.

incB S1 and *incC* S1 PCR products were cloned into pGEM-T vector and positive colonies were selected and checked for orientation of insertion.

(i) Lanes 1-4 were positive *incB* clones digested using *Nde I* to yield fragment of 390 bp and linearized pGEM-T vector. Lane 5 was λDNA/ HindIII ladder (NEB).

(ii) Lanes 1-4 were positive *incC* clones digested using *Eco RI* to yield fragment of 551 bp and linearized pGEM-T vector. Lane 5 was λDNA/ HindIII ladder (NEB).

Single pass automated sequencing of the pGEM-T clones that contained the CT *incB and incC* full length inserts was performed using gene specific primers (Fig. 2.6). Results showed that the sequences generated were sufficient to find the homologous regions of the CT serovar D by BLAST searches of the databases. BLAST analysis revealed homology for *incB* and *incC* with that of reported sequences in NCBI.

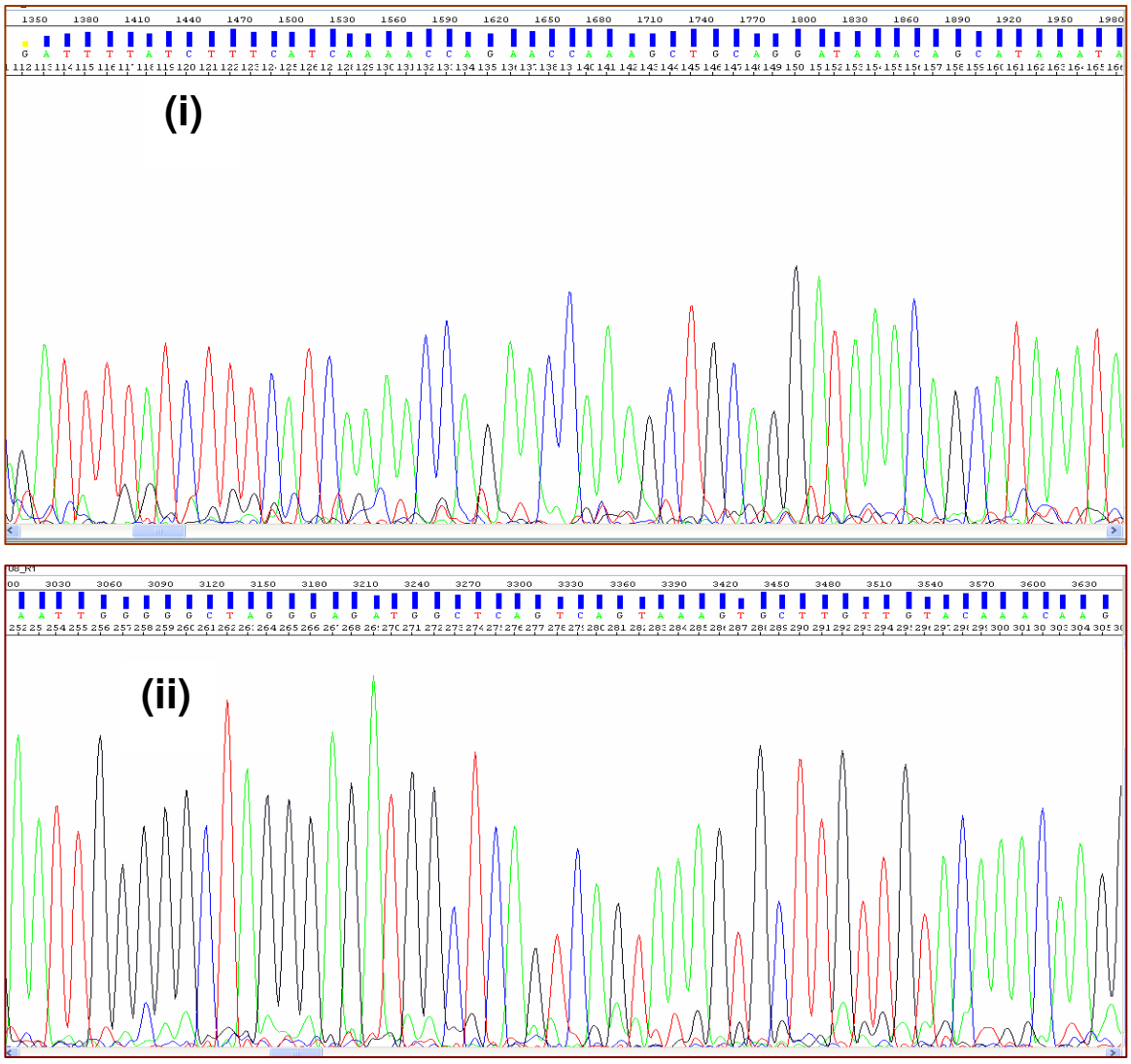


Fig 2.6. Representative pictures showing sequencing of the pGEM-T clones containing (i) CT *incB and* (ii) *incC* PCR amplicons

Table 2.3 Pairwise alignment and BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) of DNA sequencing of (i) *incB* and (ii) *incC* clones

(i) Features in this part of subject sequence:
hypothetical protein
Inclusion Membrane Protein B

Score = 399 bits (216), Expect = 3e-108

Query
ATGGTTCATTCTGTATACAATTCATTGGCTCCAGAAGGTTTTAGCCAAGTCTC
TATTCAA
||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct
ATGGTTCATTCTGTATACAATTCATTGGCTCCAGAAGGTTTTAGCCAAGTCTC
TATTCAA

Query
CGCTGTCAGAATCCAATCAGCAATTCAAGTAATGATTGCGATAATGACTCTT
TTTGCACT
| | |||||| ||||| |||||| ||||||||||||||||||||||||||||||||
Sbjct CCCAGTCAGATTCCAACCAGCAA-
AAAAGTAATGATTGCGATAATGACTCTTTTTGCACT

Query
CACAGCCATTGGAGCAATACGTGGTATTCCTATCGTTTACAGTTAGTCGAGG
TTTTCCTC
|||||||||||| ||||||| || | |||| |||| |||||||| || |||| ||||||
Sbjct CACAGCCATTGCAGCAATA-GTCCT-TTCC-ATCG-
TTACAGTTTGTGGAGGGTTTCCTT

Query
TTCATGTTGCTGCACTTAACACGGTAACTATTGGTGCATGCGTATCCTTGCCG
GTATTCA
||| | ||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct
TTCTTCTTGCTGCACTTAACACCGTAACTATTGGTGCATGCGTATCCTTGCCG
GTATTCA

Query CTTGCATAGCTACAACGTTATTTCTTCTTTGTCTCCGTAATATCG
|||||||||||||||||||| ||||||||||||||||||||

Sbjct CTTGCATAGCTACAACGTTATTACTTCTTTGTCTCCGTAATATCG

(ii) Features in this part of subject sequence:

hypothetical protein
Inclusion Membrane Protein C

Score = 761 bits (412), Expect = 0.0

Query
ATGACGTACTTTATATTCGATCTAGCCCACATATCTGATAATTCTAATCCGAC
GTCTCCC
||||||||| ||||| |||| |||| |||| |||||||| ||||||||| |||||||||
Sbjct
ATGACGTACTCTATATCCGATATAGCACACAAATCTGATATTTCTAATCCCAC
GTCTCCC

Query
GCTGGCATCAAGAAAACGAGGATCCTTTCCCCCACAATCTCCTTCTGCCGTG
GGCTCTTT
||| ||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct GCT-
CCATCAAGAAAACGAGGATCCTTTCCCCCACAATCTCCTTCTGCCGTGGGCT
CTTT

Query
AGAGGGAGCTACTATCTCTACTTGGGGGCCATGCCCCTTGTGCACTGTCCCA
GTTTATCC
|||||||||| | ||||||| ||||||||| ||||||| | ||||||||| ||||||||
Sbjct
AGAGGGAGCTAATTTCTCTACATGGGGGCCAGGCCCCTTCTTCACTGTCCCT
GTTTATCC

Query
ACAACAACTCGCTGCAATGCAAAACAACCTTTTTACATTGCAAACAGAGGTT
TCTGCTCT
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Sbjct
ACAACAACTCGCTGCAATGCAAAACAACCTTTTTACATTGCAAACAGAGGTT
TCTGCTCT

Query
CAAGAAAAAATTAGTTCAGTCTAGTCAGACACGCGGATCTTTAGCACTCGGC
CCGCAGTT
|||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct
CAAGAAAAAATTAGTTCAGTCTAGTCAGACACGCGGATCTTTAGGACTCGGC
CCGCAGTT

Query
TTTAGCGGCATGCTTAGTTGCTGCGACAATCCTTGCAGTAGCTGTTATCGTAC
TTGCTTC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct
TTTAGCGGCATGCTTAGTTGCTGCGACAATCCTTGCAGTAGCTGTTATCGTAC
TTGCTTC

Query
CTTAGGACTTGGCGGTGTTCTTCCTTTTGTCCTTGTTTGTCTGGCTGGGTCAAC
TAATGC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct
CTTAGGACTTGGCGGTGTTCTTCCTTTTGTCCTTGTTTGTCTGGCTGGGTCAAC
TAATGC

Query AATTTGGGCTATTGTGAGCGCCTCCATCACTACACTGATTT
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Sbjct AATTTGGGCTATTGTGAGCGCCTCCATCACTACACTGATTT

Expression of IncB and IncC proteins

IPTG induction of recombinant clones showed presence of a 38 kDa band for IncB and a 44.4 kDa band for IncC (Fig. 2.7). Presence of fusion proteins were further confirmed by Western blot analysis using anti-GST antibody (Fig. 2.8)

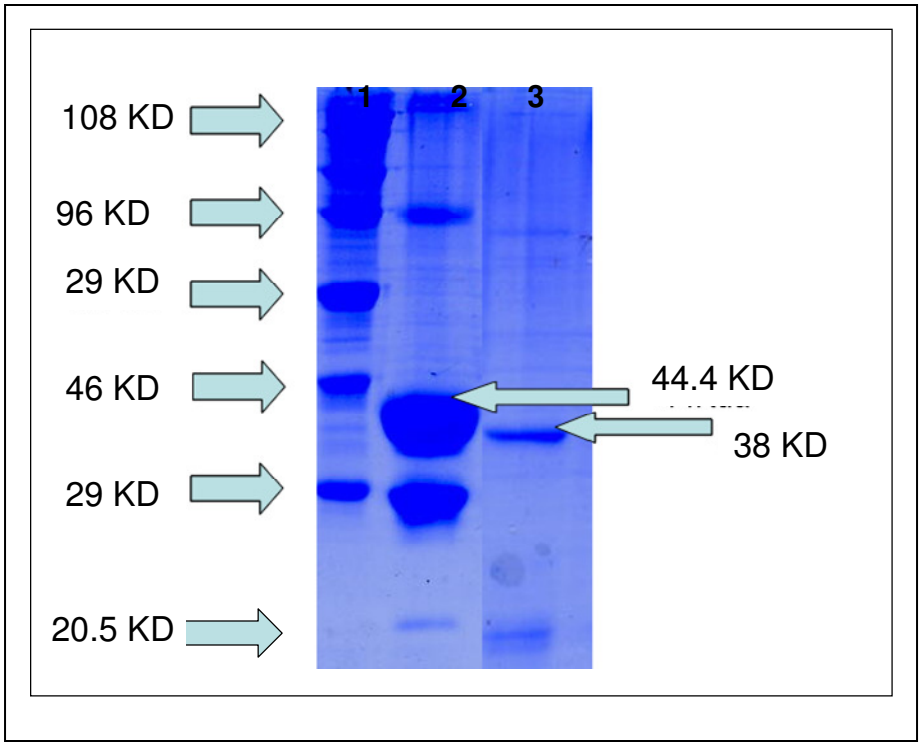

Fig 2.7. 12% SDS-PAGE analysis of GST-tagged *Inc B* and *Inc C* proteins.

Lane1 was Protein Marker; lane 2 was expression of recombinant Inc C;

lane 3 was expression of recombinant IncB.

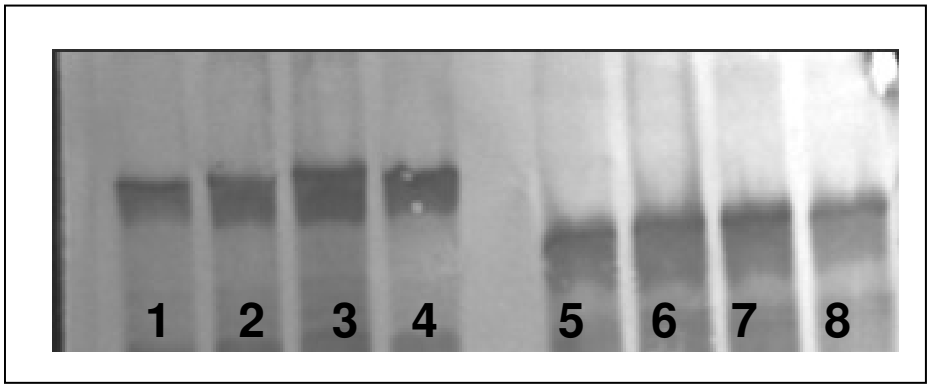

Fig 2.8. Characterization of IncB and IncC by Western Blotting using anti-GST antibody.

Lanes 1-4 Detection of recombinant GST-IncB clones; Lanes 5-8 Detection of recombinant GST-IncC clones.

Discussion

PCR is considered the most sensitive diagnostic method of detecting infectious agents including CT [Jensen *et al.*, 2003; Hardick *et al.*, 2004; Boel *et al.*, 2005]. In this study we detected 41/ 124 (31.8%) samples positive for CT using a plasmid-based (pCT) PCR. Samples positive for CT were confirmed by using real-time PCRs targeting pCT and *ompA* gene. We were further able to detect the presence of CT Inc encoding genes *incB* and *incC* in these CT-positive patients by using gene specific PCRs. To the best of our knowledge, this is the first report from India on the detection of chlamydial Incs in CT-positive patients. The *inc* PCRs showed high sensitivity and specificity of CT detection in positive samples and were able to detect *inc* amplicons in CT-positive DNA which had an initial template concentration of 7.5 pg/ml. The *inc* PCR amplicons were further characterized by restriction digestion analyses and yielded digested products as

predicted by restriction digestion maps using gene sequences of *incB* and *incC* available in published CT genome database (available from http://chlamydia-www.berkeley.edu). CT IncB and IncC have been considered to be of great significance for physiology of chlamydiae, owing to their conservativeness in genomic make-up which is particularly characteristic of proteins involved in crucial cellular processes [Kostrjukova *et al.,* 2005]. CT Incs have been proposed to be key players in host-CT interactions [Rockey *et al.,* 2000]. IncB recombinant peptides of 101 amino acid residues have been able to detect antibodies in *C. psittaci* infected sera [Bannantine *et al.,* 1998]. Further, carboxy-terminal fragments of the CT IncA and IncG have been able to generate antibodies to these proteins and have shown that the all functional features of Incs are only due to the hydrophobic fragment characteristic of Inc-proteins [Scidmore-Carlson *et al.,* 1998]. The hydrophilic amino-terminal domains of Inc-proteins have been functionally responsible for secretion of CT Incs by the *Shigella flexneri* type III system [Subtil *et al.,* 2001]. Phosphorylation studies on IncA and IncG have shown specific host cellular machinery-Inc interaction with each other.

It is also to be mentioned here that we have also reported the existence of a plasmid-less clinical isolate of CT in India [Gupta *et al.*, 2008]. The occurrence of such strains globally are very helpful in our understanding of chlamydia biology [Ripa *et al.,* 2006, Herrmann *et al.,* 2007, Schacter 2007]. The fact that pCT is positively selected during evolution confers its role in aiding the pathogen in host infection, replication and survival by modulation of host's immune system. Hence, availability of such strains would favour further studies in pCT role in pathogenesis of the organism. Plasmid-deficient *C muridarum* strains have been reported to display altered plaque morphology

and reduced infectivity in cell culture [O'Connell *et al.,* 2006]. Further there are reports on clinical samples which lack expression of CT IncA strains and show formation of multiple inclusions [Rockey *et al.,* 2002]. These mutants have been reported to grow more slowly, produced subclinical infections more often and alter host-cell functions [Bannantine *et al.,* 2000]. However there is no data available on the functionalities of Incs in plasmid-less strains. Studies of this nature will be helpful for understanding the role of Incs in disease pathogenesis. Overall there is accumulating evidence of the close association of chlamydial Incs and host cell components and hence deserves further investigations to decipher their role in generating host cell immune responses.

Introduction

Chlamydia trachomatis (CT) is the most common bacterial sexually transmitted infection worldwide, especially among young adults [WHO, 2001]. Chlamydial infections are asymptomatic in majority of patients and hence often remain undiagnosed. Undiagnosed and untreated chlamydial infections can ascend to the upper genital tract, where they colonize the endometrial mucosa and the fallopian tubes, leading to pelvic inflammatory disease (PID). Early detection is hence judicious for preventing established infection within the human host.

There is evidence on host humoral immune responses being involved in chlamydial control [Morre *et al.*, 2002]. The association of serovar-specific antibodies in local secretions with protective immunity after CT infections of humans and non-human

primates has been documented [Nichols *et al.*, 1973; Barenfanger *et al.*, 1974]. Serovar and serogroup-specific monoclonal antibodies (mAbs) against the major outer membrane protein (MOMP) are reported to neutralize chlamydial infectivity in tissue culture *in vitro*, protect mice from chlamydial toxicity when passively administered and prevent infection of monkey conjunctivae [Zhang *et al.*,1987; Peterson *et al.*, 1991]. It has also been reported that local (cervical) but not systemic (serum) anti-chlamydial immunoglobulin (Ig) A correlated with decreased shedding of chlamydiae in women with CT cervical infection [Brunham *et al,* 1983]. Studies in the guinea-pig and mouse models of chlamydial genital infection have demonstrated that antibodies contribute to protective immunity [Rank *et al.*, 1987; Rank *et al.*, 1989; Cotter *et al.*, 1995; Su *et al.*, 1997].

The several chlamydial inclusion proteins termed 'Incs' [Rockey *et al.*, 2002] localized to the inclusion membrane have the potential to play key roles in this host-pathogen interaction and thus have become an important area of research. Expression of chlamydial Incs early in the infectious process suggests that their involvement in inclusion modification is crucial to the outcome of host-chlamydiae interactions [Hackstadt *et al.*, 1997]. Incs are considered to be mediators of host-chlamydiae interactions as their hydrophilic domains localized on the outer surface of the inclusion membrane are phosphorylated by host kinases [Rockey *et al.*, 1997; Scidmore *et al.*, 2001]. Chlamydial incs are secreted through the type III secretion apparatus [Guy *et al.*, 2006] and incorporated into the host phagosomal membrane [Hackstadt *et al.*, 1999]. Although these proteins may provide contact with the host cell, their role in the development of host immunity against infection is yet to be clearly elucidated.

With the recent availability of literature on comparative immunogenicity of chlamydial inclusion proteins [Li *et al.*, 2008], we assessed the role of 2 incs of CT, namely inclusion proteins B and C (IncB and IncC respectively) in humoral immune responses in CT-infected women diagnosed with cervicitis or PID/ infertility. These two proteins, with homologues in *C. pneumoniae* [Shirai *et al.*, 2000], *C. psittaci* [Bannantine *et al.*, 1998], *C. muridarum* [Read *et al.*, 2000], and *C. abortus* [Thomson *et al.*, 2005], belong to the early phase of infection where their gene expression begins within a half hour post infection of the host cell and is simultaneous with inclusion formation and its transportation into the perinuclear space, and evasion of fusing with early lysosomes [Scidmore *et al.,* 2000]. These features suggest that IncB and IncC might play a significant role at early stages of chlamydial infection development and provide necessary elements in processes of inclusion formation.

We evaluated the prevalence of *C. trachomatis* infection by measuring antibody levels of IncB and IncC with respect to anti-MOMP antibodies in sera obtained from symptomatic women. We correlated the titres of antibodies against IncB and IncC with severity of disease in these CT-positive women diagnosed with cervicitis or PID/infertility.

Materials

Agarose, Bovine Serum Albumin, Glycine, Glutamine, HEPES, Streptomycin, Gentamycin sulphate, Adenosine, Tris base, RPMI-1640, Kanamycin sulphate, Ponceau S, Diaminobenzamide, Coomassie, and other fine chemicals were from Sigma Chemicals, USA. Fetal bovine serum was obtained from Biological Industries, Israel. Terrific Broth, phenyl methyl sulfonyl fluoride, aprotinin, leupeptin and pepstatin were

purchased from Amresco, USA. Horse radish peroxidase (HRP)- conjugated rabbit anti-human IgG (whole and subtypes) antibodies were purchased from Bangalore Genei, India). *Taq* polymerase, dNTPs, $MgCl_2$, were from Invitrogen, USA. Cytokine ELISA were purchased from eBiosciences, San Diego, USA .

Experimental Methods

1. Study population

A total of 355 women (aged 22 to 36 years) attending the outpatient department of Safdarjang Hospital, New Delhi, India, for gynaecological complaints (discharge, lower abdominal pain, pelvic pain and infertility) were enrolled in the study. They were confirmed as symptomatic after careful physical examination. Of these, 163 patients were diagnosed with cervicitis (presented with mucopus in endocervical exudates) while 76 had PID/ infertility. Findings at diagnostic laparoscopy/ hysterosalpingogram, viz. tubal patency, adhesions, hydrosalpinx formation and endometriosis were noted for infertile patients. Infertile women were identified as those, which lack recognized conception after 1.5 to 2 years of regular intercourse without the use of contraception. The study was approved by the hospital ethical committee and informed written consent was obtained from each patient. On recruitment, a detailed history was taken from each patient.

2. Collection of samples

Endocervical samples were collected from patients and detection of CT and other STD pathogens was performed as described previously in **Chapter 4**. Non-heparinised venous blood was drawn from patients; the serum was separated and then stored at –

70°C for the detection of antibodies against MOMP, IncB and IncC and for measuring serum cytokines.

2. Expression of CT IncB and IncC proteins

Recombinant full length proteins were expressed and purified from pGEX expression vectors (Amersham Pharmacia Biotech Inc., NJ, USA) as described in C**hapter 4**. Bacterial lysate samples that showed a prominent band at the expected molecular weight position on an SDS-PAGE were separated and frozen at -80°C to be used in further assays.

3. Detection of antibodies against CT MOMP, IncB and IncC

The MOMP, IncB and IncC specific antibody titre in sera were determined by ELISA as previously described [Dutta *et al.*, 2007]. Briefly, 96- well plates were coated with 1 µg antigen/ well and 100 µL patient sera samples were added per well in serial dilutions. Free GST was coated in separate wells to serve as negative controls. After incubation for 2 hours at 37°C and subsequent washing with PBS-Tween 20 (PBS-T), plates were incubated with horse radish peroxidase (HRP) - conjugated rabbit anti-human IgG (1:10,000 dilutions), IgG_1, IgG_2, IgG_3 and IgG_4 (all 1:2000 dilutions) antibodies (Bangalore Genei, Bangalore, India). The binding was measured in an ELISA reader using OPD (o-phenylenediamine dihydrochloride) as the substrate. Positive samples were defined as those yielding an absorbance (OD) value at least two standard deviations (SDs) above the mean value obtained from the panel of samples taken from the negative subjects.

4. Quantification of serum cytokines

Quantification of interleukin-1 (IL-1ß), IL-4, IL-10, IL-12, tumor necrosis factor-(TNF-α), and interferon-(IFN-γ) was done using ELISA kits (eBiosciences, San Diego, CA, USA), in accordance with the manufacturer's instructions. A log- log standard curve was generated, and unknowns were interpolated. The minimum detectable cytokine concentrations for these assays were- IL-1ß (4 pg/ml), IL-4 (2 pg/ml), IL-10 (2 pg/ml), IL-12 (4 pg/ml), IFN-γ (4 pg/ml) and TNF-α (4 pg/ml). Results from test samples were compared with controls-sera obtained from 25 healthy women attending the family planning department.

5. Statistical analysis

Statistical Analysis was performed with Graphpad Prism Version 5 (La Jolla, CA, USA). Spearman's rank method was used to find any correlation between anti-chlamydial antigens. The level of significance among groups was compared using the $\chi 2$ test. Significance of antibody titres was calculated by independent t-test.

Results

Diagnosis of STD pathogens in the cervix

Cervical CT infection was diagnosed in 108 (30.2%) patients. These patients were found to be uninfected with other STD pathogens. Among the CT-negative patients, 13 (5.1%) were infected with Candida spp., 28 (11.3%) had bacterial vaginosis, 24 (9.4%) were infected with *M. hominis*, and 61 (24.7%) with *U. urealyticum*. None of the patients had *N. gonorrhoeae or T. vaginalis*.

Detection of antibodies against MOMP, IncB and IncC

ELISA results for anti-MOMP, anti-IncB and anti-IncC IgG antibodies in patients' sera showed significantly higher (P<0.001) OD values in CT-positive patients than in CT-negative patients (median values: 1.660 v/s 0.130, 0.770 v/s 0.067 and 0.690 v/s 0.110, respectively; Fig. 3.1). All samples positive for anti-MOMP, anti-IncB and anti-IncC IgG antibodies had OD values greater than the mean +2SD of that of negative samples. Out of the 108 CT-positive patients, 73 (67.59%) showed ELISA positivity for anti-MOMP IgG, 67 (62.03%) for anti-IncB IgG and 64 (59.25%) for anti-IncC IgG. Wells coated with GST alone served as negative controls and showed very low antibody titres in CT-positive and CT-negative patients (Mean ± standard deviation; 0.039± 0.0012; 0.043± 0.0019 respectively)

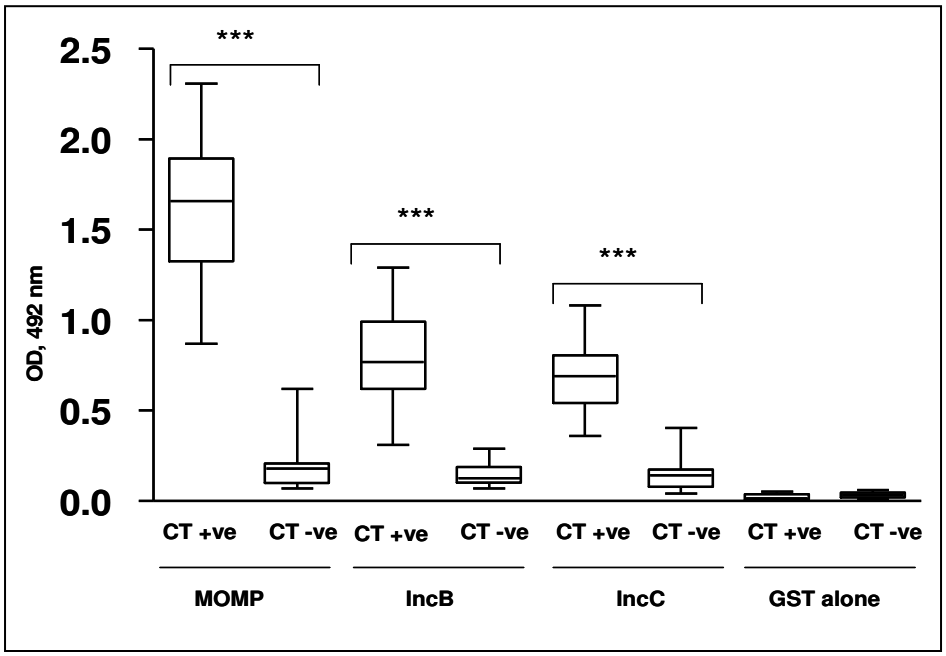

Fig. 3.1 Quantitation of anti chlamydial antibodies in patients' sera by ELISA using recombinant chlamydial proteins. Median values of antibodies against MOMP, IncB and IncC are measured in sera from *Chlamydia trachomatis* positive (CT +ve) and CT negative (CT – ve) sera obtained from women. Wells coated with GST alone served as negative controls. *** represents P<0.0001 *i.e.* highly significant. NS, Not Significant. Y axis = Optical Density (OD) of anti chlamydial antibodies measured at 492 nm; X axis = chlamydial antigens in CT +ve and CT-ve sera.

Anti-MOMP, anti-IncB and anti-IncC antibody titres

CT-positive sera were serially diluted (1:10, 1:100, 1:1000, 1:10,000, 1:100,000 and 1:1000, 000) and assayed for antibody titres for anti-MOMP, anti-IncB and anti-IncC. Anti-MOMP antibody titre was found to significantly higher (P<0.001) compared to that of anti-IncB and anti-IncC at 1:100, 1:1000 and 1:10,000 dilutions. Further, anti-IncB titre was found to be higher than anti-IncC at 1:100 (P=0.0028) and at 1:1000 (P=0.0189) (Fig. 3.2).

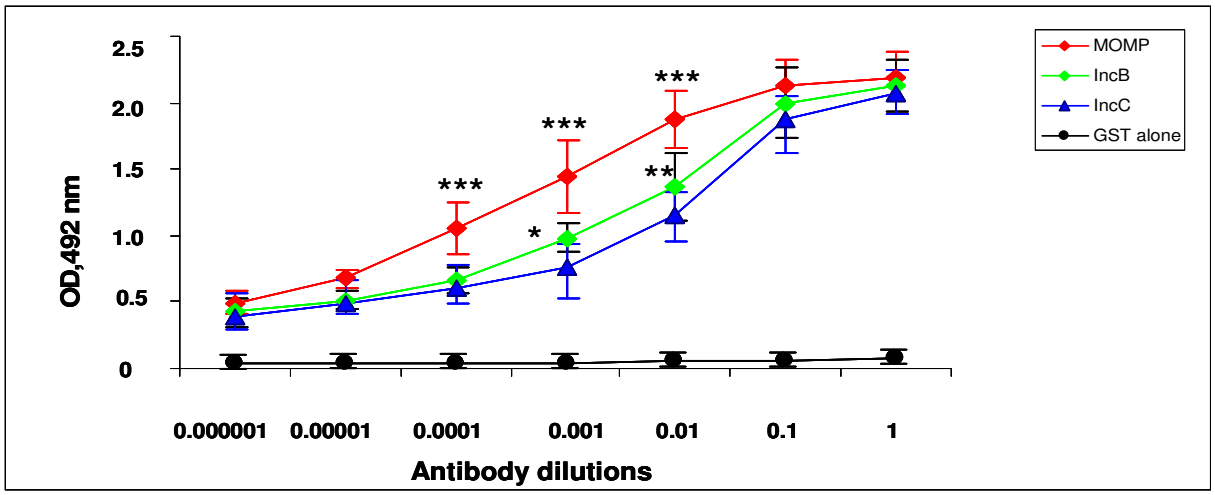

Fig. 3.2 Antibody titres against CT proteins in serially diluted CT-positive patients'

sera.

CT-positive patients' sera (n=108) were serially diluted and antibody titres of MOMP, IncB and IncC were measured and expressed as mean (OD values) ± standard deviation. Statistically significant differences in OD values between anti-MOMP and anti-IncB or anti-IncC were detected at 1:100, 1:1000 and 1:10,000 ($P<0.0001$ ***). Anti-IncB antibody titres were significantly higher than that of anti-IncC at 1:100 ($P= 0.0028$ **) and 1:1000 ($P= 0.0189$ *) Y axis = Optical Density (OD) of anti chlamydial antibodies measured at 492 nm; X axis = Serial dilutions of sera obtained from CT –positive patients.

Correlation between anti-MOMP, anti-IncB and anti-IncC antibody titres

A highly significant positive correlation ($r=0.3275$, $P=0.0068$) was seen between anti-MOMP and anti-IncB antibodies in CT-positive sera (Figure 3.3A). Similarly, anti-MOMP and anti-IncC antibody titres were positively correlated ($r=0.3608$, $P=0.0034$) (Fig. 3.3B). The anti-IncB and anti-IncC antibody titres were also positively correlated ($r=0.2827$, $P=0.0236$) in these sera (Fig. 3.3C). There was a negative and insignificant correlation between antibody titres against MOMP, IncB and IncC in CT-negative sera.

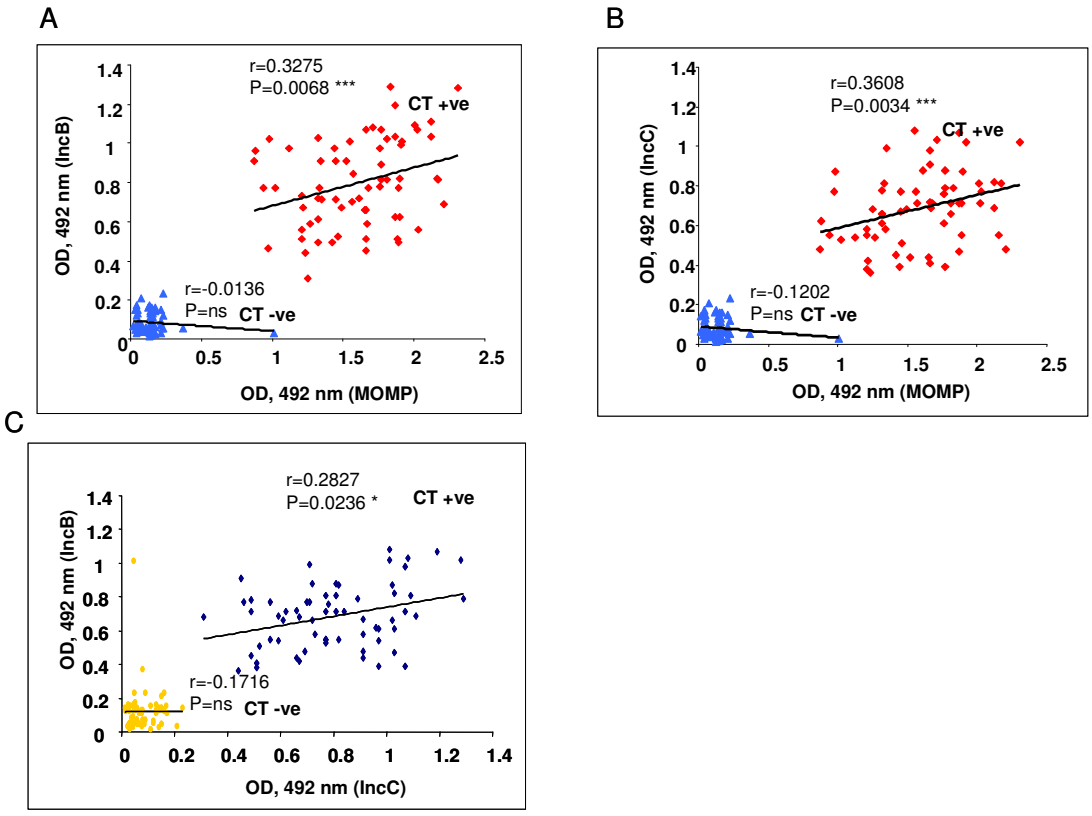

Fig. 3. 3 Correlation of antibody titres against MOMP and Incs in CT-positive and CT-negative patients' sera.

Scatter plot showing the correlation of the serological response to immunogenic chlamydial proteins: (A) anti-MOMP versus anti-IncB; (B) anti-MOMP versus anti-IncC and (C) anti-IncB and anti-IncC. There is positive correlation (r) between respective antibody titres in CT-positive sera and negative, insignificant correlation in CT-negative sera.

Anti-IncB and anti-IncC antibody titres in cervicitis and PID/ infertility sera

In CT-positive, IncB ELISA-positive patients (n=67), 21 (31.34%) were diagnosed with PID/ infertility while 38 (56.71%) had cervicitis. In the IncC ELISA-positive patients (n=64), 17 (25.37%) had PID/ infertility and 45 (67.16%) had cervicitis. Sera from these inc-positive women were serially diluted (1:10, 1:100, 1:1000, 1:10,000, 1:100,000 and 1:1000, 000) for comparing antibody titres between the cervicitis and PID/ infertility groups. Anti-IncB antibody titres were significantly higher in sera of cervicitis patients with respect to those with PID/ infertility at dilutions 1:10,000 (P=0.0017), 1:1000 (P=0.0154) and 1:100 (P= 0.0002) (Fig 4A). Similar trends were seen for anti-IncC antibody titres; however titres were significantly higher at sera dilutions 1:1000 (P=0.0179) and 1:100 (P=0.0178) (Figure 4B). Anti-MOMP antibody titres were also significantly high in sera of cervicitis patients with respect to those with PID/ infertility at dilutions 1:10,000 (P= 0.0014), 1:1000 (P= 0.0135) and 1:100 (P= 0.0034)

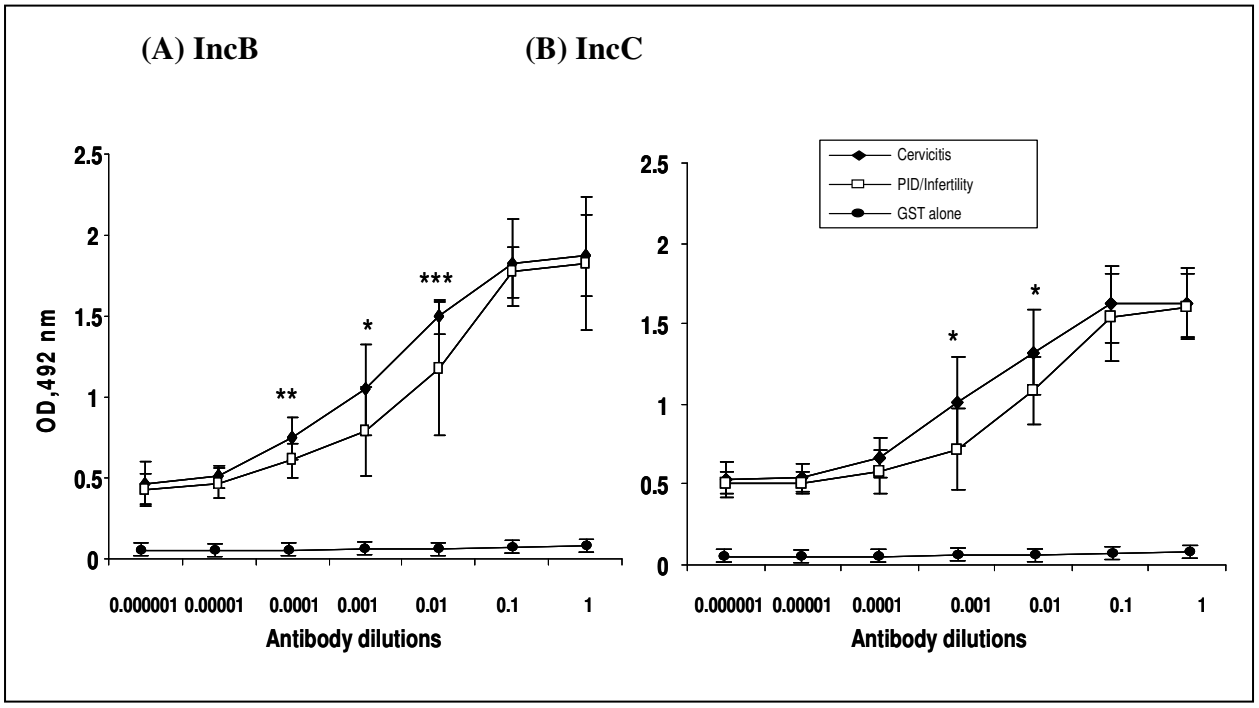

Fig 3. 4 Antibody titres against CT Incs in serially diluted sera of CT-positive patients diagnosed with cervicitis or PID/Infertility.

CT-positive sera (n=108) were serially diluted and antibody titres of IncB and IncC were compared between cervicitis and PID/infertility cases: A) Comparison of OD values of anti-IncB showed significantly high titre values in cervicitis sera at 1:100 (P=0.0002***),1:1000 (P=0.0154*) and 1:10,000 (P=0.0017**) B) OD values for anti-IncC antibodies showed statistically significant high titre values in cervicitis sera at serial dilutions 1:100 (P=0.0178*) and 1:1000 (P=0.0179*)

Antibody subtype titres against IncB and IncC in CT-positive and CT-negative sera

In CT-positive sera, anti-IncB IgG_2 and anti-IncB IgG_3 levels were significantly higher (P<0.001) than that in CT-negative sera. Also anti-IncB IgG_2 produced significantly higher titres (P<0.001) than anti-IncB IgG_3 levels in CT-positive sera.

Similar trends were seen for anti-IncC IgG_2 and anti-IncC IgG_3 (Fig. 3.5). Low antibody titres (0.01-0.046) were detected in negative controls wells coated with free GST.

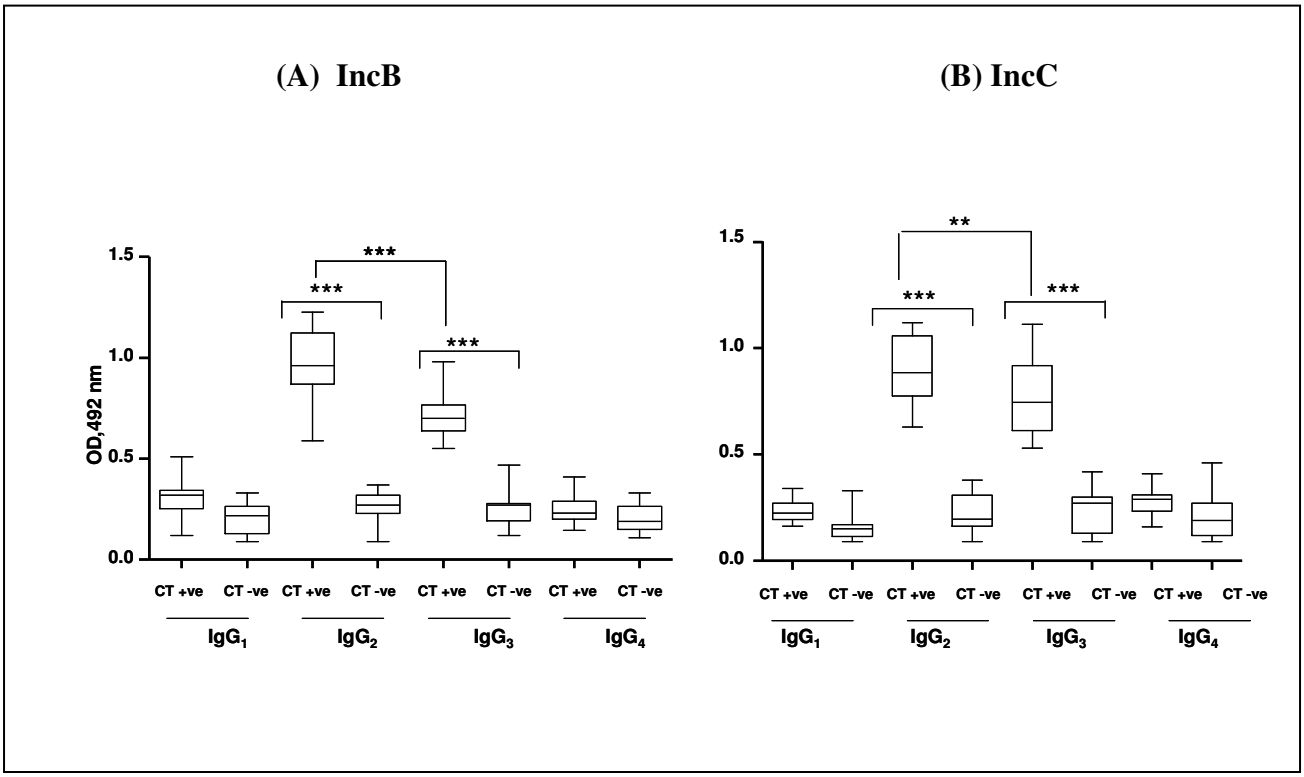

Fig. 3. 5 Antibody subtype titres against CT Incs in patients' sera

Quantitation of (A) anti-IncB and (B) anti-IncC specific IgG subtypes in CT-positive and CT- negative sera. Median values of IgG_1, IgG_2, IgG_3 and IgG_4 were compared between CT-positive and CT-negative sera. *** represents P<0.0001 *i.e*. highly significant or ** represents P<0.05 *i.e* significant. IgG subclass titre values for GST alone were very low in both CT-positive and CT- negative sera.

Cytokines concentrations in Inc-positive cervicitis and PID/ infertility sera

Median concentrations of cytokines in serum samples of MOMP-positive, inc-positive women with cervicitis or PID/ infertility are given in Table 3.1. Median IFN-γ, IL-12 and TNF-α levels were higher in CT IncB or CT IncC positive cervicitis women with women diagnosed with PID/Infertility. Serum IL-4 levels were under the detection limit in all serum samples. No significant difference was observed between levels of IL-1ß in IncC-positive patients. Cytokine levels in MOMP-positive, inc-positive women were higher than that in CT-negative patients or healthy controls.

Table 3. 1 Cytokine levels (pg/mL) in serum samples of CT-positive, Inc-positive women diagnosed with cervicitis or PID/ infertility.

	MOMP+ve, IncB+ve Cervicitis (n=38)	**MOMP+ve, IncB+ve PID/ Infertility (n=38)**	**P value**	**MOMP+ve, IncC+ve Cervicitis (n=38)**	**MOMP+ve, IncC+ve PID/ Infertility (n=38)**	**P value**
IL-1β	16.9 (7.8-26.7)	21.2 (9.7-36.2)	0.0158	22.7 (12.3-32.3)	23.3 (16.1-30.7)	NS
IFN-γ	83.4 (66.2-132.4)	27.8 (16.8-54.3)	<0.0001	93 (69.5-138.7)	24.2 (12.6-44.3)	<0.0001
TNF-α	54.85 (26.3-72.1)	31.8 (16.8-53.7)	<0.0001	61.1 (31.6-78.1)	24.3 (12.6-44.3)	<0.0001
IL-10	63.14 (37.4-78.9)	81 (36.8-91.2)	0.0231	57.3 (35.7-76.3)	79.4 (45.5-99.6)	0.038
IL-12	79.3 (45.6-89.5)	47 (36.8-61.4)	0.0059	68.9 (57.4-77.9)	48.2 (32.1-65.3)	0.0043

Note. Data are median cytokine levels in picograms per millilitre unless otherwise stated. Numbers in parentheses denote range.

Discussion

Chlamydiae actively modify the vesicular interactions of the inclusion very early in the infectious process to create a protected intracellular niche. Many of these interactions are controlled by chlamydial proteins located at the cytoplasmic face of the inclusion membrane [Hackstadt *et al.*, 2000]. Several Inc proteins have been identified in CT and there is recent literature on the characterization and location of Incs [Li *et al.*, 2008] but their role in host pathogen interaction is not well elucidated. Further, there is lack of information on the probable association of CT incs with disease pathologies in patients with genital chlamydial infection.

There is evidence on the role of antibodies following CT infection, and the immunoprotective role of antibody-mediated immunity [Thomson *et al.,* 2003]. It has been suggested that animals infected with live chlamydial organisms can develop higher titres of antibodies against inclusion membrane proteins than animals immunized with dead organisms. The first inclusion membrane protein, IncA, was identified by taking advantage of the animal antisera raised with live organism infection [Hackstadt *et al.*, 2000]. It has also been reported that antibodies from women with urogenital tract CT infection are likely generated in response to live infection [Sharma *et al.*, 2004], predominantly recognized inclusion membrane-localized proteins, suggesting that the inclusion membrane proteins are immunodominant during natural infection in humans. Further it has been shown that the C-terminal fragments of many inclusion membrane proteins are believed to be exposed at the host cell cytoplasmic surface

[Rockey *et al.*, 1997] and are dominantly recognized by human antibodies [Li *et al.*, 2008].

The results of this study agreed with previous data which showed 23-30% positivity for chlamydial infection in the lower genital tract [Mittal *et al.*, 1993; Singh *et al.*, 2002; Singh *et al.*, 2003]. Using an anti-recombinant protein antibody approach, we were able to detect antibodies against IncB and IncC in 62.03% and 59.25% respectively in CT-positive women. Further, antibody titres against IncB were higher than anti-IncC in these sera at dilutions 1:100 (P=0.0028) and 1:1000 (P=0.0189), however, MOMP titres were the highest suggesting that MOMP is more immunogenic than Incs.

There was significant positive correlation between antibodies against incs and MOMP in CT-positive sera suggesting thereby that incs are expressed simultaneously during chlamydial infection and also with established infection. It has been reported by Bannantine *et al.*, that *C. psittaci incB* and *incC* are co-transcribed in an operon and that there is identical arrangement of these genes in the CT genome with homologous sequence identity matches [1998]. Thus, as seen in our data, simultaneous expression of both Inc proteins could be attributed to their respective genes being activated simultaneously.

In a bid to find an association between disease pathology and seroprevalence of incs the present study found high IncB and IncC antibody titres in cervicitis patients in comparison to those with PID. Significantly high titres of antibodies against IncB in CT-positive cervicitis sera in comparison to PID/ infertility was detected, at up to a dilution of 1:10,000 whereas that of IncC was detectable at up to a 1:1000 dilution of the same. Differential immunogenic properties of inclusion proteins and their involvement in particular disease pathologies could be a result of multiple factors like subcellular

localization, cytoplasmic exposure and spatial arrangements on the inclusion membrane or within the inclusion needs further research.

We also found significant levels of anti-incB and anti-incC IgG_2 and IgG_3 isotypes in CT-positive sera in comparison to IgG_1, IgG_3 and IgG_4 in CT indicating that there is predominant Th_1 response. Since the relation between the production of IgG subclasses and T helper cytokines in humans is not as well defined as in the mouse our findings regarding a Th_1 mediated protection can only be confirmed by measurement of Th_1 related cytokines from PBMCs isolated from these patients and stimulated with CT inclusion proteins B and C. *Pal et al* had previously reported that to establish the protective role of sera IgG_{2a} antibodies in 3 strains of mice against a chlamydial genital challenge, IFN-γ and IL-4 responses were monitored and correlated to the initial findings [*Pal et al*, 1996; 2003]. Our data showed significantly higher levels of pro-inflammatory (IL-12 and TNF-α) and inflammatory cytokine, IFN-γ in Inc-positive sera from cervicitis sera with respect to PID/Infertility. This suggests that there is a protective role towards infection clearance at the systemic level within infected host cells. Thus, inflammatory cytokines secreted by the infected immune cells may play an essential role in immunity and in the immunopathogenesis of chlamydial infection by initiating and regulating inflammation as well as the immune responses. Further characterization of B cell epitopes of these proteins will help in our understanding of whether these peptides have a role in humoral responses generated by these proteins.

Introduction

Chlamydial infection and propagation within host genital mucosal epithelial cells rely upon a unique biphasic developmental cycle inside a membrane-bound vacuole termed inclusion. Host cell components are capable of interacting and modifying segments of inclusion membrane proteins (Incs) which are exposed to the cytosolic face of the inclusion [Rockey *et al.,* 1997; Scidmore *et al.,* 2001]. Incs have been reported to generate humoral immunity in infected humans and animals [Bannantine *et al.,* 1998a; Li *et al.,* 2008; Rockey *et al.,* 1995; Bannantine *et al.,* 1998b; Scidmore-Carlson *et al.,* 1999] and also cellular immunity by eliciting MHC class I-restricted $CD8^+$ T cell responses [Fling *et al.,* 2001; Wizel *et al.,* 2002; Starnbach *et al.,* 2003]. Studies on the involvement of CT IncA in homotypic membrane fusion via N-terminal SNARE-like motifs [Delevoye *et al.,* 2004; Delevoye *et al.,* 2008] and IncA mutant stains have been instrumental in elucidating the role of incs in disease pathogenesis and inducing modified host-cell transcriptional responses [Xia *et al.,* 2005]. CT IncB and IncC with homologues in *C. pneumoniae* [Shirai *et al.,* 2000], *C. psittaci* [Bannantine *et al.,* 1998], *C. muridarum* [Read *et al.,* 2000], and *C. abortus* [Thomson *et al.,* 2005], are expected to be involved in processes like inclusion formation, transportation to perinuclear space and evasion of early lysosomal fusion as their corresponding genes are expressed within 0.5 hours of the infection cycle and coincides with these events. Several reports on the interactions of chlamydial Incs with host cell components [Rockey *et al.,* 1997; Scidmore *et al.,* 2001], suggests that these proteins might be critically involved in interaction between infected host cells and the chlamydial developmental forms. There is however, no evidence on the role of incs and host immune responses and the resulting clearance or establishment of infection in the genital tract.

We assessed immune responses to IncB and IncC in CT-infected fertile and infertile women. Gene-specific PCRs were used for detection of *incB* and *incC* genes in endocervical samples. Cellular proliferation using MTT assay and cellular damage using LDH assay were assayed in IncB or IncC stimulated cervical cells and peripheral blood mononuclear cells (PBMCs) in enrolled women. Effect of IncB and IncC on cytokine production upon stimulation of cervical cells and PBMC was evaluated by cytokine specific real-time reverse-transcriptase (RT)-PCR assay, ELISA and ELISPOT.

Materials

Reagents and Chemicals

Agarose, Bovine Serum Albumin, Glycine, Glutamine, HEPES, Streptomycin, Gentamycin sulphate, Adenosine, Tris base, RPMI-1640, Kanamycin sulphate, Ponceau S, Diaminobenzamide, Ficoll-Hypaque, Coomassie, MTT and other fine chemicals were from Sigma Chemicals, USA. Fetal bovine serum was obtained from Biological Industries, Israel. Terrific Broth, phenyl methyl sulfonyl fluoride, aprotinin, leupeptin and pepstatin were purchased from Amresco, USA. Horse radish peroxidase (HRP)-conjugated rabbit anti- human IgG and IgA antibodies were purchased from Bangalore Genei, India. LDH-Cytotoxicity Assay Kit was purchased from BioVision Research Products, CA, USA. RNeasy Mini Kit was from Qiagen, CA, USA. SuperScript™ First-Strand Reverse Transcriptase kit and Trizol, *Taq* polymerase, dNTPs, $MgCl_2$, DTT, Superscript II reverse transcriptase, oligo dT_{20}, RNasin were from Invitrogen, USA. DyNAmo™ SYBR® Green qPCR Kit was purchased from Finnzymes, Espoo, Finland. MACS MicroBeads® was obtained from Miltenyi Biotec, CA, USA. Bradford assay and

polyvinyl difluoride (PVDF) membrane was purchased Bio-Rad Laboratories, CA, USA. Cytokine ELISA were purchased from eBiosciences, San Diego, USA and ELISPOT kits were available from Diaclone, Cedex, France. All HPLC purified primers were commercially synthesized from MWG-Biotech AG, Ebersberg, Germany.

Experimental methods

1. Study population

After obtaining informed written consent, 296 patients attending the gynaecology outpatient department, Safdarjung Hospital, New Delhi, India were enrolled for the study. Thirty-six healthy age-matched controls attending the family planning department for birth control measures were also enrolled. The study received approval from the hospital's ethics review committee.

Fertile women were those having their last child birth within the last 4 months to 1 year and testing positive for CT during last pregnancy. Infertile women were identified as those who lacked recognized conception after 1.5 to 2 years of regular intercourse without the use of contraception and were laparoscopically confirmed. Patients with endometriosis and male factor related infertility were not included in this group. Since variations in sex hormones are known to influence cytokines concentrations and immune cell population, cervical samples were collected during mid-cycle (median 13 days, range 11th to 15th day of the menstrual cycle). None of the patients had sexual intercourse 3 days or more prior to collection of samples. All samples were collected as described in **Chapter 5**. No samples were collected from patients with friable cervix and contact bleeding to ensure collection of cervical mononuclear cells. 5ml of heparinized blood (for isolation of PBMCs) and 2 ml non-heparinized blood (for separating serum)

was also collected. Samples were then stored at 4 °C, transported to the laboratory and processed within 1 h.

2. Laboratory diagnosis and detection of CT *incB* and *incC* genes

Endocervical samples were tested for positivity of CT and other STD pathogens as described in **Chapter 4**. PCR amplification of both CT incB (CT 232) and incC (CT 233) genes in endocervical samples were carried out using conditions as mentioned in **Chapter 4**.

3. Detection of antibodies against CT IncB and IncC

CT IncB and IncC specific IgG and IgA titres in cervical washes and sera were determined by ELISA as previously in **Chapter 5**. Positive samples were defined as those yielding an absorbance (OD) value at least two standard deviations (SDs) above the mean value obtained from the panel of samples taken from the negative subjects.

CT IncB and IncC specific IgG in cervical washes and sera were further determined by Western blot assay. 2 µg Incs were electrophoresed on 12% (SDS)-polyacrylamide gels, transferred to polyvinyl difluoride (PVDF) membrane (Bio-Rad) and reversibly stained with Ponceau S (Sigma-Aldrich) to confirm complete transfer. Membranes were then blocked with 5% non-fat dry milk in PBS-T and strips of membranes were incubated with individual patient's cervical washes or sera (1:100). Membranes were further incubated with HRP-conjugated rabbit anti- human IgG (1:1000 dilutions) antibodies and developed by Diaminobenzamide (DAB) (Sigma-Aldrich) as a detection system. Images were analyzed with the help of ImageJ software (http://rsb.info.nih.gov/ij/).

4. Cell cultures

Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll-Hypaque density gradient centrifugation (Sigma-Aldrich) whereas endocervical cells were isolated and counted as described earlier [Agrawal *et al.,* 2007]. Cells were washed three times with Hank's balanced salt solution (Sigma-Aldrich) and suspended in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated human AB serum. The cells were then cultured in duplicate (0.6×10^5 cells/well) in round-bottomed 96-well plates (for mRNA expression, estimation of secreted cytokines, cell proliferation assays and isolation of T-cell subsets) with or without stimulants in a total volume of 200 µl. Subsequently cultures were incubated in humidified 5% CO_2 at 37°C for 12h (for real-time RT-PCR) and 72h (for ELISA and cell proliferation assays).

5. Stimulants

Cells were stimulated with/without CT serovar D whole EBs at multiplicity of infection 2 (to serve as CT positive control), IncB (1 µg/ml) and IncC (1 µg/ml). IncB and IncC were expressed and purified as described in **Chapter 4**. Phytohaemaglutinin (PHA 2 µg/ml) (Sigma-Aldrich) and free GST were used as positive control mitogen and negative control respectively in each experiment. CT serovar D was grown on confluent McCoy cell monolayers as described previously [Mittal *et al.,* 1993] and EBs were harvested and purified on Renograffin gradients. Optimum concentrations of antigens and mitogen were determined in preliminary experiments as minimum concentrations giving maximal proliferation at different time intervals post stimulation.

6. Cell proliferation assay

A colorimetric assay based on MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide) (Sigma-Aldrich) was performed to measure the proliferative activity of cervical cells and PBMCs upon stimulation with incs. In brief, at 72 h post stimulation, 10 µl of 5 mg/ml MTT solution was added to cell cultures, which was incubated for 3 h at 37 ^{o}C. The MTT reaction was terminated by the addition of acidic isopropanol (40 mM HCl). Readings were taken at 570/650 nm using a µQuant microplate spectrophotometer (BioTek Instruments, Winooski, VT). The MTT results were expressed as stimulation indices (SI) and an SI value of 2 or more was considered a positive response.

7. Cell cytotoxity assay

A colorimetric assay based on measurement of lactate dehydrogenase (LDH) release was performed to measure the cytotoxicity of cervical cells and PBMCs upon stimulation with incs. Release of LDH from damaged cells was measured using LDH-Cytotoxicity Assay Kit (BioVision Research Products, CA, USA) according to manufacturer's instructions. Readings were taken at 500/600 nm (for LDH) using a µQuant microplate spectrophotometer (BioTek Instruments, Winooski, VT). For LDH, positive samples were those yielding an absorbance (OD) value > mean ± 2SD than that of controls.

8. RNA extraction and real-time RT-PCR analysis for cytokines

Total RNA from stimulated cervical cells and PBMCs was isolated using RNeasyMini Kit (Qiagen, CA, USA), in accordance with the manufacturer's instructions and stored at -70°C. Complementary DNA (cDNA) was prepared using a SuperScript™ First-Strand Reverse Transcriptase kit (Invitrogen), in accordance with the manufacturer's instructions. The cDNA solution was diluted to 150 µl and stored at –

20°C. All samples were reverse transcribed in a single batch and were all analysed for a given primer set in the same PCR run. The PCR amplification employed reagents supplied in a DyNAmo™ SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland), and each reaction volume (20 µl total) contained 5 µl of cDNA, and 0.5 µM of both primers. Sequences for endogenous control (ß-actin) and cytokine genes (IL-1ß, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-α, IFN-γ and GM-CSF) used in this study were same as mentioned earlier and is listed in Table 4.1 [Jasper *et al.,* 2006]. All HPLC purified primers were commercially synthesized (MWG-Biotech AG, Ebersberg, Germany). The negative control included in each reaction consisted of H_2O substituted for cDNA. PCR amplification was performed in an Applied Biosystems 7000 Real-Time PCR System (Applied Biosystems, CA, USA) under universal cycling parameters for relative quantification of cytokine expression in target samples according to the manufacturer's instructions (Applied Biosystems User Bulletin #2: Relative Quantitation of Gene Expression). For data analysis, the $2^{-\Delta\Delta Ct}$ method was used to calculate fold change [Livak *et al.,* 2001]. ß-actin expression was unaffected under all conditions and was used as a reference gene for normalization of threshold cycles (Ct).

Table 4.1 Primers used in this study

No.	Cytokine gene	5' -3' sequence
1	GM-CSF F*	AGC CCT GGG AGC ATG TGA
	GM-CSF R**	GAG TAG AGA CAC TGC TGC TGA GAT G
2	IL-β1 F	GCT GAT GGC CCT AAA CAG ATG
	IL-β1 R	ACGAAT CTC CGA CCA CCA CTA
3	IFNγ F	GAA ACG AGA TGA CTT CGA AAA GCT
	IFNγ R	ATG TCC AAC GCA AAG CAA TAC A

4	IL-12 F	TCG CGT TCA CAA GCT CAA GT
	IL-12 R	CA AAC CTG ACC CAC CCA AGA
5	TNF-α F	GCC CGA CTA TCT CGA CTT TGC
	TNF-α R	A ACC TTC CCA AAC GCC TCC
6	IL4 F	CCA CGG ACA CAA GTG CGA TAT
	IL4 R	CGT AAC AGA CAT CTT TGC TGC C
7	IL5 F	AAA GGC AAA CGC AGA ACG TT
	IL5 R	CTC TTG GAG CTG CCT ACG TGT
8	IL6 F	ACT CAC CTC TTC AGA ACG
	IL6 R	AGT AGT GAG GAA CAA GCC
9	IL10 F	GGG AGA ACC TGA AGA CCC TCA
	IL10 R	AAC AAG AGC AAG GCC GTG G
10	β-actin F	TGT GAT GGT GGG TAT GGG TC
	β-actin R	TAC AAT GAG CTG CGT GT

Note *F-forward primer **R-reverse primer

9. Quantification of secreted cytokines

Quantification of IL-1ß, IL-4, IL-5, IL-6, IL-10, IL-12, IFN-γ, TNF- α and GM-CSF in culture supernatants of stimulated cervical cells and PBMCs was performed by commercially available ELISA kits (eBiosciences, San Diego, USA), in accordance with the manufacturer's instructions. The absorbance was read at 450 nm, log–log standard curves were generated and unknowns were interpolated. The minimum detectable cytokine concentrations for these assays were- IL-1ß (4 pg/ml), IL-4 (2 pg/ml), IL-5 (4 pg/ml), IL-6 (2 pg/ml), IL-10 (2 pg/ml), IL-12 (4 pg/ml), IFN-γ (4 pg/ml), TNF- α (4 pg/ml) and GM-CSF (2.5 pg/ml).

10. Antigen specific $CD4^+$ T cell responses

Since T-helper (Th) responses are important for anti-chlamydial immunity, the role of $CD4^+$ T cells in modulating of cytokines after stimulation with IncB and IncC was examined. For purification of $CD4^+$ T cells, $CD8^+$ T cells were positively selected from cervical cells and PBMCs using CD8 MACS MicroBeads® (Miltenyi Biotec, CA, USA) according to manufacturer's instructions. In brief, $CD8^+$ cells were magnetically labelled with CD8 microbeads and the cell suspension was loaded onto a MACS® column which was then placed in the magnetic field of a MACS separator. The magnetically labelled $CD8^+$ T cells were retained in the column while the unlabelled cells which passed through the column were collected; this cell fraction was thus CD8 depleted and contained $CD4^+$ T cells including antigen presenting cells. This cell fraction was used for further assays and termed as $CD4^+$ T cells in the rest of the manuscript. These cells were further gently pelleted and suspended in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated human AB serum. $CD4^+$ T cell cultures (7.5×10^4 cells/ well) were then stimulated with IncB or IncC and incubated in humidified 5% CO_2 at 37°C for 12h (for RT-PCR) and 18h (for ELISPOT). The numbers of IL-4 and IFN-γ producing cells were measured by ELISPOT assay (Diaclone, Cedex, France) as per the manufacturer's instructions and the number of cytokine-secreting cells were counted on a Bioreader® 4000 ELISpot Reader (Biosys, Leeds, UK). To detect transcripts at mRNA levels, real-time RT-PCR analysis of cytokines (IL-4 and IFN-γ) was performed as described above.

11. Statistical analysis

The Kruskal–Wallis non parametric test was used to compare continuous variables among multiple groups. The Mann–Whitney U test was used for comparing two groups. Categorical variables were compared using χ^2 test. The results were presented with 95% confidence interval (CI) and $P < 0.05$ was considered significant. All statistical analyses were performed with Graphpad Prism Version 5 (La Jolla, CA, USA).

Results

Study population

Cervical CT infection was diagnosed by PCR in 89 /296 (30.06%) patients. Fifteen of these patients were found to be co-infected either with *Candida sp.*, bacterial vaginosis, *T. vaginalis*, *M. hominis*, *U. urealyticum* or *N. gonorrhoeae* in the cervix and were thus excluded from the study. Seven CT-positive patients and two controls were excluded as the count of cervical cells was less than 2 million cells. All healthy controls tested negative for a current CT infection (absence of CT IgM and IgG antibodies). Three healthy controls, which were positive for CT IgG antibodies with no current chlamydial infection, were also excluded from the study. Based on clinical history and diagnosis, the patients were categorized into three groups. Group I (GI, n=31) comprised of CT-uninfected healthy controls with no infertility problems, Group II (GII, n=38) comprised of fertile CT-positive women and Group III (GIII, n=29) comprised of CT-positive infertile women. The median ages of women with CT-positive infertile and fertile women and controls were comparable (26, 27 and 24 years, respectively) (Table 4.2A).

Detection of incB and incC genes

Detection of *incB* and *incC* was carried out in 89 patients positive for CT infection. Visualization of 357 bp (S1) and 360 bp (S2) amplicons for *incB* gene and 545 bp (S1) and 554 bp (S2) amplicons for *incC* gene in a 1.2% (w/v) agarose gel was considered as a positive result for amplification of inc genes. 88/89 (98.87%) and 87/89 (97.75%) of CT-positive patients showed positivity for *incB* and *incC* genes respectively.

Detection of antibodies against IncB and IncC

ELISA results showed that IgG and IgA antibodies to both IncB and IncC were significantly higher ($P < 0{\cdot}05$) in cervical washes and sera of CT-positive fertile and infertile patients compared to controls. The prevalence of IncB and IncC antibodies was significantly higher in cervical washes of CT-positive fertile women as compared to CT-positive infertile women. There was no significant difference ($P > 0.05$) between IgG and IgA antibodies to both IncB and IncC in sera obtained from CT-positive fertile and infertile women (Table 4.2A). Significant correlation was observed between antibodies to incs in cervical washes and sera in CT-positive fertile and infertile women (Table 4.2B).

Antibody recognition of IncB and IncC proteins was confirmed by Western blot assay (Fig. 4.1). Western blot assay showed presence of a 38 kDa band for IncB and a 44.4 kDa band for IncC in CT-positive samples. No corresponding bands were observed in cervical washes and sera obtained from controls.

Table 4.2 A
Prevalence of IncB and IncC IgG and IgA antibodies in sera and cervical washes of study groups

Groups	Age	IncB IgG^{+ve}		IncB IgA^{+ve}		IncC IgG^{+ve}		IncC IgA^{+ve}	
	Median (Range)	Serum	Cervical Washes	Serum	Cervical Washes	Serum	Cervical Washes	Serum	Cervical Washes
		n (%)							
Group I (n=31)	24 (21-28)	2(6)	1(3)	1(3)	1(3)	2(6)	1(3)	1(3)	1(3)
Group II (n=38)	26 (22-29)	36(95)[a]	24(63)[b]	23(61)[c]	29(77)[d]	34(89)[e]	27(71)[f]	26(68)[g]	32(84)[h]
Group III (n=29)	27 (22-31)	27(93)[i]	11(38)[j]	17(59)[k]	15(52)[l]	26(90)[m]	13(45)[n]	28(96)[o]	17(59)[p]

Note values in parenthesis represent corresponding percentages unless otherwise stated.

[a] P=NS as compared to GIII; [b] P=0.0428 as compared to GIII; [c] P=NS as compared to GIII; [d] P=0.0378 as compared to GIII; [e] P=NS as compared to GIII; [f] P=0.0320 as compared to GIII, [g] P=NS as compared to GIII; [h] P=0.0378 as compared to GIII;

[i, j, k, l, m, n, o, p] P<0.0001 as compared to corresponding GI where,

Group I (GI) comprised of CT-uninfected healthy controls with no infertility problems Group II (GII) comprised of CT-positive fertile women, Group III (GIII) comprised of CT-positive infertile women. All categorical variables were compared using the $\chi 2$ test.

Table 4.2 B
Correlation of IncB and IncC specific IgG and IgA antibodies in sera and cervical washes of women enrolled in the study.

Antibodies	GII (n=38) r, *p*	GIII (n=29) r, *p*
IncB IgG and IncC IgG in sera	0.47 , 0.0047	0.54 , 0.0148
IncB IgG and IncC IgG in cervical washes	0.57 , 0.0040	0.94 , <0.0001
IncB IgA and IncC IgA in sera	0.56 , 0.0049	0.92 , 0.0003
IncB IgA and IncC IgA in cervical washes	0.43 , 0.0172	0.60 , 0.018

Note. Table shows the correlation of optical densities of IncB and IncC IgG and IgA antibodies in sera and cervical washes of CT-positive women. Significant correlations was observed in GII and GIII where, Group II (GII) comprised of CT-positive fertile women, Group III (GIII) comprised of CT-positive infertile women.
Correlation was tested with Spearman's correlation coefficient (r).n- number of patients, r- Spearman's correlation coefficient.

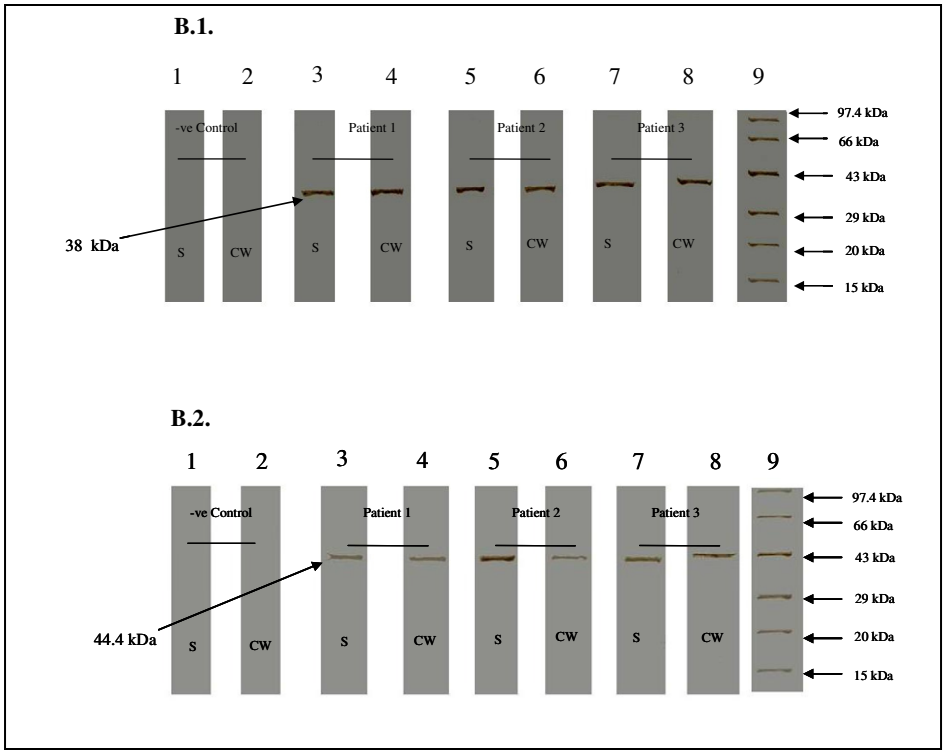

Fig. 4. 1 . Western blot assay for detection of anti-IncB and anti-IncC IgG in sera and cervical washes of CT-positive patients and controls.

B.1. Detection of anti-IncB IgG antibodies in sera (S) and cervical washes (CW) obtained from CT-positive patients and controls. Strips 3, 5, 7 - Detection of IncB IgG antibodies in sera (S) of patients' 1, 2, 3 respectively; strips 2, 4, 6- Detection of IncB IgG antibodies in cervical washes (CW) of patients' 1, 2, 3 respectively; strips 1, 2 - Serum and cervical wash respectively obtained from CT-negative patient (which served as negative controls for assay). Strip 9 - Molecular weight marker (Bangalore Genei). A 38kDa band corresponding to IncB specific IgG was detected in sera and cervical washes obtained from CT-positive patients. No bands observed in negative control.

B. 2. Detection of anti-IncC IgG antibodies in sera (S) and cervical washes (CW) obtained from CT-positive patients and controls. Strips 3, 5, 7 - Detection of IncB IgG antibodies in sera(S) of patients' 1, 2, 3 respectively; strips 2, 4, 6- Detection of IncC IgG antibodies in cervical washes (CW) of patients' 1, 2, 3 respectively; strips 1, 2 - Serum and cervical wash respectively obtained from CT-negative patient (which served as negative controls for assay). Strip 9 - Molecular weight marker (Bangalore Genei). A 44.4 kDa band corresponding to IncC specific IgG was detected in sera and cervical washes obtained from CT-positive patients. No bands observed in negative control.
IncB- CT Inclusion membrane protein B; IncC- CT Inclusion membrane protein C; CT- *Chlamydia trachomatis*; kDa- Kilodaltons; S- Serum; CW- Cervical washes

Cell proliferation

MTT assay was used to evaluate cellular proliferation of cervical cells and PBMCs upon stimulation with IncB and IncC. Proliferative responses (SI > 2) to IncB and IncC was statistically significant (P < 0·05) in cervical cells (Fig. 4.2A) and PBMCs (Fig. 4.2B) from CT-positive fertile women compared to CT-positive infertile women. Stimulation with free GST (which served as negative controls) showed no significant differences in proliferative responses in any group.

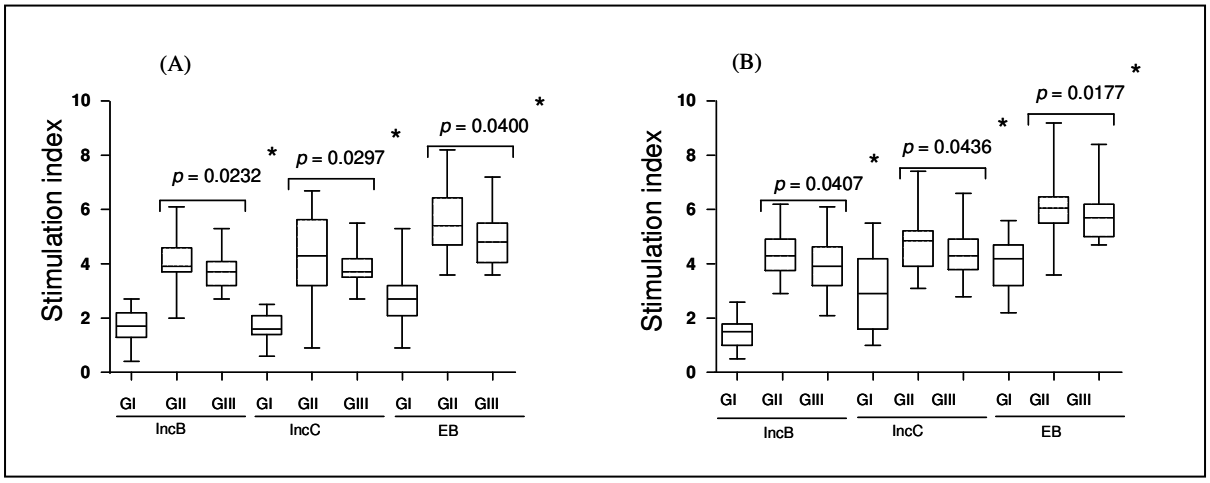

Fig. 4.2. MTT assay showing proliferative responses
Proliferative responses (stimulation indices) of (A) cervical cells and (B) PBMCs isolated from GI, GII, GIII on stimulation with IncB, IncC and CT EB were estimated by MTT assay.
(A) Proliferative responses (stimulation index) of cervical cells upon stimulation with IncB, IncC and EB. Significant difference in proliferative responses in GII with respect to GIII (P = 0.0232, P = 0.0297 and P = 0.0400 upon stimulation with IncB, IncC and EB respectively)
(B) Proliferative responses (stimulation index) of PBMCs upon stimulation with IncB, IncC and CT EBs.
*Significant difference in proliferative responses in GII with respect to GIII (P = 0.0407, P = 0.0436 and P = 0.0177 upon stimulation with IncB, IncC and CT EBs respectively)
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., *Significant; PBMCs- Peripheral blood mononuclear cells; IncB- CT Inclusion membrane protein B;

IncC- CT Inclusion membrane protein C; CT- *Chlamydia trachomatis*; EB- Elementary Bodies; n- Number. Proliferative responses between groups were evaluated using Mann–Whitney U test.

Cell cytotoxity assays

Damage to plasma membranes of cells stimulated with incs was evaluated by measuring the release of LDH, a stable cytoplasmic enzyme into culture supernatants. No significant difference ($P > 0.05$) was observed in the number of positive responders for LDH release in cervical cells and PBMCs isolated from CT-positive patients and controls (Table 4.2C). Free GST did not produce significant differences in LDH in any group.

Table 4.2C Lactate dehydrogenase (LDH) assay for cell cytotoxicity

Groups	**No. of patients' positive for LDH release from cells stimulated with chlamydial antigens**					
	Cervical cells			**PBMCs**		
	IncB	**IncC**	**EBs**	**IncB**	**IncC**	**EBs**
	n (%)					
GI	2(6.45)	3(9.67)	2(6.45)	2(6.45)	2(6.45)	2(6.45)
GII	3(7.89)[a]	4(10.52)[b]	3(7.89)[c]	2(5.26)[d]	4(10.52)[e]	5(13.15)[f]
GIII	4(13.79)	3(10.34)	5(17.24)	4(13.79)	4(13.79)	3(10.34)

Note. Table shows the number of patients (n) positive for LDH release in culture supernatants of stimulated cells. Cervical cells and PBMCs were isolated from patients of GI, GII and GIII and stimulated with chlamydial antigens IncB, IncC and EBs. The number of patients positive for LDH release into culture supernatants post stimulation was noted. Values in parentheses represent corresponding percentages.
[a, b, c, d, e, f] P not significant as compared to corresponding GI and GIII. All categorical variables were compared using the $\chi 2$ test.

Cytokine mRNA expression levels in stimulated cervical cells and PBMCs

Messenger RNA for IL-1ß, IL-4, IL-5, IL-6, IL-10, IL-12, TNF- α, IFN-γ and GMCSF was detected in stimulated cervical cells and PBMCs in all patients' groups. On stimulation of cervical cells (Fig. 4.3 A) and PBMCs (Fig. 4.3 B) with incs, significant increase in mRNA expression levels of IFN- γ, IL-12, and GM-CSF ($p < 0.05$) was observed in cervical cells and PBMCs obtained from CT-positive fertile women compared to other groups. In contrast, IL-1ß, IL-4, IL-5, IL-6 and IL-10 mRNA expression levels were significantly higher ($P < 0.05$) in cells obtained from CT-positive infertile women compared to other two groups. There were no changes in cytokine mRNA expression in cervical cells and PBMCs stimulated with free GST.

Fig. 4. 3. mRNA expression in Inc stimulated cells

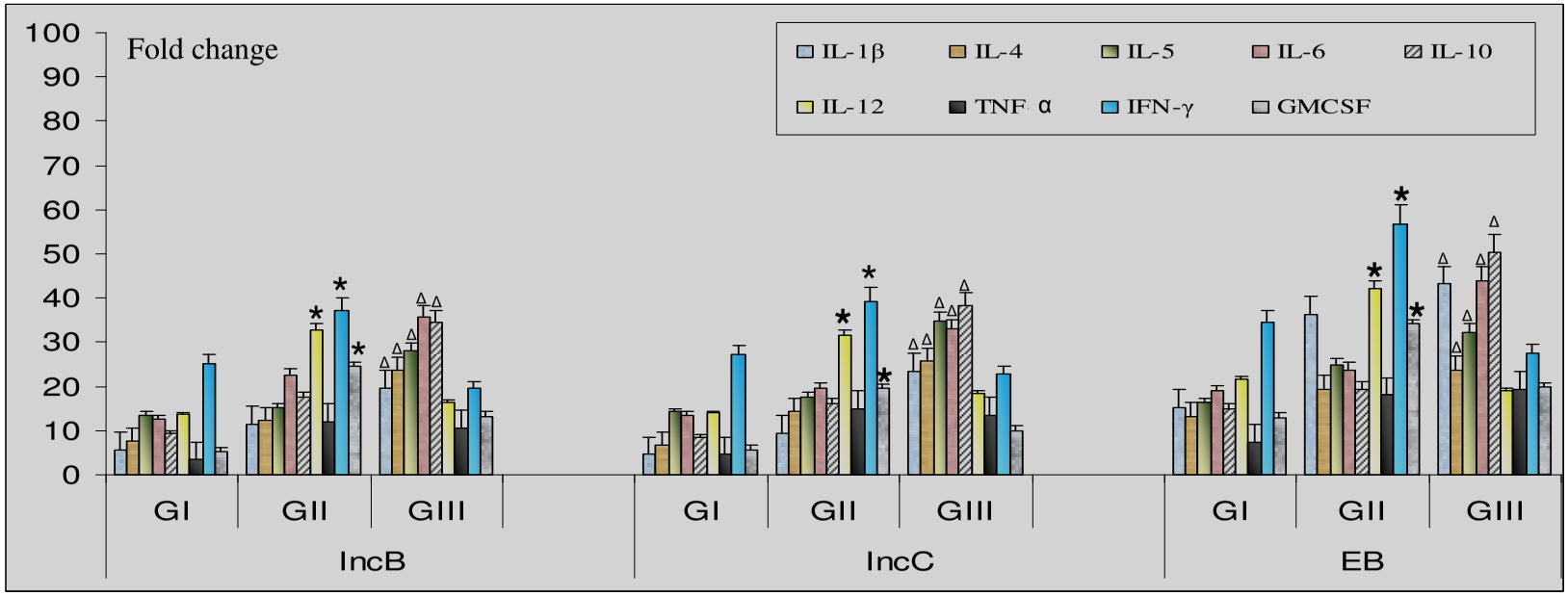

Fig. 4. 3A. Real-time RT-PCR for cytokine mRNA expression in stimulated cervical cells
Estimation of mRNA expression of IL-1ß, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-α , IFN- γ and GMCSF in 0.6×10^5 cervical cells after *invitro* stimulation with IncB, IncC and CT EB.
Real-time RT-PCR analysis of mRNA levels was done at 12 hours post infection in cervical cells isolated from GI, GII and GIII where,
Group I (GI) comprised of healthy women with no CT infection,
Group II (GII) comprised of CT-positive fertile women,
Group III (GIII) comprised of CT-positive infertile women,
* $P < 0.05$ Expression of cytokine mRNA in GII compared to corresponding levels in GI and GIII by Kruskal Wallis test.
Δ $P < 0.05$ Expression of cytokine mRNA in GIII compared to corresponding levels in GI and GII by Kruskal Wallis test.
RT-PCR- Reverse-Transcriptase PCR; IncB- CT Inclusion membrane protein B; IncC- CT Inclusion membrane protein C;
CT- *Chlamydia trachomatis*; EB-Elementary bodies; IL-Interleukin; ß-Beta; IFN- γ -Interferon gamma;
TNF-α -Tumor Necrosis factor alpha; GMCSF- Granulocyte macrophage colony-stimulating factor.
All cytokines were normalised against corresponding levels of ß-actin endogenous gene.
The graph show results as fold change represented by bars. Bars represent mean±s.e.m. for all experiments.
X-axis- Stimulants used in the study; Y-axis- Fold change in RNA expression under different conditions.

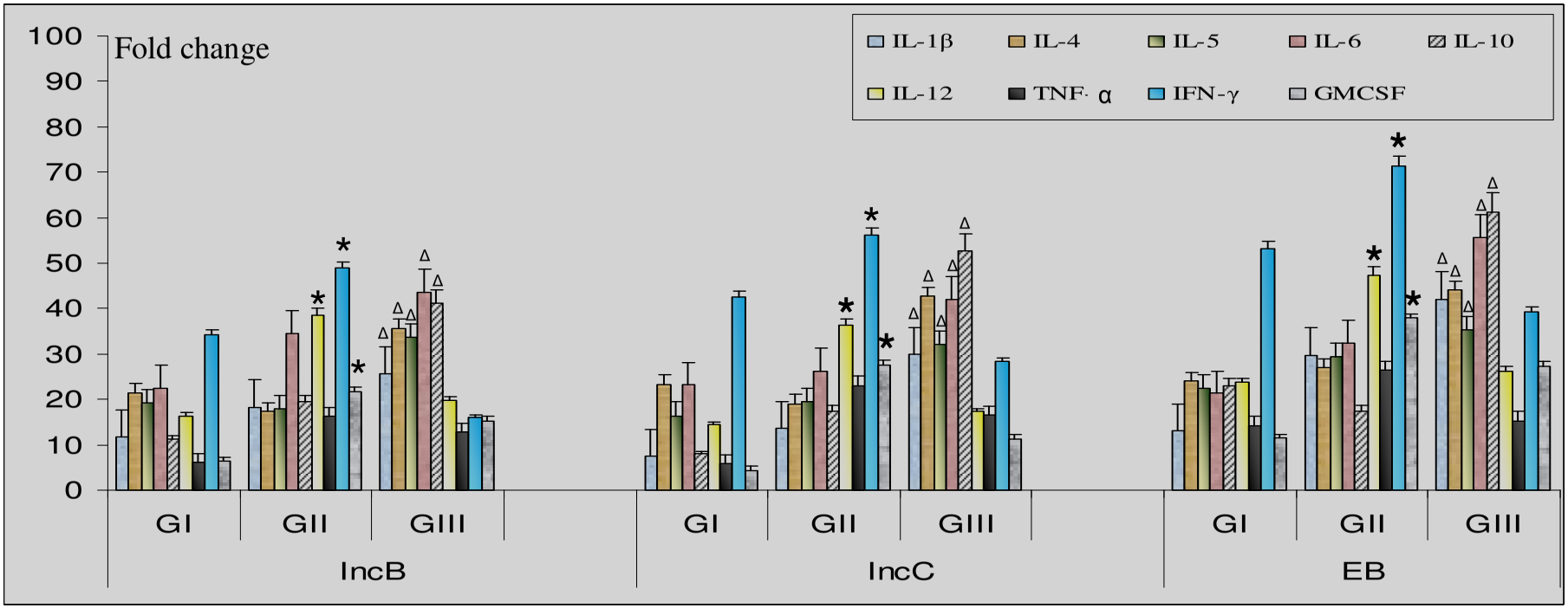

Fig. 4. 3B. Real-time RT-PCR for cytokine mRNA expression in stimulated PBMCs

Estimation of mRNA expression of IL-1ß, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-α , IFN- γ and GMCSF in 0.6×10^5 PBMCs after *invitro* stimulation with IncB, IncC and CT EB.

Real-time RT-PCR analysis of mRNA levels was done at 12 hours post infection in PBMCs isolated from GI, GII and GIII where,

Group I (GI) comprised of healthy women with no CT infection,

Group II (GII) comprised of CT-positive fertile women,

Group III (GIII) comprised of CT-positive infertile women,

* $P < 0.05$ Expression of cytokine mRNA in GII compared to corresponding levels in GI and GIII by Kruskal Wallis test.

Δ $P < 0.05$ Expression of cytokine mRNA in GIII compared to corresponding levels in GI and GII by Kruskal Wallis test.

RT-PCR- Reverse-Transcriptase PCR; IncB- CT Inclusion membrane protein B; IncC- CT Inclusion membrane protein C;

CT- *Chlamydia trachomatis*; EB-Elementary bodies; IL-Interleukin; ß-Beta; IFN- γ -Interferon gamma;

TNF-α -Tumor Necrosis factor alpha; GMCSF- Granulocyte macrophage colony-stimulating factor.

All cytokines were normalised against corresponding levels of ß-actin endogenous gene.

The graph show results as fold change represented by bars. Bars represent mean±s.e.m. for all experiments.

X-axis- Stimulants used in the study; Y-axis- Fold change in RNA expression under different conditions.

ELISA for cytokines in cell supernatants of stimulated cervical cells and PBMCs

Significantly higher levels of IL-1ß, IL-6 and IL-10 were observed upon Inc stimulation of cervical cells (Table 4.3A) and PBMCs (Table 4.3B) from CT-positive infertile women as compared to CT-positive fertile women or controls ($P < 0.05$). In contrast, cervical cells and PBMCs obtained from CT-positive fertile women secreted significantly higher levels of IL-12, IFN-γ and GM-CSF compared to CT-positive infertile women or controls ($P < 0.05$). Significantly high levels of TNF- α and IL-6 levels were secreted in cervical cells and PBMCs from CT-positive fertile women compared to controls. IL-4 and IL-5 was below detection limit in all culture supernatants. Cervical cells and PBMCs cultures stimulated with free GST showed no significant differences in levels of secreted cytokines.

Table 4.3A Cytokine concentrations (pg/ml) in culture supernatants of stimulated cervical cells

	GI			GII			GIII		
	IncB	**IncC**	**EBs**	**IncB**	**IncC**	**EBs**	**IncB**	**IncC**	**EBs**
IL-1β	**78** (49.5-235.9)	**93** (58.6-286.9)	**143.6** (52.1-362.76)	**143** (61.8-381.5)	**167.2** (55.2-417.3)	**268** (48.6-473.6)	**293.2**[a] (77.5-731.4)	**327.5**[b] (65.3-821.8)	**521.1**[c] (86.2-921.1)
IL-6	**111** (38.2-371.5)	**127.4** (52.1-443.4)	**178.65** (67.9-871.76)	**398.4**[d] (82.8-671.8)	**431.56**[e] (97.6-544.9)	**631**[f] (111.7-931.6)	**732.3**[a] (241.3-1121.62)	**756.61**[b] (222.9-1006.8)	**1107.1**[c] (341-1621.8)
IL-10	**158.6** (100.1-267.2)	**172.2** (97.6-342.5)	**183.4** (92.6-512.46)	**366.9** (112.5-548.9)	**387.9** (126.1-561.9)	**537.8** (221-712.5)	**956.9**[a] (347.9-1231)	**869.7**[b] (358.7-1331.9)	**1259.8**[c] (568.7-1712.89)
IL-12	**152.5** (97.4-306.8)	**168.9** (88.8-277.1)	**251.8** (113.7-342.7)	**289.8** [g] (134.6-450)	**297.6**[h] (156.9-430.7)	**566.5**[i] (189.4-821.6)	**141.4** (68.9-281.8)	**156.8** (75.8-312.8)	**171.6** (126.9-400)
IFN-γ	**312.5**[j] (78.8-454.3)	**321.8**[k] (91.3-431.6)	**426.7**[l] (122.6-600.1)	**512.5** [g] (346.1-861.4)	**571.3**[h] (311.9-814.3)	**843.8**[i] (424.8-1272.5)	**168.2** (107.9-575.3)	**187.3** (142.7-612.3)	**313.6** (163.4-621.9)

TNF-α	**129.3**	**136.9**	**143.6**	**256.3[d]**	**234.6[e]**	**411.7[f]**	**226.4**	**267.8**	**320.4**
	(43.6-212.5)	(38.9-232.8)	(54.1-312.6)	(115.7-385.4)	(114.8-412.6)	(134.2-532.7)	(89.7-352.7)	(97.3-411.9)	(118.9-543.1)
GM-CSF	**116.1**	**117.6**	**135.2**	**245.1 [g]**	**222.3[h]**	**300.8[i]**	**147.9**	**132.4**	**204.1**
	(55.8-143.7)	(48.3-129.8)	(76.8-176.9)	(76.3-311.1)	(83.9-288.7)	(123.4-389.8)	(63.6-176.4)	(72.5-166.9)	(89.2-237.1)

Note. Data represents median values (bold). Values in parentheses depict range. All values in pg/ml unless otherwise stated. Along the horizontal axis are the stimulants IncB, IncC and EBs used in cells obtained from GI, GII and GIII and vertical axis are the cytokines secreted in cell cultures assayed using specific ELISAs.

[a, b, c] $P<0.05$ Level of secreted cytokine in GIII compared to respective levels in groups I and II by Mann Whitney U test.

[d, e, f] $P<0.01$ Level of secreted cytokine in GII compared to respective levels in group I by Mann Whitney U test.

[g, h, i] $P<0.05$ Level of secreted cytokine in GII compared to respective levels in groups I and III by Mann Whitney U test.

[j, k, l] $P<0.05$ Level of secreted cytokine in GI compared to respected levels in group III by Mann Whitney U test.

Table 4.3B Cytokine concentrations (pg/ml) in culture supernatants of stimulated PBMCs

	GI			GII			GIII		
	IncB	**IncC**	**EBs**	**IncB**	**IncC**	**EBs**	**IncB**	**IncC**	**EBs**
IL-1β	**109.4** (56.7-258.3)	**119.7** (59.6-324.1)	**159.9** (73.1-462.69)	**172.6** (68.4-393.8)	**183.2** (65.5-407.5)	**289** (73.1-491.4)	**413.36[a]** (82.1-786.3)	**432.5[b]** (95.1-729.8)	**568.9[c]** (76.4-821.7)
IL-6	**132.7** (54.3-457.9)	**146.3** (67.21-547.1)	**201.65** (87.9-719.82)	**487.6[d]** (88.86-791.1)	**473.58[e]** (98.6-564.7)	**668[f]** (121.4-956.8)	**813.6[a]** (300.2-1421.4)	**793.45[b]** (312.9-1201.4)	**1335.47[c]** (436.1-1578.3)
IL-10	**183.4** (106.3-383.6)	**196.3** (87.3-446.3)	**328.4** (88.1-612.46)	**462.7** (189.2-631.3)	**411.21** (166.8-661.7)	**554.9** (246-745.2)	**1005[a]** (414.2-1426.3)	**956.2[b]** (437.7-1431.6)	**1472.8[c]** (336.2-1803)
IL-12	**204.8** (113.7-342.7)	**198.7** (96.8-327.1)	**342.7** (234.7-515.1)	**466.5[g]** (173.9-712.6)	**450.1[h]** (167.2-572.8)	**621.1[i]** (188.3-745.8)	**167.4** (75.8-312.8)	**156.8** (88.3-345.8)	**186.36** (129.3-621.3)
IFN-γ	**453.7[j]** (88.5-549.3)	**511.3[k]** (87.3-631.6)	**578.8[l]** (311.9-714.3)	**726.6[g]** (351.3-1128.4)	**671.3[h]** (454.8-1272.5)	**884.7[i]** (557.4-1377.2)	**241.7** (142.-612.3)	**196.8** (157.6-661.7)	**263.6** (163.4-721.9)
TNF-α	**143.6** (54.1-312.6)	**156.3** (48.9-331.5)	**264.2** (127.4-423.7)	**314.7[d]** (119.7-523.8)	**293.6[e]** (127.4-623.7)	**311.7[f]** (113-452.9)	**289.9** (97.3-411.9)	**277.2** (113-452.9)	**289.3** (125.6-543.7)

GM-CSF	**128.7** (63.4-156.8)	**136.4** (71.3-149.4)	**152.4** (89.4-201.1)	**263.1**[g] (125.3-291.2)	**248.1**[h] (111-321.2)	**320.4**[i] (136-520)	**151.2** (76.1-184)	**163.4** (91.3-201)	**268.7** (100.1-306.9)

Note. Data represents median values (bold). Values in parentheses depict range. All values in pg/ml unless otherwise stated. Along the horizontal axis are the stimulants IncB, IncC and EBs used in cells obtained from GI, GII and GIII and vertical axis are the cytokines secreted in cell cultures assayed using specific ELISAs.
[a, b, c] $P<0.05$ Level of secreted cytokine in GIII compared to respective levels in groups I and II by Mann Whitney U test.
[d, e, f] $P<0.01$ Level of secreted cytokine in GII compared to respective levels in group I by Mann Whitney U test.
[g, h, i] $P<0.05$ Level of secreted cytokine in GII compared to respective levels in groups I and III by Mann Whitney U test.
[j, k, l] $P<0.05$ Level of secreted cytokine in GI compared to respected levels in group III by Mann Whitney U test

Antigen specific $CD4^+$ T cell responses

IL-4 and IFN-γ spot forming cells (SFCs) were enumerated in inc-stimulated $CD4^+$ T cells (isolated from cervical cells and PBMCs) obtained from CT-positive groups and controls. As shown in Table 4.4, purified $CD4^+$ T cells (from cervical cells and PBMCs) from CT-positive fertile women exhibited significantly elevated numbers of IFN-γ SFCs, in a dose-dependent fashion, upon stimulation with 0.5 µg/ml or 1 µg/ml of both IncB and IncC, as compared to cells from CT-positive infertile women. Cells stimulated with EBs (positive control) showed maximal IFN-γ SFCs whereas cells stimulated with free GST (negative control) showed low IFN-γ production. Further, frequency of IFN-γ secreting cells was highest among $CD4^+$ T cells (from cervical cells and PBMCs) from CT-positive fertile women followed by controls. In CT-positive infertile women, significantly ($P < 0.05$) lower number of IFN-γ SFCs was present. There was no detectable IL-4 SFCs in any of the cell culture supernatants.

Table 4.4. IFN-γ ELISPOT assay in IncB or IncC stimulated $CD4^+$ T cell cultures

	GI				**GII**				**GIII**			
	IncB (µg/ ml)		**IncC (µg/ ml)**		**IncB (µg/ ml)**		**IncC (µg/ ml)**		**IncB (µg/ ml)**		**IncC (µg/ ml)**	
	0.5	1	0.5	1	0.5	1	0.5	1	0.5	1	0.5	1
Cervical cells	**111** (48-214)	**126** (67-263)	**91** (29-167)	**142** (43-211)	**132**[a] (47-312)	**157**[b] (56-343)	**112**[c] (38-112)	**178**[d] (77-329)	**43** (12-59)	**49** (12-88)	**38** (17-63)	**59** (26-76)
PBMCs	**121** (38-200)	**134** (45-212)	**103** (48-176)	**172** (44-176)	**212**[a] (56-273)	**226**[b] (76-301)	**243**[c] (91-322)	**251**[d] (100-316)	**56** (38-97)	**67** (43-115)	**63** (59-108)	**81** (60-149)

Note. Median number of IFN-γ SFCs per 7.5×10^4 $CD4^+$ T cells at 18 h post stimulation with IncB or IncC (0.5µg/ml and 1µg/ml) counted using ELISPOT. Figures in parenthesis depict range of SFCs for each group. [a, b, c, d] $P<0.05$ Number of IFN-γ in GII compared to respective levels in group III by Mann Whitney U test.

Real time RT-PCR analysis showed significant increase in IFN-γ mRNA in purified $CD4^+$ T cells (from cervical cells and PBMCs) from CT-positive fertile women compared to cells from CT-positive infertile women. Increase in IFN-γ expression in these cells was dose-dependent with increasing concentrations of IncB and IncC (Fig. 4.4). Relative expression of IFN-γ was higher in CT-positive fertile women and controls

with respect to CT-positive infertile women. Expression of IL-4 was detected in 26-43% of stimulated cells obtained from CT-positive women and controls. IL-4 expression levels were low and not significant. Free GST failed to produce significant differences in expression levels of cytokines in induced cell cultures.

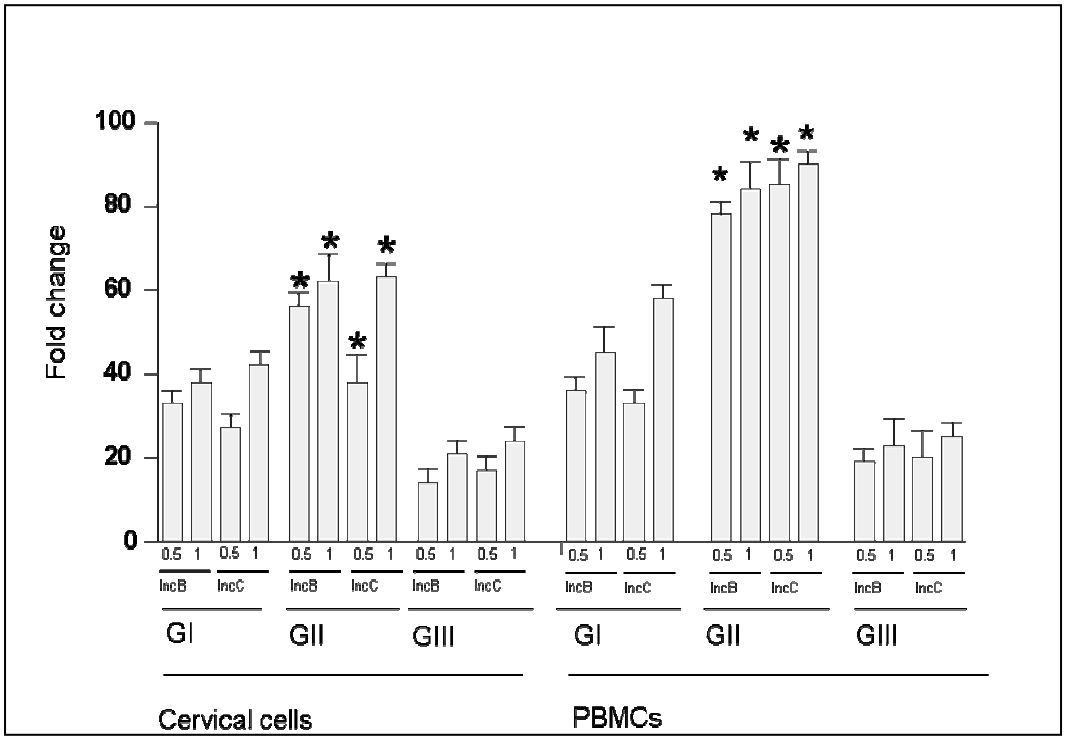

Figure 4.4 Estimation of IFN-γ mRNA relative expression in 7.5×10^4 $CD4^+$ T cells isolated from cervical cells and PBMCs after *in vitro* stimulation with IncB or IncC.
Real-time RT-PCR analysis of IFN-γ mRNA levels was done at 12 hours post stimulation in $CD4^+$ T cells isolated cervical cells and PBMCs isolated from GI, GII and GIII where,
* $P<0.05$ mRNA expression of IFN-γ in GII compared to respective levels in group III by Mann Whitney U test. All cytokines were normalised against corresponding levels of ß-actin endogenous gene. The graph show results as fold change represented by bars. Bars represent mean±s.e.m. for all experiments.

Discussion

To the best of our knowledge, this is the first study on mucosal and peripheral immune responses to chlamydial inclusion proteins in CT-positive women with or without reproductive sequalae from the same population and geographic region. This study could be helpful in providing insights into the contribution of these proteins in immuno-protective/pathological consequences of genital chlamydial infection.

Our knowledge of bacterial antigens and defence mechanisms that lead to immunity against CT has increased substantially in recent years, but still needs an improved and comprehensive understanding of all the elements of the immune system that act in concert to control chlamydial growth and facilitate pathogen clearance without causing immunopathology. Although several inc proteins have been identified in different chlamydial species, their role in chlamydial biology, especially in the aspect of generating host immunity is not well understood. Earlier reports by our group have shown that mucosal and peripheral immune responses are useful for understanding the immune mechanisms operating during chlamydial infection [Vats *et al.,* 2007; Agrawal *et al.,* 2009]. Thus in this study, we evaluated the mucosal and peripheral immune responses in terms of antibody production, cellular proliferation and cytokine production by incs in CT-positive women with or without pathological sequalae.

Using reported [Kostrjukova *et al.,* 2006], and unique primer sets, we were able to detect *incB* and *incC* genes in CT-positive endocervical samples. The prevalence of antibodies against IncB and IncC in cervical washes were detected significantly higher in CT-positive fertile women compared to CT-positive infertile women, which shows their involvement in initial infection than in established disease pathology. Our results are in accordance with previous findings where antibodies from women with urogenital tract *C. trachomatis* infection are generated significantly at the initial infection site in response

to live infection [Sharma *et al.,* 2004]. We did not find any significant difference between prevalence of antibodies to IncB and IncC in sera obtained from CT-positive fertile women compared to CT-positive infertile women. The differential humoral responses of chlamydial proteins in disease condition is previously documented. In a previous study, the seroprevalence of antibodies to CT surface components was found to be higher in women with recurrent chlamydial infections [Agrawal *et al.,* 2007]. The differential immunogenic properties of inclusion proteins and their involvement in particular disease pathologies could be attributed to multiple factors like subcellular localization, cytoplasmic exposure, domain and spatial arrangements on the inclusion membrane or within the inclusion [Li *et al*., 2008]. Seroprevalence of antibodies to chlamydial incs in CT-positive women diagnosed with cervicitis and infertility has been recently reported from our laboratory [Gupta *et al.,* 2009]. High prevalence of antibodies against CT MOMP antigen in patients with primary chlamydial infections has also been reported [Agrawal *et al.,* 2007]. In addition to their roles in neutralizing infectious organisms, antibodies to CT surface elements are known to dramatically enhance opsonization of dendritic cells and to promote strong Th1 immune responses [Shaw *et al.,* 2002]. Conversely, antibodies against cHSP60 antigen in patients with recurrent infections [Agrawal *et al.,* 2007] and against recombinant cHSP60 proteins in women with fertility disorders have also been reported [Srivastava *et al.,* 2008]. Serological data have shown a strong association between antibody responses to cHSP60 and sequalae of CT infection, including PID, tubal infertility, ectopic pregnancy, and scarring trachoma [Eckert *et al.,* 1997; Peeling *et al.,* 1998; Gaur *et al.,* 1999; Peeling *et al.,* 1999; Kinnunen *et al.,* 2003].

Cellular proliferation using MTT assay showed higher stimulation indices of IncB or IncC stimulated cervical cells and PBMCs isolated from CT-positive fertile women with respect to controls and CT-positive infertile women. There is previous evidence on the role of chlamydial antigens in stimulating immune-mediated inflammation. Previous studies on lymphocyte proliferation to chlamydial antigens, including MOMP and heat shock protein 60, have shown enhanced responses in individuals who spontaneously resolve trachoma infection as compared to those with persistent infections [Holland *et al.,* 1993]. Relative depression in lymphocyte proliferation in response to CT antigens in subjects with persistent clinical signs of inflammatory trachoma and in subjects with severe trachomatous scarring has been reported too [Holland *et al.,* 1996]. It is to be mentioned here that a recent study by Alzhanov et al has shown that inclusion protein, CT223 in transfected HeLa or McCoy cells causes reduction in host cell cytokinesis and facilitates centromere supranumeracy defects [Alzhanov *et al.,* 2009]. This study further showed that maximal effect was attributed to the operon coding gene cluster of CT 223-227 and less pronounced with CT IncC. Our data on the number of positive responders for LDH release into supernatants of inc-stimulated cervical cell and PBMC-cultures showed no significant differences between CT-positive patients and controls. Thus both incs failed to cause any disruption of cellular integrity of stimulated cervical cells and PBMCs thereby suggesting that these incs can initiate specific cellular immune responses.

Results in this study suggest that exposure to incs could significantly affect host immune function by modifying the release of cytokines. On comparing cytokine expression by cells obtained from CT-positive fertile women with controls and CT-positive infertile women, cells from CT-positive infertile women secreted significantly

higher levels of IL-1ß. The probable role of IL-1 in fallopian tube destruction during CT infection has been reported [Hvid *et al.,* 2007]. Further, it is also shown that synovial tissues from chronic arthritis patients with synovial *C. pneumoniae* infection have significant levels of mRNA for IL-1ß [Gerard *et al.,* 2002]. These results collectively confirm that IL-1ß can be one of the cytokines that are responsible for chlamydial pathology.

Both incs induced high levels of IL-6 in cells from CT-positive fertile women with or without fertility in comparison to controls. CT-positive infertile women produced highest levels of IL-6 upon induction. In CT-infected cells, IL-6 has been reported to act synergistically with IL-12, for inducing protective immune responses [Yu *et al.,* 2003]. However, our data also showed elevated IL-6 levels in CT-positive infertile patients suggesting the pathogenic role of IL-6. High levels of IL-6 levels have been previously reported to be present in the tubal fluids of patients with infertility caused by chlamydial infections [Li *et al.,* 2000].

IL-10 was found to be up-regulated in both cervical cells and PBMCs stimulated with incs in CT-positive infertile women, with levels being significantly higher than CT-positive fertile women and controls. In a previous report on a longitudinal study of genital CT infection, *in vitro* IL-10 production by PBMCs stimulated with recombinant chlamydial antigens was the greatest risk factor for recurrent infections [Cohen *et al.,* 2005]. In murine models of chlamydial infection, high levels of IL-10 impede pathogen clearance [Yang *et al.,* 1999] whereas IL-10 mutant mice clear infection faster than genetically intact controls [Yang *et al.,* 1996; Redpath *et al.,* 2001; Igietseme *et al.,* 2000]. IL-10 has been found to be associated with susceptibility to chlamydial infection and typical pathological changes caused by the infection such as granuloma formation

and fibrosis [Conti *et al.,* 2003]. Excessive levels of IL-10 levels in conjunctiva are a risk factor for scarring and blindness during CT-associated trachoma. IL-10 contributes to the pathogenesis of fibrotic responses [Yang *et al.,* 1996; Barbarin *et al.,* 2005], and is involved in local matrix remodelling during healing and repair of inflammation-induced injury [Lee *et al.,* 2002]. Thus, in accordance with these reports, our data suggests that IL-10 over-expression in cells from CT-positive infertile women could partly contribute to slow or non-clearance of CT resulting in establishment of tubal pathologies.

Our data shows that in CT-positive fertile women- cases where no apparent clinical pathological symptoms is detected, IFN-γ is the major cytokine to be secreted whereas; in CT-positive women with fertility disorder- with established clinical pathologies, IFN-γ is downregulated. Debattista *et al.*, have reported that women with chlamydial PID or a history of repeated CT infection had PBMCs that produced less IFN-γ in response to CHSP60 than women with a single episode of CT infection [2002]. Subjects with scarring trachoma have shown to produce reduced IFN-γ levels during persistent chlamydial infection [Holland *et al.,* 1996]. Also, co-occurrence of IL-10 and IFN-γ producing cells in synovial tissues from CT-positive arthritis patients have suggested that excessive IL-10 production suppresses IFN-γ and mediates persistence [Kotake *et al.,* 1999]. These results along with ours suggest a protective role of IFN-γ during active chlamydial infection.

Levels of IL-12 were significantly higher in CT-positive fertile women compared to controls and CT-positive infertile women. IL-12 is primarily produced by activated macrophages and dendritic cells and further induces IFN-γ production to favour the differentiation of Th1 cells [Trinchieri *et al.,* 2003; Heufler *et al.,* 1996]. Along with TNF- α, IL-12 is reported to co-stimulate IFN-γ by killer T cells [Tripp *et al.,* 1993]. It

has also been previously reported that clearance of chlamydial infection from female adolescents has been associated with decrease in IL-12 concentrations in endocervical samples suggesting its role in protective immune responses against infection [Wang *et al.,* 2005].

Levels of GM-CSF were significantly higher in CT-positive fertile women compared to CT-positive infertile women and controls. GM-CSF is known to activate macrophages and up-regulate CD14 and MHC class II expression [Caulfield *et al.,* 1997]. As a result, macrophages present antigen more efficiently for the subsequent development of protective immunity. In a murine model of CT mouse pneumonitis lung infection and intrapulmonary adenoviral GM-CSF transfection, it has been demonstrated that the expression of GM-CSF in the airway compartment significantly enhanced systemic Th1 cellular and local IgA immune responses following immunization with inactivated organisms [Lu *et al.,* 2002]. In contrast, GM-CSF which is known to be important for successful pregnancy has been shown to be down-regulated by IFN-γ in the reproductive tract. This coupled with the chlamydiostatic effects of IFN-γ on infected cells, GM-CSF proved to be one of the key elements in the persistence of *C. psittaci* in infected sheep and subsequent abortion of the foetus [Entrican *et al.,* 1999]. However our results suggest that GMCSF expression levels following stimulation with IncB and IncC in CT-positive fertile women could confer protection and clearance of infection.

Stimulation of cervical cells and PBMCs with IncB and IncC significantly increased mRNA expression levels of IL-4 in cells obtained from CT-positive infertile women in comparison to the other two groups. It has previously been reported that individuals with severe trachomatous scarring had PBMCs that produced IL-4 to CHSP60 more frequently than did PBMCs from matched community control subjects

without scarring [Holland *et al.,* 1996]. In this study we failed to detect IL-4 protein levels in cell supernatants. This could be due to the fact IL-4 secreting cells may be quenched or short lived in peripheral circulation or its secretion was downregulated by other factors in the mixed cell population [Holland *et al.,* 1996]. Depressed levels of IL-4 detected in CT-infected HIV positive patients using TaqMan™ based real time PCR has been reported [Scott *et al.,* 2006].

Expression levels of IL-5 mRNA were elevated in cells obtained from CT-positive infertile women in comparison to controls and CT-positive fertile women. Elevated levels of IL-4 and IL-5 by splenic lymphocytes from IFN-γ knock-out mice following chlamydia-specific and polyclonal T cell activation compared with that in wild-type mice has been reported. This Th2 mediated delayed hypersensitivity was not protective in controlling local chlamydial infection and associated with serious tissue damage and cellular inflammation and failing to prevent dissemination of the pathogen to other organs in these mice [Wang *et al.,* 1999].

Overall our data on cytokine modulation in cervical cells and PBMCs on *in vitro* stimulation with IncB or IncC suggests that women (CT-negative women) who encounter chlamydial antigens for the first time (thereby mimicking *in vivo* naïve cells) or those with no pathological damage (CT-positive fertile women) produce higher amounts of IFN-γ, IL-12 and GM-CSF but in women with reproductive sequalae to chlamydial infection, the cells secrete higher levels of IL-1ß, IL-4, IL-5, IL-6 and IL-10. Although it would be presumptuous to assume that the clinical condition of women with fertility disorders could be completely attributed to chlamydial infections yet it is not wrong to say that the changes in cytokine patterns can be responsible for development of various pathological consequences to chlamydial infection. Further, to this end the role

of IncB and IncC in maintaining a differential cytokine surveillance milieu within infected cells is of interest.

Our data on $CD4^+$ T cells isolated from cervical cells and PBMCs from CT-positive women with and without fertility disorders displayed high levels of IFN-γ secreting cells and mRNA expression and minimal IL-4 production upon *in vitro* dose dependent stimulation with IncB and IncC, demonstrating the induction of an antigen-specific Th1 cellular response. There is accumulated evidence to suggest that Th1 responses and IFN-γ production are important for optimal resolution of genital chlamydial infection [Brunham *et al.,* 2005; Morrison *et al.,* 2002]. It has been demonstrated that *Chlamydia*-specific Th1 clones, but not Th2 clones, are capable of adoptively transferring anti-chlamydial immunity [Igietseme *et al.,* 1994; Igietseme *et al.,* 1993; Ramsey *et al.,* 1991], and that MHC class II deficient, but not MHC class I deficient animals were incapable of resolving a primary chlamydial challenge [Williams *et al.,* 1997]. Mice with a targeted disruption in -/- IFN-γ production (IFN-γ -/- mice) have been shown to unable to resolve chlamydial infection and prevent bacterial dissemination [Wang *et al.,* 1999]. In this regard, the bactericidal effect of IFN-γ on intracellular *Chlamydia* in epithelial cell cultures has been shown to occur via the indoleamine-2, 3-dioxygenase pathway in human cells versus p47 GTPases in murine cells [Nelson *et al.,* 2005]. Thus, our data shows that IncB and IncC could be able to mount a protective Th1 cellular response for clearance of CT from infected host cells.

Since chlamydial inclusion proteins are believed to be involved in biochemical cross-talk between host cells and infecting pathogens, this data on host immunity to these proteins would therefore be helpful in our understanding of biological functioning of this elusive pathogen. Further in-depth efforts are needed to understand the role of particular

cell populations which are specifically involved in providing cell mediated immunity against chlamydial inclusion proteins in infected host cells.

Introduction

Genital infections due to *Chlamydia trachomatis* (CT) is a world-wide health problem. It has been established that acquired immunity to chlamydial infection, involves both humoral and cell-mediated immune responses in infected individuals [Brunham and Rey-Ladino, 2005]. Further, there is accumulating evidence through murine and human studies, that the antichlamydial protective immunity is mediated by T helper type 1 (Th1) response, involving the induction and recruitment of Th1 cells into the local mucosae [Igietseme *et al.,* 1993; Su and Caldwell 1995; Cotter *et al.,* 1997; Stagg *et al.,* 1998; Yang *et al.,* 1998; Igietseme *et al.,* 1998]. Humoral immune responses through secretory and systemic antibodies are able to generate protective immunity [Bailey *et al.*, 1993; Johansson *et al.*, 1997] or aid in Th1-driven T cell immunity by modifying the disease severity or path of reinfection [Cotter *et al.*,1995; Morrison *et al.*, 1995; Yang and Brunham, 1998]. Furthermore, antigen presenting cells (APCs) like dendritic cells (DCs) contribute to antichlamydial immunity and preferentially activate Th1 responses with the help of interleukin (IL)-1, IL-12, intercellular adhesion molecule (ICAM-1), lymphocyte function-associated antigen 3 (LFA-3), CD40 and B7 molecules [Jenkins *et al.*, 1993; King *et al.*, 1993; Ojcius *et al.*,1998].

Adaptive immune protection to CT involves recruitment of $CD4^+$ or $CD8^+$ T cells at sites of infection by adoptive transfer in mice models [Igietseme *et al.,* 1993; Igietseme *et al.,* 1994]. Furthermore, $CD8^+$ T cells play a minor protective role compared

to that of $CD4^+$ cells in genital chlamydial infection [Magee *et al.,* 1993; Su and Caldwell 1995]. T cell recognition of infected cells leads to secretion of cytokines capable of restricting intracellular pathogen growth. Cytokines produced by T cell subsets in response to CT infection are known to influence each other through stimulatory or inhibitory pathways and finally determine the clinical course of infection by resulting in successful clearance of CT or associated pathology [Debattista *et al.*, 2003]. Emerging data from our laboratory have suggested that CT IncB and IncC modulate host immune responses and may have a role in protection/ pathogenesis of genital chlamydial infection in women. Therefore, in this study we used cytokine-specific real-time reverse-transcriptase (RT)-PCR assay and ELISA to evaluate the role of IncB and IncC stimulation on Th1 and Th2 cytokine release in $CD4^+$ T cells from CT-infected fertile or infertile women.

Materials

Reagents and Chemicals

Agarose, Bovine Serum Albumin, Glycine, Glutamine, HEPES, Streptomycin, Gentamycin sulphate, Adenosine, Tris base, RPMI-1640, Kanamycin sulphate, Ponceau S, Diaminobenzamide, Ficoll-Hypaque, Coomassie, MTT and other fine chemicals were from Sigma Chemicals, USA. Fetal bovine serum was obtained from Biological Industries, Israel. Terrific Broth, phenyl methyl sulfonyl fluoride, aprotinin, leupeptin and pepstatin were purchased from Amresco, USA. Horse radish peroxidase (HRP)-conjugated rabbit anti- human IgG_2 and IgA antibodies were purchased from Bangalore Genei, India. LDH-Cytotoxicity Assay Kit was purchased from BioVision Research

Products, CA, USA. RNeasy Mini Kit was from Qiagen, CA, USA. SuperScript™ First-Strand Reverse Transcriptase kit and Trizol, *Taq* polymerase, dNTPs, $MgCl_2$, DTT, Superscript II reverse transcriptase, oligo dT_{20}, RNasin were from Invitrogen, USA. DyNAmo™ SYBR® Green qPCR Kit was purchased from Finnzymes, Espoo, Finland. MACS MicroBeads® was obtained from Miltenyi Biotec, CA, USA. Phycoerythrin conjugated CD4 and CD8 antibodies were purchased from Becton Dickinson, San Diego, USA. Bradford assay and polyvinyl difluoride (PVDF) membrane was purchased Bio-Rad Laboratories, CA, USA. Cytokine ELISA kits were purchased from eBiosciences, San Diego, USA and ELISPOT kits were available from Diaclone, Cedex, France. All HPLC purified primers were commercially synthesized from MWG-Biotech AG, Ebersberg, Germany.

Experimental methods

1. Study population

After obtaining informed written consent, 35 patients attending the gynaecology outpatient department, Safdarjung Hospital, New Delhi, India were enrolled for the study. 25 healthy age-matched controls attending the family planning department for birth control measures were also enrolled. All samples were collected as mentioned in **Chapter 6**.

2. Laboratory diagnosis

As described in **Chapter 4**, endocervical samples were tested for chlamydial positivity by PCR analysis using CT specific 200 base pair (bp) plasmid primers. PCR detection of CT *incB* (CT 232) and *incC* (CT 233) genes in all endocervical samples was also performed as in **Chapter 4**.

3. Antibody assays

Cervical washes of patients and controls were assayed for immunoglobulin G (IgG) antibodies to CT surface components using a commercially available ELISA (Ridascreen, R-Biopharm AG, Darmstadt, Germany). Results were obtained as mean absorbance of duplicated samples at A_{450}. An OD>1.1 was considered positive.

CT IncB and IncC specific IgG_2 titres in cervical washes were determined by ELISA as described in **Chapter 5**. Positive samples were defined as those yielding an absorbance (OD) value at least two standard deviations (SDs) above the mean value obtained from the panel of samples taken from the negative subjects.

CT IncB and IncC specific IgG in cervical washes was further determined by Western blot assay according to protocol mentioned in **Chapter 6**. Patients who showed presence of a 38 kDa band for IncB and a 44.4 kDa band for IncC were considered positive by western blot assay.

4. Cell cultures

Endocervical cells were isolated and counted as described [Agrawal *et al.,* 2007]. In brief, endocervical cells were isolated from the cytobrush by vigorously rotating it along the sides of the transport tube after incubating the sample with 5mM $_{DL}$-dithiothreitol (Sigma-Aldrich) at 37 ^{o}C for 10 min (to reduce mucus content of the sample). The cell suspension was then filtered through a sterile 70-µm nylon cell strainer (BD Biosciences, San Diego, USA) and pelleted down at 1,500 rpm for 10 min to yield endocervical cells. The resulting cell pellet was washed three times with Hank's balanced salt solution (Sigma-Aldrich) and suspended in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated human AB serum. The viability of cells was determined by a trypan blue exclusion assay and an aliquot of cells was used for flow-

cytometric analysis while the rest of the cells were used for separation of T cell subsets as described below.

5. Flow cytometry

To quantify percentage of different T cell in cervical samples flow cytometry was done by standard methodology as described [Reddy *et al.,* 2004]. Endocervical cells obtained were stained with fluorescein isothiocyanate (FITC)-conjugated anti CD3 (for T cell identification) antibodies, and phycoerythrin (PE)-conjugated CD4 and CD8 antibodies (Becton Dickinson) for 25 min on ice. Preparations were then washed with buffer (PBS supplemented with 0.1% NaN_3 and 2% FBS) and acquired using FACS Caliber (BD Biosciences). A total of 10,000 events were acquired. Appropriate isotype matched control antibodies were used to rule out nonspecific fluorescence.

6. Isolation of T cell subsets

$CD8^+$ T cells were positively selected from cervical cells using CD8 magnetic microbeads, MACS MicroBeads® (Miltenyi Biotec, CA, USA) according to manufacturer's instructions as described in **Chapter 6.** The CD8 depleted cell fraction contained $CD4^+$ T cells and other cell populations including antigen presenting cells. This cell fraction was used for further assays and will be termed as $CD4^+$ T cells to simplify explanation in the rest of the chapter. Cells were gently pelleted and suspended in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated human AB serum. Purified $CD4^+$ T cells (2×10^5 cells/ well) were stimulated and cultures were incubated in humidified 5% CO_2 at 37°C for 12h (for RT-PCR) and 18h (for ELISpot).The purity of $CD4^+$ T cells was determined by flow cytometry on a FACS

Caliber (BD Biosciences) using a PE-conjugated anti-CD4 monoclonal antibody (Becton Dickinson, San Jose, USA). Purity of $CD4^+$ T cells was more than 90%. The $CD4^+$ T cells were then cultured in triplicate (2×10^5 cells/well) in round-bottomed 96-well plates (cell proliferation assays, for cytokine and transcription factors' mRNA expression and estimation of secreted cytokines) with or without stimulants in a total volume of 200 µl. Subsequently cultures were incubated in humidified 5% CO_2 at 37°C for 12h (for real-time RT-PCR) and 72h (for cell proliferation assays and cytokine specific ELISA and ELISPOT).

7. Stimulants

$CD4^+$ T cells were stimulated with/without CT serovar D whole EBs at multiplicity of infection 2 (to serve as CT positive control), IncB (1 µg/ml) and IncC (1 µg/ml). IncB and IncC were expressed and purified as described in **Chapter 4**. Phytohaemaglutinin (PHA 2 µg/ml) (Sigma-Aldrich) and free GST were used as positive control mitogen and negative control respectively in each experiment. Optimum concentrations of antigens and mitogen were determined in preliminary experiments as minimum concentrations giving maximal proliferation at different time intervals post stimulation.

8. Cell proliferation assay

A colorimetric assay based on MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide) (Sigma-Aldrich) was performed to measure the proliferative activity of $CD4^+$ T cells on stimulation with IncB and IncC using methodology described in **Chapter 6**.

9. RNA extraction and real-time RT-PCR analysis for cytokines and transcription factors

Total RNA from IncB or IncC-stimulated $CD4^+$ T cells was isolated using RNeasyMini Kit (Qiagen, CA, USA), in accordance with the manufacturer's instructions and stored at -70 °C. Real-time RT-PCR analysis for cytokines and transcription factors were performed according to protocol in **Chapter 6**.

Table 5.1 Primers used in this study

No.	**Cytokine gene primers**	**5'-3' sequence**
1	GM-CSF F*	AGC CCT GGG AGC ATG TGA
	GM-CSF R**	GAG TAG AGA CAC TGC TGC TGA GAT G
2	IL-β1F	GCT GAT GGC CCT AAA CAG ATG
	IL-β1R	ACGAAT CTC CGA CCA CCA CTA
3	IFNγ F	GAA ACG AGA TGA CTT CGA AAA GCT
	IFNγ R	ATG TCC AAC GCA AAG CAA TAC A
4	IL-12 F	TCG CGT TCA CAA GCT CAA GT
	IL-12 R	CA AAC CTG ACC CAC CCA AGA
5	TNF-α F	GCC CGA CTA TCT CGA CTT TGC
	TNF-α R	A ACC TTC CCA AAC GCC TCC
6	IL4 F	CCA CGG ACA CAA GTG CGA TAT
	IL4 R	CGT AAC AGA CAT CTT TGC TGC C
7	IL5 F	AAA GGC AAA CGC AGA ACG TT
	IL5 R	CTC TTG GAG CTG CCT ACG TGT
8	IL6 F	ACT CAC CTC TTC AGA ACG

	IL6 R	AGT AGT GAG GAA CAA GCC
9	IL10 F	GGG AGA ACC TGA AGA CCC TCA
	IL10 R	AAC AAG AGC AAG GCC GTG G
10	T-bet(TBX21) F	AAC ACA GGA GCG CAC TGG AT
	T-bet(TBX21) R	ATT GTG CTC CAG TCC CTC CA
11	GATA3 F	AGA TGG CAC GGG ACA CTA CCT
	GATA3 R	ATT AAG CCC AAG CGA AGG C
12	B-actin F	TGT GAT GGT GGG TAT GGG TC
	B-actin R	TAC AAT GAG CTG CGT GT

* forward primer ** reverse primer

10. Quantification of secreted cytokines

Quantification of IL-1ß, IL-4, IL-6, IL-10, IL-12, IFN-γ, TNF-α, GM-CSF and IL-23 in culture supernatants of IncB or IncC-stimulated $CD4^+$ T cells was performed by commercially available ELISA kits (eBiosciences, San Diego, USA), in accordance with the manufacturer's instructions. The absorbance was read at 450 nm, log–log standard curves were generated and unknowns were interpolated. The minimum detectable cytokine concentrations for these assays were- IL-1ß (4 pg/ml), IL-4 (2 pg/ml), IL-6 (2 pg/ml), IL-10 (2 pg/ml), IL-12 (4 pg/ml), IFN-γ (4 pg/ml), TNF-α (4 pg/ml), GM-CSF (2.5 pg/ml) and IL-23 (15 pg/ml).

11. Cytokine analysis using ELISPOT assay

The numbers of IL-10, IFN-γ, IL-12 and IL-4 producing cells in culture supernatants of IncB or IncC-stimulated $CD4^+$ T cells was measured by ELISPOT assay (Diaclone, Cedex, France) as per the manufacturer's instructions and the number of

cytokine-secreting cells were counted on a Bioreader® 4000 ELISpot Reader (Biosys, Leeds, UK).

12. Statistical analysis

The Kruskal–Wallis non parametric test was used to compare continuous variables among multiple groups. The Mann–Whitney U test was used for comparing two groups. Categorical variables were compared using χ^2 test. The results were presented with 95% confidence interval (CI) and $P < 0.05$ was considered significant. All statistical analyses were performed with Graphpad Prism Version 5 (La Jolla, CA, USA).

Results

Study population

Cervical CT infection was diagnosed by PCR in 32 patients. Three of these patients were found to be co-infected either with *Candida sp.*, bacterial vaginosis, *T. vaginalis*, *M. hominis*, *U. urealyticum* or *N. gonorrhoeae* in the cervix and were thus excluded from the study. Based on clinical history and diagnosis, the patients were categorized into three groups. Group I (GI, n=25) comprised of CT-uninfected healthy controls with no infertility problems, Group II (GII, n=14) comprised of CT-positive fertile women and Group III (GIII, n=18) comprised of CT-positive infertile women. The median ages of CT-positive infertile or fertile women and controls were comparable (28, 26 and 27 years, respectively).

Detection of *incB* and *incC* genes

Detection of *incB* and *incC* was carried out in 32 patients positive for CT infection. Visualization of 357 bp amplicon for *incB* gene and 545 bp amplicon for *incC* gene in a 1.2% (w/v) agarose gel was considered as a positive result for amplification of inc genes.

Detection of antibodies against IncB and IncC

ELISA results showed that IgG_2 antibodies to both IncB and IncC were significantly higher in cervical washes of CT-positive fertile or infertile women compared to controls. The prevalence of IncB and IncC IgG_2 was significantly higher in CT-positive fertile women as compared to CT-positive infertile women (Table 5.2).

Antibody recognition of IncB and IncC proteins was confirmed by Western blot assay. Western blot assay showed significantly higher positivity in cervical washes of CT-positive fertile or infertile women compared to controls. Significantly higher number of women positively detected IncB and IncC IgG in CT-positive fertile women as compared to CT-positive infertile women (Table 5.2). No bands were observed in cervical washes obtained from controls.

Table 5.2 Detection of *C. trachomatis* positivity in study population by PCR, ELISA and Western blot

	ELISA			PCR		Western blot	
	CT IgG	IncB IgG_2	IncC IgG_2	IncB	IncC	IncB IgG	IncC IgG
GI (n=25)	1 (4)	1(4)	2 (8)	0	0	1(4)	1(4)
GII (n=14)	12 (86)[a, i]	10 (72)[b, j]	11 (79)[c, k]	12 (86)[d, l]	12 (86)[e, m]	9 (64)[f, n]	11 (79)[g, o]
GIII (n=18)	9 (50)[q]	8 (44)[r]	7 (39)[s]	17 (94)[t]	18 (100)[u]	8 (44)[v]	7 (39) [w]

Note Table shows the number of patients who were positive for tests mentioned. Values in parenthesis denote percentage unless otherwise stated.

[a] P=0.029 as compared to GIII; [b] P=0.010 as compared to GIII; [c] P=0.007 as compared to GIII; [d] P=NS as compared to GIII; [e] P=NS as compared to GIII; [f] P=0.007 as compared to GIII, [g] P=0.006 as compared to GIII; [i, j, k, l, m, n, o, q, r, s, t, u, v, w] $P<0.05$ as compared to corresponding GI where,

Group I (GI) comprised of healthy women with no CT infection, Group II (GII) comprised of CT-positive fertile women, Group III (GIII) comprised of CT-positive infertile women. All categorical variables were compared using the $\chi 2$ test.

Immune cell population in cervical mucosa

Flow-cytometric analysis demonstrated the presence of $CD3^+$ $CD4^+$ T cells and $CD3^+$ $CD8^+$ T cells in all the CT-positive groups. There was significant increase in the mean number of $CD4^+$ T cells per 10,000 events in cervical mucosa of CT-positive

fertile women as compared to CT-positive infertile women and controls (3446 versus 1632, and 433 respectively; $P < 0.05$) (Fig. 5.1). In contrast, in the cervical mucosa the $CD8^+$ T cell population was found to be increased during chlamydial infection but not significantly ($P < 0.05$).

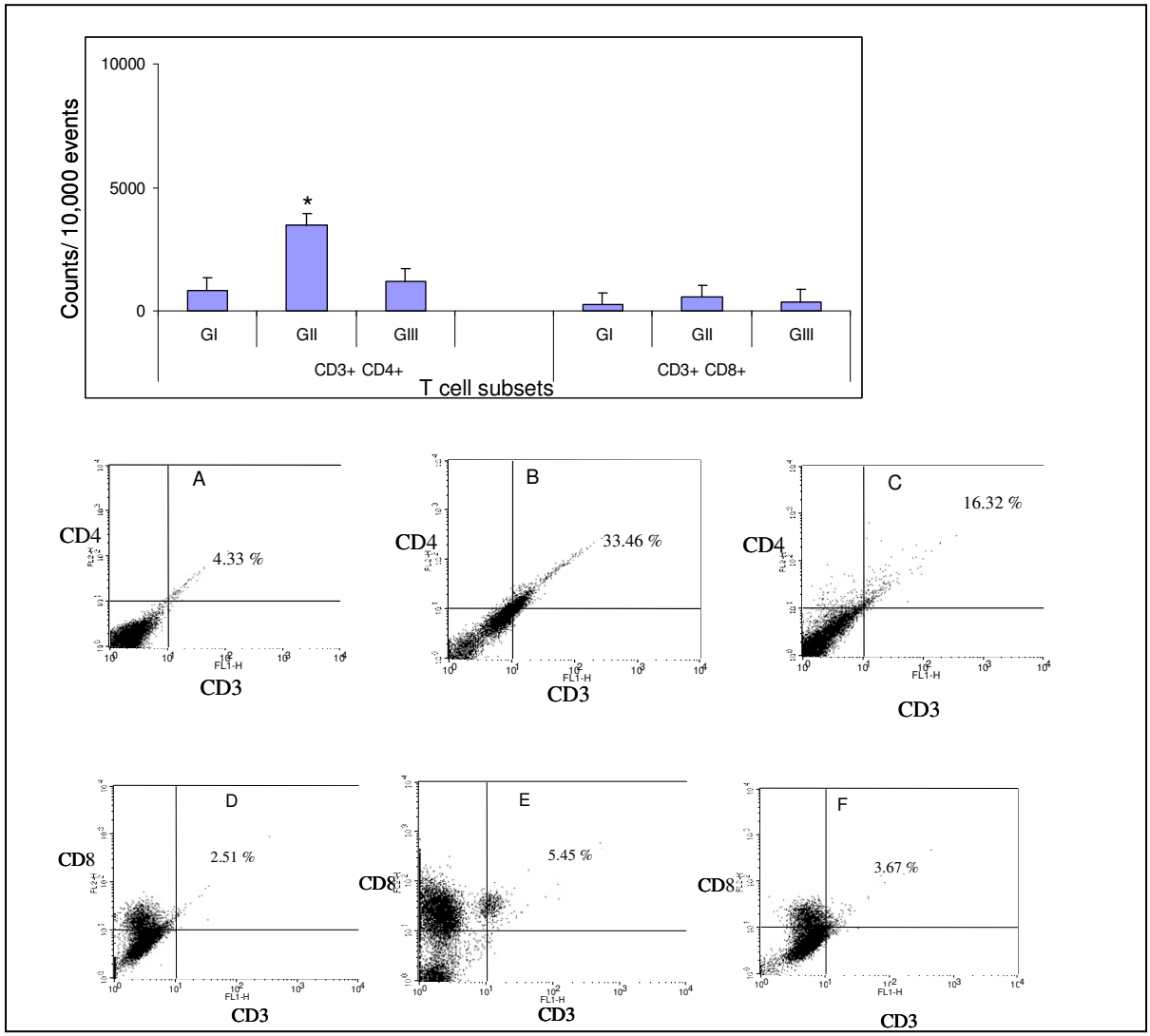

Fig. 5.1. Flow-cytometric analysis to detect the presence of $CD3^+$ $CD4^+$ T cells and $CD3^+$ $CD8^+$ T cells in CT-positive fertile and infertile women and controls.

There was significant increase (*) in the mean number of $CD3^+CD4^+$ T cells per 10,000 events in cervical mucosa of CT-positive fertile women (B) as compared to CT-positive infertile women (C) and controls (A). In contrast, in the cervical mucosa the $CD3^+CD8^+$

T cell population was found to be increased in CT-positive fertile (E) and CT-positive infertile women (F) compared to controls (D) but not significantly ($P < 0.05$)

Cell proliferation

MTT assay was used to evaluate cellular proliferation of $CD4^+$ T cells on stimulation with IncB and IncC. Positive proliferative responses (SI > 2) to IncB and IncC were statistically significant ($P < 0{\cdot}05$) in $CD4^+$ T cells obtained from CT-positive fertile women compared to CT-positive infertile women or controls (Fig. 5.2). Stimulation with free GST (which served as negative controls) showed no significant differences in proliferative responses in any group.

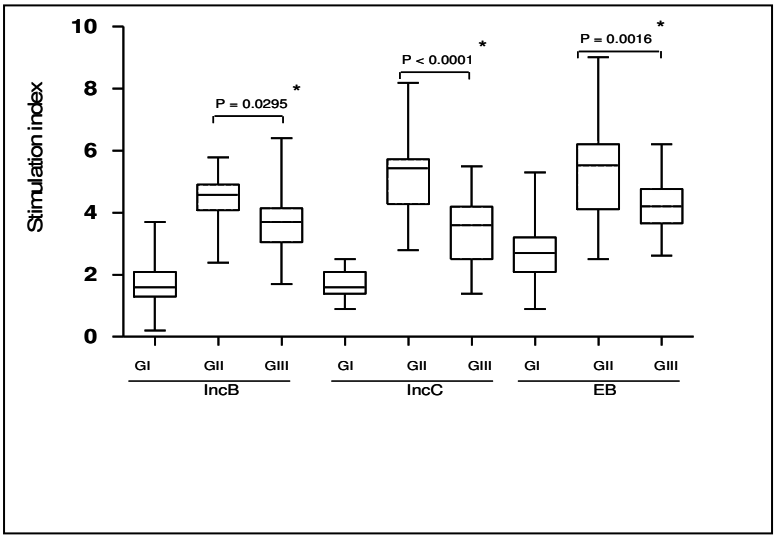

Fig. 5.2. Proliferative responses (stimulation indices) of $CD4^+$T cells isolated from GI, GII, GIII and stimulation with IncB, IncC and CT EB were estimated by MTT assay. The horizontal line in the middle of the box is the median value of the responses and the

lower (upper) is the 25th (75th) percentile, *Significant; Proliferative responses between groups were evaluated using Mann–Whitney *U* test.

Cytokine mRNA expression levels in stimulated CD4$^+$ T cells

mRNA for cytokines viz. IL-1ß, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-□, IFN-γ and GMCSF and transcription factors, T-Bet and GATA3 was detected in stimulated CD4$^+$ T cells in all patients' groups (Fig. 5.3A). On stimulation of CD4$^+$ T cells with IncB or IncC, significant increase in mRNA expression levels of IFN- γ, IL-12, GM-CSF and ($P < 0.05$) was observed in cells obtained from CT-positive fertile women compared to controls and CT-positive infertile women (Fig. 5.3B). In contrast, IL-1ß, IL-4, IL-5, IL-6, IL-10 mRNA expression levels were significantly higher ($P < 0.05$) in cells obtained from CT-positive infertile women compared to controls and CT-positive fertile women (Fig. 5.3C). On stimulation of CD4$^+$ T cells with IncB or IncC, IFN- γ and T-Bet levels were found to positively correlated in CT-positive fertile women (Fig. 5.3D) whereas IL-4 mRNA levels were positively correlated to GATA3 expression (Fig. 5.3E). There were no changes in cytokine mRNA expression in cervical cells stimulated with free GST.

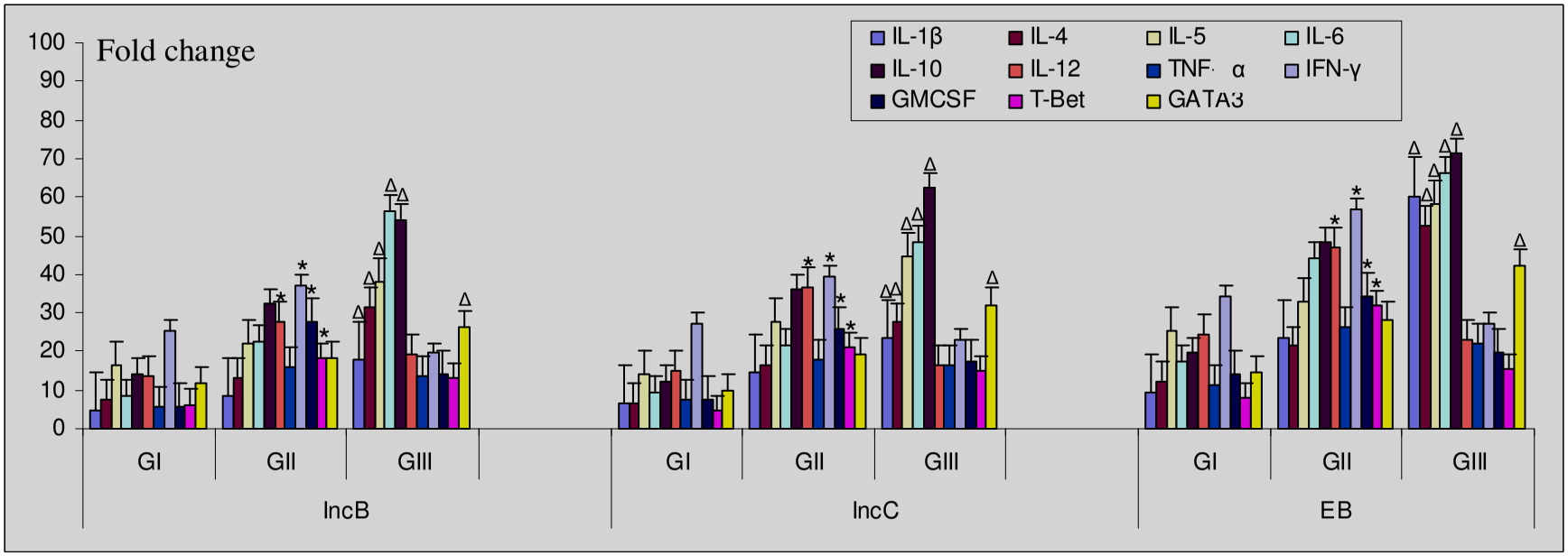

Fig. 5. 3A. Real-time RT-PCR for cytokine mRNA expression in stimulated mucosal $CD4^+$ T cells.
Estimation of mRNA expression of IL-1ß, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-α , IFN- γ and GMCSF in 2×10^5 PBMCs after *invitro* stimulation with IncB, IncC and CT EB.
Real-time RT-PCR analysis of mRNA levels was done at 12 hours post infection in PBMCs isolated from GI, GII and GIII where,
Group I (GI) comprised of healthy women with no CT infection,
Group II (GII) comprised of CT-positive fertile women,
Group III (GIII) comprised of CT-positive infertile women,
* $P < 0.05$ Expression of cytokine mRNA in GII compared to corresponding levels in GI and GIII by Kruskal Wallis test.
Δ $P < 0.05$ Expression of cytokine mRNA in GIII compared to corresponding levels in GI and GII by Kruskal Wallis test.
RT-PCR- Reverse-Transcriptase PCR; IncB- CT Inclusion membrane protein B; IncC- CT Inclusion membrane protein C;
CT- *Chlamydia trachomatis*; EB-Elementary bodies; IL-Interleukin; ß-Beta; IFN- γ -Interferon gamma;
TNF-α -Tumor Necrosis factor alpha; GMCSF- Granulocyte macrophage colony-stimulating factor.
All cytokines were normalised against corresponding levels of ß-actin endogenous gene.
The graph show results as fold change represented by bars. Bars represent mean±s.e.m. for all experiments.
X-axis- Stimulants used in the study; Y-axis- Fold change in RNA expression under different conditions.

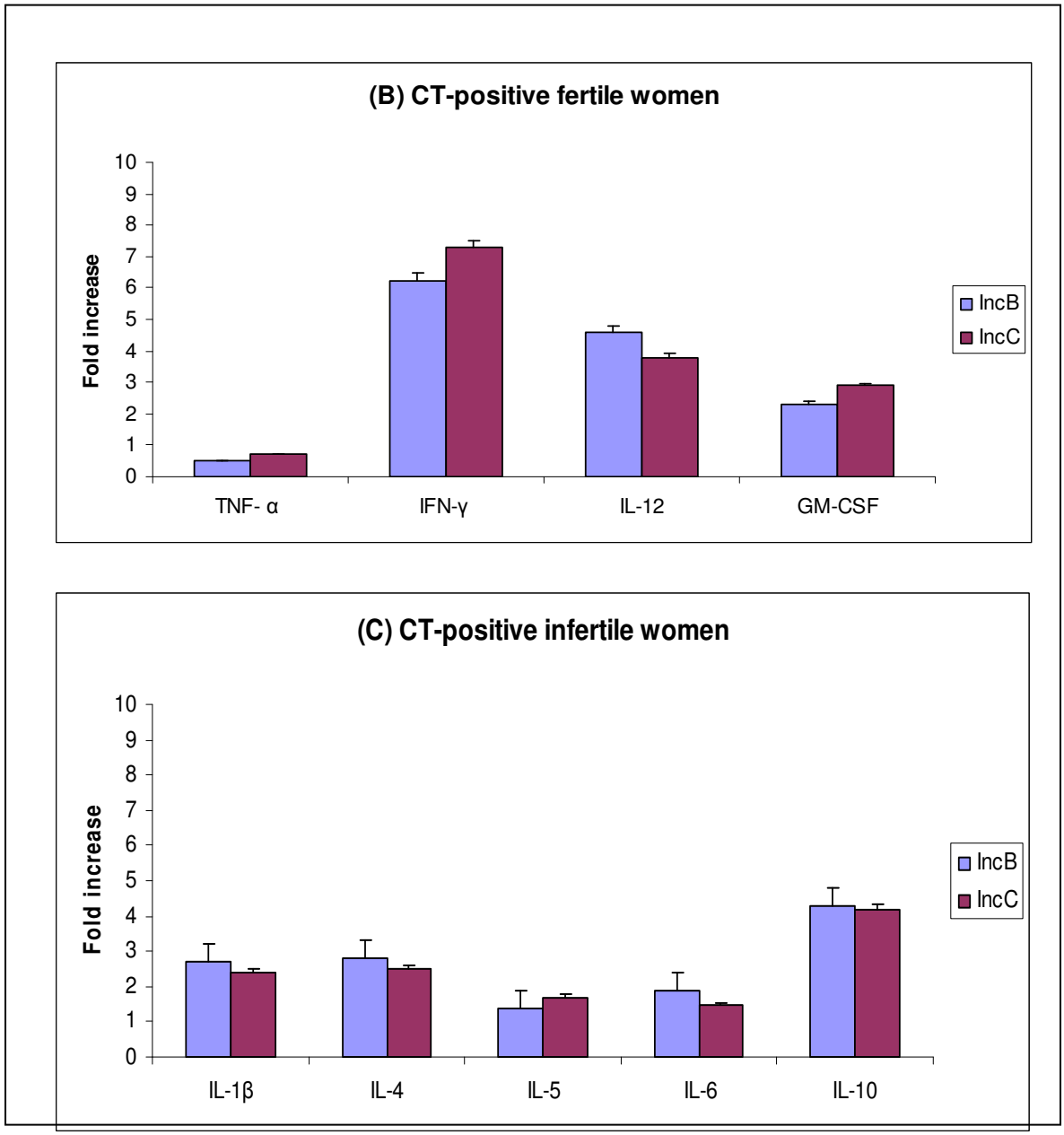

Fig. 5.3. Graph shows fold increase in expression of (**B**) TNF- α, IFN- γ, IL-12 and GMCSF in CT-positive fertile women and (**C**) IL-1ß, IL-4, IL-5, IL-6, IL-10 in CT-positive infertile women. 2×10^5 cervical $CD4^+$ T cells were *invitro* stimulated with IncB, IncC and real-time RT-PCR analysis of cytokine mRNA levels was done at 12 hours post infection. All cytokines were initially normalised against corresponding levels of ß-actin endogenous gene. Post normalization, cytokines which were significantly increased upon Inc-stimulation were further normalized against corresponding levels in other

patient groups. The graph show results as fold change represented by bars. Bars represent mean±s.e.m. for all experiments. X axis- cytokines increased in particular patient group; Y-axis- Fold increase in RNA expression on stimulation.

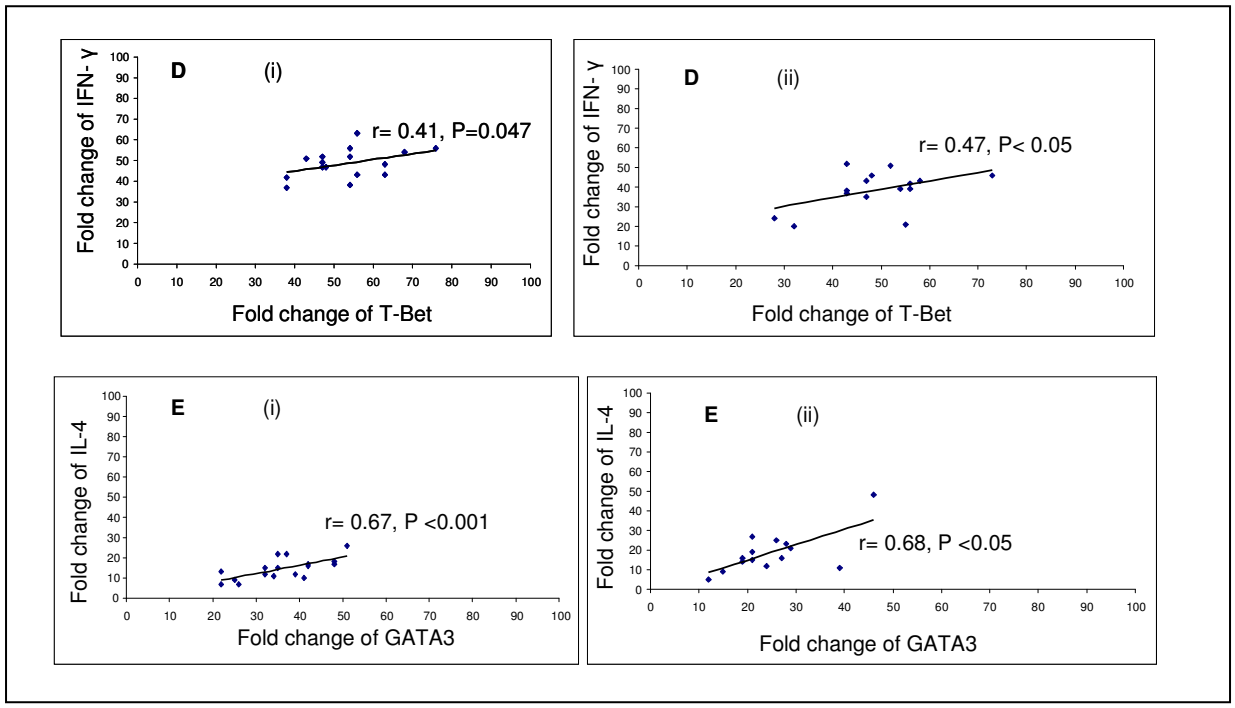

Fig. 5.3. Scatter plot showing the correlation between transcription factors and cytokines in **(D)** CT-positive fertile and **(E)** infertile women. A positive correlation is observed between IFN-γ and T-Bet in **D (i)** IncB and **D (ii)** IncC stimulated cells from CT-positive fertile women whereas a positive stimulation was observed between IL-4 and GATA3 in **E (i)** IncB and **E (ii)** IncC stimulated cells from CT-positive infertile women.

ELISA for cytokines in cell supernatants of stimulated $CD4^+$ T cells

Significantly higher levels of IL-1ß, IL-6 and IL-10 were secreted from IncB or IncC stimulated $CD4^+$ T cells obtained from CT-positive infertile women as compared to CT-positive fertile women or controls ($P < 0.05$). In contrast, IncB and IncC stimulated $CD4^+$ T cells obtained from CT-positive fertile women secreted significantly higher levels of IL-12, IFN-γ, GM-CSF and IL-23 compared to CT-positive infertile women or controls ($P < 0.05$). Significantly high levels of TNF- α and IL-6 levels were secreted in

$CD4^+$ T cells from CT-positive fertile women compared to controls (Table 5.3). IL-4 was below detection limit in all culture supernatants. $CD4^+$ T cells stimulated with free GST showed no significant differences in levels of secreted cytokines.

Table 5.3. Cytokine concentrations (pg/ml) in culture supernatants of stimulated mucosal $CD4^+$ T cells

	GI			GII			GIII		
	IncB	**IncC**	**EBs**	**IncB**	**IncC**	**EBs**	**IncB**	**IncC**	**EBs**
IL-1β	**141.8** (109.5-335.5)	**169.3** (98.8-368.5)	**243.6** (145.1-467.9)	**312.3** (109.8-489.2)	**407.1** (135.2-444.2)	**538.3** (124.8-767.3)	**692.9**[a] (167-941.4)	**825.3**[b] (182.8-982.3)	**900**[c] (288.6-1231.3)
IL-6	**146.7** (74.9-400.3)	**159.4** (87.3-548.5)	**299.92** (98.67-981.32)	**604**[d] (132.2-871.6)	**612.87**[e] (156.5-716.9)	**751**[f] (187.97-1093.2)	**900.63**[a] (441.4-1054.72)	**878.41**[b] (422.9-1078.78)	**1241.07**[c] (656.4-1878.3)
IL-10	**278.4** (132.7-254.2)	**287.9** (143.66-267.5)	**313.7** (159.4-623.9)	**500.3** (267.9-702)	**587.1** (249.3-734.2)	**643.9** (338.2-742.6)	**1132.1**[a] (562.6-1441.9)	**1278.3**[b] (600.3-1502.4)	**1538.4**[c] (768.2-1709.07)
IL-12	**187.6** (112.6-336.4)	**193.6** (123.2-414.1)	**265.9** (132.7-451.1)	**345.9** [g] (231.4-552)	**365.2**[h] (256.3-462.2)	**463.2**[i] (259.7-606.3)	**200.2** (168.4-378.4)	**189.7** (179.3-334.8)	**243** (182.1-356.2)
IFN-γ	**443.6**[j] (125.1-600.8)	**467.28**[k] (141-580)	**580.3**[l] (154.2-671.4)	**703.1** [g] (332.6-988.61)	**571.3**[h] (311.9-814.3)	**859.2**[i] (342.1-1460.5)	**229.5** (110.1-300.8)	**285.4** (154.2-412.6)	**453.3** (189.5-432.9)

TNF-α	**156.1** (72.5-254.3)	**167.3** (73.2-269.1)	**199.3** (82.2-343.5)	**298.3[d]** (138.2-400)	**271.9[e]** (128.3-438.6)	**486.1[f]** (188.2-569.2)	**248.6** (132.6-378.1)	**263.2** (128.97-350.6)	**411.2** (164.5-588.6)
GM-CSF	**181.2** (85.8-243.7)	**117.6** (93.6-229.7)	**233.1** (106.4-259.9)	**278.2 [g]** (127.5-333.6)	**282.5[h]** (148.3-313.4)	**356.2[i]** (172.3-421.6)	**172.4** (99.8-223.8)	**186.7** (103.7-245.7)	**228.9** (141.6-279.6)
IL-23	**59.3** (43-176.5)	**76.2** (57.2-217.4)	**126.7** (105.6-211.3)	**153.2[g]** (65.7-200.1)	**134.7[h]** (87.2-213.2)	**187.4[i]** (148.2-231)	**75.2** (74.1-211)	**88.6** (68.9-202.6)	**147.5** (118.9-156)

Note. Data represents median values (bold). Values in parentheses depict range. All values in pg/ml unless otherwise stated. Along the horizontal axis are the stimulants IncB, IncC and EBs used in cells obtained from GI, GII and GIII and vertical axis are the cytokines secreted in cell cultures assayed using specific ELISAs.
[a, b, c] P<0.05 Level of secreted cytokine in GIII compared to respective levels in groups I and II by Mann Whitney U test.
[d, e, f] P<0.01 Level of secreted cytokine in GII compared to respective levels in group I by Mann Whitney U test.
[g, h, i] P<0.05 Level of secreted cytokine in GII compared to respective levels in groups I and III by Mann Whitney U test.
[j, k, l] P<0.05 Level of secreted cytokine in GI compared to respected levels in group III by Mann Whitney U test.

ELISPOT analysis of IL-10, IFN-γ, IL-12 and IL-4 producing cells in stimulated $CD4^+$ T cells

IL-10, IFN-γ, IL-12 and IL-4 spot forming cells (SFCs) were enumerated in IncB or IncC stimulated $CD4^+$ T cells obtained from CT-positive women and controls. Median number of IL-10 SFCs was comparable in controls and CT-positive fertile women but were significantly higher (P = 0.01) among $CD4^+$ T cells obtained from CT-positive

infertile women. Frequency of IFN-γ secreting cells was highest among cells obtained from CT-positive fertile women followed by cells from controls. In CT-positive infertile women, significantly ($P < 0.05$) lower number of IFN-γ secreting cells was present. For IL-12, median SFC count was significantly higher in CT-positive women compared to controls but was much higher among $CD4^+$ T cells from CT-positive fertile women (Table 5.4). There was no detectable IL-4 SFCs in any of the cell culture supernatants. Free GST failed to produce significant differences in expression levels of cytokines in induced cell cultures.

Table 5.4. ELISPOT assay to determine the frequency of cytokine SFCs in mucosal $CD4^+$ T cells stimulated with IncB or IncC.

	GI		GII		GIII	
	IncB	**IncC**	**IncB**	**IncC**	**IncB**	**IncC**
IFN-γ	**147**	**163**	**214****	**236****	**82**	**91**
	(93-284)	(89-217)	(103-412)	(129-363)	(33-106)	(51-124)
IL-12	**78**	**103**	**220****	**212****	**87**	**76**
	(38-110)	(48-136)	(164-309)	(126-273)	(61-142)	(48-127)
IL-10	**43**	**48**	**66**	**74**	**123*****	**124*****
	(38-76)	(27-91)	(43-89)	(46-98)	(61-142)	(74-146)

Note. Median number of cytokine SFCs per 2×10^5 $CD4^+$ T cells isolated from cervical cells at 18 h post stimulation counted using ELISPOT. Data represents median values (bold). Values in parentheses depict range. ** $P < 0.05$ Number of cytokine SFCs in GII compared to respective levels in group III by Mann Whitney U test. *** $P < 0.05$ Number of cytokine SFCs in GIII compared to respective levels in group II by Mann Whitney U test.

Discussion

There is accumulating evidence on the role of CD4+ T cells in providing vigorous protective immunity aiding clearance of chlamydiae from host cells [Su and Caldwell, 1993]. We evaluated the mucosal immune responses to chlamydial incs in $CD4^+$ T cells (from cervical cells) obtained from CT-positive fertile and infertile women and controls.

incB and *incC* genes were detected in CT-positive endocervical samples using gene specific PCRs. Antibodies to IncB and IncC were detected in cervical washes and was significantly higher in CT-positive fertile women compared to CT-positive infertile women. Antibodies are induced and are present in the genital tract secretions following

CT infection, and the immunoprotective role of antibody-mediated immunity has been reported [Morrison *et al.*, 2002]. Seroprevalence of antibodies to chlamydial incs in CT-positive women diagnosed with cervicitis or infertility has been reported [Gupta *et al.,* 2009]. CT IncA, B and C have been reported to be identified by their reactivity to convalescent sera from experimentally infected guinea pigs [Rockey *et al.,* 1995; Bannantine *et al.,* 1998]. A major role for antibodies in chlamydial immunity is the enhancement of Th1 cell activation via Fc receptor (FcR)-mediated processes involving DCs. Moore *et al.* have reported that *in vitro* anti chlamydial antibodies increase the rate of Th1 cell activation by FcR $^{+/+}$ but not FcR $^{-/-}$ antigen-presenting cells, providing a mechanistic basis for the need for both T-cell and humoral immune responses in protective immunity to chlamydial reinfection [2002].

Stimulation of $CD4^+$ T cells with IncB and IncC showed higher stimulation indices in cells from CT-positive fertile women with respect to controls and CT-positive infertile women thereby showing an antigen specific immune recognition in these cells. Our data on cellular proliferation is in accordance with a recent report where CT IncC had an insignificant effect on reduction in cell cytokinesis in transfected HeLa or McCoy cells whereas other inclusion proteins, CT223-227 caused maximal reduction of cell cytokinesis and caused centromere supranumeracy defects [Alzhanov *et al.,* 2009].

The importance of cell mediated immunity in immune protection against chlamydial genital tract infection has been reported previously [Mahoney *et al.*, 2001]. Major histocompatibility complex (MHC)-class II-restricted $CD4^+$ T cells play a central role both by responding to, or by orchestrating, the activation of other immune components including cytokine production. In this study we found high levels of IL-1ß, IL-6, IL-4, IL-5 and IL-10 in IncB or IncC-stimulated $CD4^+$ T cells from CT-positive

infertile women compared to CT-positive fertile women and controls. It is reported that along with IL-1α, IL- ß and IL-6 are induced in murine lungs in response to CT mouse pneumonitis EBs [Magee *et al.,* 1992]. The role of IL-1ß in CT induced chronic infection [Bobo *et al.,*1996] and also in the co-induction of scarring associated proteins, matrix metalloproteins (MMPs) and collagen has been reported [Burton *et al.*, 2004; Faal *et al.,*2005; Natividad *et al.,* 2006]. Further, in trachoma subjects, the prolonged production of TNF-α and IL-1ß together with a reduction in IL-1Ra inhibitory pathways may promote the development of scarring and progression to trachomatous trichiasis [Skwor *et al.,* 2008]. In addition to IL-1, TNF-α, alone or along with IFN-γ and GM-CSF are known to be involved in inducing IL-6 [Akira *et al.,* 1990; Van Snick 1990]. Although pleiotropic in nature, IL-6 is strongly correlated with pathogenesis in chlamydial infection [Gabey *et al.,* 2006]. Elevated IL-6 production for all grades of CT induced trachoma in an endemic group of Nepali patients has been reported [Hessel *et al.,* 2005]. $CD4^+$ T cells are considered the principle producers of IL-4 in an immune response [Mossmann *et al.,*1986], although other cell types, like mast cells, basophils, and $CD4^-$ T cells, can produce IL-4 as well [Bradding *et al.,*1992, Brunner *et al.,* 1993]. IL-4 itself has been shown to be a dominant factor for the induction of IL-4 expression in resting T cells. Further, expression of IL-4 can be induced in Th cells independent of IL-4 from non-Th cells [Romagnani 1992; Scott 1993]. High levels of IL-4 have been reported to be produced in CT-infected trachomatous scarring patients [Gerard *et al.,* 2004]. Elevated levels of IL-4 and IL-5 in splenic lymphocytes from IFN-$\gamma^{--/--}$ mice following chlamydia-specific challenge and the inability in controlling local chlamydial infection and associated tissue damage and cellular infiltration has also been reported [Wang *et al.,* 1999]. IL-10 is known to limit inflammatory and fibrotic damage and might enhance

chlamydial persistence [Conti *et al.,* 2003]. In the genital tract, elevated levels of IL-10 have been found in infertile women with documented CT infections [Yang *et al.,* 1996; Reddy *et al.,* 2004]. Kinjyo *et al.* have characterized IL-10 as a Th3/Tr1 regulatory cytokine, showing the over production of IL-10 and TGF-ß in the absence of a Th2 polarizing gene, SOC3, the suppressor of cytokine signalling [2006]. Faal *et al.*, found elevated levels of a T cell regulatory gene, forkhead box 3 (FOXP3), during active trachoma [2006]. Additionally, Mark *et al.*, had demonstrated diminished FOXP3 and IL-10 levels with early clearing of CT infection in a murine model [2007]. The antagonistic role of IL-10 towards production of IFN- γ is also known. Yang *et al.*, have reported that excessive IL-10 production in BALB/c mice inhibits Th1-like responses, including IFN-γ expression and the delayed-type hypersensitivity response following chlamydial infection, and consequently delays resolution of the infection [1999].

We found high levels of IL-12, IL-23, IFN-γ and GM-CSF in IncB or IncC-stimulated $CD4^+$ T cells from CT-positive fertile women compared to CT-positive infertile women and controls. IL-12, which is derived from monocytes and DCs is known to be important for the initial clearance of bacteria [Mittal *et al.,* 1996; Kelly *et al.,* 1996; Kelly *et al.,* 2000; Kelly *et al.,* 2003] and for promoting IFN-γ production by NK cells [Tseng *et al.,* 1998]. It has also been previously documented that clearance of chlamydial infection from female adolescents has been associated with decrease in IL-12 concentrations in endocervical samples [Wang *et al.*, 2005]. We have previously reported the increased levels of IL-12 in cervical secretions of CT-positive infertile women compared to cells in upper genital tract [Reddy *et al.,* 2004]. Further in another report, a high percentage of plasmocytoid DCs in cervical secretions of CT-positive infertile women [Agrawal *et al.,* 2009] has suggested a possible mechanism recognition

of CD4 or CD8 antigens on APCs such as DCs. Phagocytosis of chlamydiae induces DCs to produce IL-12, which in turn promotes Th1 response and induces the presentation of chlamydial antigens to $CD4^+$ T-cells [Stagg, 1992]. IL-23 is known to activate macrophages to produce pro-inflammatory cytokines and to improve antigen presentation by DCs [Bastos *et al.,* 2004]. In addition, IL-23 has been described as acting indirectly by inducing the production of Th1 polarizing cytokines, such as IL-12 and IFN-γ in murine DCs [Belladonna *et al.,* 2002]. IL-23 is composed of the p40 subunit of IL-12 paired with a unique chain, p19 (IL-23a), which is related distantly to IL-12 p35 and might act during the induction of Th 1-cell development and the acute reiteration of IFN-γ production, in cooperation with IL-18. It has been reported that 60-kDa heat shock protein of *C. pneumoniae* promotes a Th1 immune response through IL-12/IL-23 production in monocyte-derived dendritic cells [Ausiello *et al.,* 2006]. Thus, IL-23 fosters the ability of IL-12 to polarize the T cell response towards a Th1 pattern, which is protective to intracellular pathogens, but can be detrimental if the infection is not eradicated or if a re-infection occurs, leading to particularly strong activation of a Th1 response. Further, it has also been reported that IL-23, plays a central role in mediating chronic inflammatory responses and autoimmunity in mice [Langrish *et al.,* 2004; Hunter *et al.,* 2005]. $CD4^+$ and $CD8^+$ T cells are reported to be sources of IFN-γ production in CT infection although the former is the major contributor [Igietseme 1998]. Further, the antichlamydial activity of $CD4^+$ T cells is primarily associated with the production of high levels of IFN-γ [Zhong *et al.,* 1989; Rank *et al.,*1993; Perry *et al.,*1997]. IFN-γ reportedly promotes the destruction of CT [Beatty *et al.,* 1993] and also triggers macrophage release of inflammatory mediators that cause fibroblast proliferation, thereby enhancing the synthesis of collagen. In addition, IFN-γ delays the

developmental cycle of CT so that chlamydial reticulate bodies 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]. Further, there is accumulated evidence to suggest that Th1 responses and IFN-γ production are important for optimal resolution of genital chlamydial infection [Brunham *et al.,* 2005; Morrison *et al.,* 2002]. GM-CSF is known to activate macrophages and up-regulate CD14 and MHC class II expression resulting in more efficient antigen presentation and development of protective immunity.

In this study we found elevated expression of transcription factor T-Bet in Inc-stimulated $CD4^+$T cells from CT-positive fertile and infertile women compared to controls. Expression of T-Bet was found to be higher in cells of CT-positive fertile women compared to CT-positive infertile women. T-bet has been identified as a Th1 cell-specific factor that induces the production of IFN-γ by developing Th2 cells. T-bet is a member of the T-box family of transcription factors and is expressed in developing and committed Th1 cells [Szabo *et al.,*2000]. T-bet is also reported to be involved in chromatin remodelling of the gene that encodes IFN-γ, induction of expression of the IL-12 receptor ß2-subunit (IL-12Rß2) and stabilizing its own expression, either through an intrinsic autocatalytic loop or the autocrine effects of IFN-γ signalling [Mullen *et al.,*2001]. Although both $CD4^+$ and $CD8^+$ T cells , as well as NK cells express T-bet, there is less dependence on T-bet for high-level expression of IFN-γ in $CD8^+$ T cells than in $CD4^+$ T cells [Szabo *et al.,*2002]. Further, the importance of T-bet for the development of Th1 responses *in vivo* is highlighted by the susceptibility of T-bet knock-out mice to challenge with *Leishmania major* and their predisposition to allergic airway disease [Szabo *et al.,*2002; Finotto *et al.,*2002]. Additionally, it has also been suggested

that T-bet might play an important role in $CD4^+$ Th1 differentiation, by promoting both IL-12Rß2 expression and IFN- γ production in *C. pneumoniae* infected B-, T-, NK-mutant mice models [Oppmann *et al.,*2002]. Thus higher expression of T-Bet in CT-positive fertile women compared to CT-positive infertile women is indicative of differentiation of naïve Th0 cells towards a $CD4^+$-Th1 mediated protective response for clearance of CT.

We found elevated expression of GATA3 transcription factor in Inc stimulated $CD4^+$ T cells from CT-positive infertile women compared to CT-positive fertile women and controls. GATA3 is a zinc-finger transcription factor and is crucial for inducing key attributes of Th2 cells including transcriptional competence of the Th2 cytokine cluster, which includes the genes encoding IL-13, IL-4 and IL-5 [Zheng and Flavell, 1997; Zhang *et al.,* 1997]. GATA3 has been shown to promote Th2 cell development strongly, when expressed by a transgene [Zhang *et al.,* 1997], or by a retrovirus [Ranganath *et al.,* 1998]. Furthermore, GATA3 induces Th2 cell development of $STAT6^{-/-}$ T cells, including the production of Th2 cell-specific cytokines and expression of c-MAF, which depend normally on STAT6. A putative dominant-negative mutant of GATA3 decreases Th2 cell-mediated pulmonary allergic responses when expressed by a transgene in mice. In addition, antisense oligonucleotide treatment directed against GATA3 can repress Th2-cell responses *in vivo*, which further implies a requirement for GATA3 for Th2-cell development [Park *et al.,* 2005]. Li *et al.,* have demonstrated low expression levels of GATA3 with marked Th1 biased protective responses in a mouse model of respiratory *C. pneumoniae* infection using subunit vaccine candidates [2006].

Overall our data on cytokine modulation in $CD4^+$ T cells upon *in vitro* stimulation with IncB or IncC suggests that women (CT-negative women) who

encounter chlamydial antigens for the first time (thereby mimicking *in vivo* naïve cells) or those with no pathological damage (CT-positive fertile women) produce higher amounts of IFN-γ, IL-12 and GM-CSF but in women with reproductive sequalae to chlamydial infection, the cells secrete higher levels of IL-1ß, IL-4, IL-5, IL-6 and IL-10. Further this study reiterates the importance of T-helper (Th) responses in anti-chlamydial immunity with emphasis on the contribution of $CD4^+$ T cells in generating cellular immunity. Our study is in contrast with previous reports on

MHC Class I restricted $CD8^+$ T cells recognition of Inc antigens derived from the cytosol [Fling *et al.,* 2001; Wizel *et al.,*2002; Starnbach *et al.,*2003]. Nonetheless, it has been well established now that both MHC Class I and II restricted pathways are involved in presenting antigens to immunocompetent cells in CT-infected subjects. It is to be noted that although the clinical condition of women with fertility disorders in this study cannot be completely attributed to chlamydial infections but the changes in cytokine patterns within infected cells can be contributors towards development of various pathological consequences to chlamydial infection. Further, to this end the role of IncB and IncC in maintaining a differential cytokine surveillance milieu and their association with specific lymphocyte subsets within infected cells is of interest.

The interplay between microbes and host immunological mechanisms in the process of infection determines the disease outcome, notably the elimination of microbes and associated immunopathologies. Chlamydia encodes 894 proteins and only a small subset of these is known to be B and T cell antigens [Stephens *et al.,* 2006]. Chlamydial inclusion proteins present at the inclusion membrane are critical players in the cross-talk between host cells and the pathogens. Data on host immunity generated by such proteins would therefore be helpful in our understanding of biological functioning of this elusive

pathogen. Further, as human chlamydial vaccine research are underway to identify immunogenic proteins containing adequate Th1 epitopes to elicit a vigorous Th1 and a sufficient Th2 response, and antibody epitopes to induce a humoral immune response required for optimal protection from reinfection, the role of incs to this end are of interest.

Conclusions and Future Scope of Work

In the present study detection of *C. trachomatis* was done in endocervical samples obtained from fertile and infertile patients attending the Gynaecology outpatient department of Safdarjang Hospital, New Delhi, India using gel-based PCR assays and real-time PCR assays and also by culture based fluorescent assay. Full length gene-specific PCRs detected *C. trachomatis* CT 232 (*incB*) and CT 233 (*incC*) in positive patients' samples. PCR amplicons were further characterized by restriction digestion analysis and *incB* and *incC* PCR products were cloned into pGEM-T vector and positive clones were confirmed by restriction digestion and colony PCR. *incB* and *incC* clones were then used for expression of recombinant IncB and IncC proteins respectively in protein expression vectors. ELISA was done to detect prevalence of antibodies to IncB and IncC in sera and cervical washes of *C. trachomatis* infected patients. Anti-IncB and anti-IncC antibodies were found to be differentially correlated with severity of disease in these *C. trachomatis*-positive fertile and infertile women. The role of IncB and IncC in generating cell mediated immune responses was determined by *in-vitro* Inc-stimulated cultures of cervical cells and peripheral blood mononuclear cells (PBMCs) from *C. trachomatis* infected patients. Using MTT assay, significantly high proliferative responses were observed in Inc-stimulated cervical cells and peripheral blood mononuclear cells (PBMCs) from CT-positive fertile women compared to CT-positive women with fertility disorders and controls. Lactate dehydrogenase (LDH)-cytotoxicity assay showed no significant differences in cellular damage to Inc- stimulated cervical cells or PBMCs from CT-positive patients and controls. Modulation of cytokines (IL-1ß, IL-4, IL-5, IL-6, IL-10, IFN- γ, IL-12, TNF-α and GM-CSF) in cervical cells and

PBMCs on stimulation with IncB and IncC was determined by real-time reverse-transcriptase PCR, ELISA and ELISPOT. IFN- γ, IL-12 and GM-CSF was found to be elevated in Inc-stimulated cervical cells and PBMCs obtained from CT-positive fertile women compared to CT-positive infertile women and controls. In contrast, IL-1ß, IL-4, IL-5, IL-6 and IL-10 levels were found to be higher in these cell populations isolated from CT-positive infertile women. IncB and IncC in dose-dependent manner stimulated-$CD4^+$ T cells isolated from cervical cells and PBMCs from CT-positive fertile and infertile women. In conclusion, this study suggests that CT incs viz; IncB and IncC modulate host immune responses and might be involved in immunoprotection/ pathogenesis of chlamydial infection.

Future Scope of Work

The protective immune response to infection with *Chlamydia trachomatis* is highly dynamic and involves all features of host immune responses. However, immune responses that are associated with persistent infection with *C. trachomatis* seem to induce pathology and tissue damage as a result of chronic inflammation and heightened immune reactivity. So, a fine balance between protective immunity and immune-associated disease pathogenesis characterizes the host response to infection with *C. trachomatis*, and this will have an impact on the future design of vaccines. The data on the role of *C. trachomatis* inclusion membrane proteins B and C in modulating host immunity and their contribution to chlamydial pathogenesis can provide valuable leads for the continuous effort of laboratories worldwide to provide a vaccine for eradication of this pathogen. Further research is warranted on defining roles of specific regions of these Inc proteins and their contribution to host immunity.

The search for a vaccine against infection with *C. trachomatis* continues to be a complex task. Nevertheless, progress has been achieved in the past few years and has led to the identification of various protective *C. trachomatis* antigens as potential vaccine candidates. Although immunization regimens involving priming with DNA vaccines and boosting with protein-based vaccines have been found to be highly protective in mice, their practical application in humans remains unclear. Given that multisubunit protein vaccines seem to be more effective than vaccines based on single antigens, in future, *C. trachomatis* vaccine candidates are likely to include various antigens. This data presented in this thesis will be helpful to this end.

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Preparation of reagents

Stock solution of commonly used reagents

1M Tris

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

0.5M EDTA

186.1gm of disodium EDTA.-$2H_2O$ was added in 800ml 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 to1liter and autoclaved.

3M sodium acetate

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

10% SDS

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

Ethidium Bromide (10 mg/ml)

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

Calcium Chloride (0.1 M)

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

IPTG (1M)

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

DEPC water

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

Sodium Phosphate (1M)

Monobasic

138gm of $NaH_2HPO_4.H_2O$ was dissolved in 800ml of ddH_2O and volume made up to 1liter.

Dibasic

268gm of $Na_2HPO_4.7H_2O$ was dissolved in 700ml of ddH_2O and volume made up to 1liter.

Phosphate Buffer Saline (PBS)

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

Ammonium persulfate (10%)

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

10 X TAE buffer (Tris acetate, EDTA)

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

10X TBE buffers (Tris borate, EDTA)

8gm of Tris base, 55 gm of boric acid and 9.3gm $Na_2EDTA.H_2O$ were dissolved in 700ml dw and the final volume made up to 1 liter.

SDS-PAGE electrophoresis buffer

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

Protein transfer buffer

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

2X SDS-PAGE sample buffer

The composition of sample buffer is as follows

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

10X Ligation buffer

Tris.Cl pH 7.8	500mM
$MgCl_2$	100mM
DTT	100mM
ATP	10mM

10 X Amplification buffer

Tris.Cl pH 8.3	100mM
$MgCl_2$	15mM
KCl	500mM
Gelatin	0.1%

DNA loading dye (6X)

0.2gm bromophenol blue, 0.2gm of xylene cyanol and 30ml 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
1.5M Tris-Cl pH 8.8	2.5 ml
dw	3.3ml
10% SDS	100µl
10% APS	100µl
TEMED	10µl

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

30% acrylamide solution	0.83ml
1.0M Tris.Cl pH 6.8	0.68 ml
dw	3.4ml
10% SDS	50µl
10% APS	50µl
TEMED	5µl

Staining solution

1gm of coomassie blue was dissolved in 450ml of methanol. 100ml of glacial acetic acid was added and the volume made up to 1liter 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)

0.2gm bromophenol blue, 0.2gm of xylene cyanol and 30ml of glycerol were dissolved and volume set to 100 by autoclaved ddH_2O.

AMPICILIN (STOCK SOLUTION):

100mg / ml ampicillin stock was prepared in autoclaved distilled water and sterilized by filtration through 0.22µm filter. 100µl aliquot was stored by freezing at -20^0C.

Antibiotics

Ampicillin

100mg/ml ampicillin stock was prepared in autoclaved double distilled water and sterilized by filtration through 0.22 µm filter. 100µl aliquots were stored by freezing at –20°C.

PLASMID MINI-PREPARATION:

Reagents:

Alkaline Lysis Solution I:

50mM Glucose

25mM Tris-Cl (pH 8.0)

10mM EDTA (pH 8.0)

Alkaline Lysis Solution II:

0.2N NaOH (prepare freshly from 10N stock solution)

1 % SDS

Alkaline Lysis Solution III:

5M Potassium acetate 60ml

Glacial acetic acid	11.5ml
Distilled water	28.5ml

Preparation of bacterial culture media

LB medium (Luria Broth)

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

LB Agar

15gm of agar powder, 10gm of Tryptone, 5gm of Yeast, 5gm of Sodium chloride, (Hi media) were dissolved in double distill water. Media were sterilized by autoclaving for 20 minutes at 15lb/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.

List of Publications

1. Dutta R, Jha R, Gupta S, **Gupta R**, Salhan S, Mittal A. 2007. Seroprevalence of antibodies to conserved regions of *Chlamydia trachomatis* heat shock proteins 60 and 10 in women in India. British Journal of Biomedical Sciences; 64:78-83.

2. Jha HC, Vardhan H, **Gupta R**, Varma R, Prasad J, Mittal A. 2007. Higher incidence of persistent chronic infection of *Chlamydia pneumoniae* among coronary artery disease patients in India is a cause of concern. BMC Infectious Diseases; 7:48.

3. Srivastava P, **Gupta R**, Jha HC, Jha R., Bhengraj AR , Salhan S, Mittal A. 2008. Serovar specific immune responses to peptides of variable regions of *Chlamydia trachomatis* Major Outer Membrane Protein in serovar D infected women. Clinical and Experimental Medicine; 8:207–215.

4. **Gupta R**, Jha R., Salhan S, Eickhoff M , Krupp G , Mittal A. 2008. Existence of plasmid-less clinical isolate of *Chlamydia trachomatis* in India is a cause for concern and demands the use of real-time PCR assays. The Internet Journal of Microbiology; 5:2

5. Agrawal T**, Gupta R** , Dutta R, Srivastava P, Bhengraj AR , Salhan S , Mittal A. 2009. Protective or pathogenic immune response to genital chlamydial infection in women - a possible role of cytokine secretion profile of cervical mucosal cells. Clinical Immunology; 130: 347-354.

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6. **Gupta R**, Salhan S, Mittal A. 2009. Seroprevalence of chlamydial inclusion membrane proteins IncB and IncC in women attending a Gynaecology outpatient department in New Delhi, India. The Journal of Infection in Developing Countries; 3: 191-198.

7. **Gupta R**, Srivastava P, Vardhan H., Salhan S, Mittal A. 2009. Host immune responses to chlamydial inclusion membrane proteins B and C in *Chlamydia trachomatis* infected women with or without fertility disorders. Reproductive Biology and Endocrinology; 7:38.

8. Vardhan H, Dutta R, Vats V, **Gupta R**, Jha R, Jha HC, Srivastava P, Bhengraj AR, Mittal A. 2009. Persistently elevated level of IL-8 in *Chlamydia trachomatis* infected HeLa 229 cells is dependent on intracellular available iron. Mediators of Inflammation *(Accepted, in press).*

9. **Gupta R**, Salhan S, Mittal A. 2009. Chlamydial inclusion membrane proteins B and C and their effect on cytokine production in mucosal CD4 positive T cells from women infected with *Chlamydia trachomatis*. *(Manuscript communicated).*

Participations

INTERNATIONAL

1. Presented poster entitled "Host immune responses against Chlamydial inclusion proteins in infected women may provide protection against establishment of infection" **Gupta R**., Salhan S. and Mittal A. at International Congress on Bio-immunoregulatory Mechanisms Associated with Reproductive Organs: Relevance in Fertility and in Sexually Transmitted Infections, 9th-13th February, 2009, National Institute of Immunology, New Delhi, India.

2. Attended Fourth Winter School of Immunology, Cochin, India from 30th July - 5th August, 2008 conducted by Dr. Vijay Khuchroo (Harvard Medical School, Boston, MA, USA) and Dr. Abul K. Abbas (University of California, San Francisco, USA).

3. Presented poster entitled "*Chlamydia trachomatis* heat shock protein 60-specific antibody and cell-mediated responses can predict tubal factor infertility in infected women" **Gupta R**., Srivastava P., Bhengraj A.R., Jha R., Jha H. C, Vardhan H., Salhan S. and Mittal A. at Sixth Meeting of the European Society for Chlamydia Research, 1st -4th July, 2008, University of Aarhus, Aarhus, Denmark.

4. Presented poster entitled "Differential cytokine responses to *Chlamydia trachomatis* infection in cervical lavages of women may indicate extent of disease progression" **Gupta R.**, Dutta R., Srivastava P., Bhengraj A.R., Salhan S. and Mittal A. at 17th Meeting of the ISSTDR and the 10th IUSTI World Congress, 29th July – 1st August, 2007, Seattle (WA), USA.

5. Presented poster entitled "Cytokine gene expression profiles in cervical lavages of *Chlamydia trachomatis* positive women" **Gupta R.**, Dutta R., Salhan S. and Mittal A. at 8th FIMSA/IIS Advanced Immunology Course: Focus on Clinical Immunology, 1st -5th March 2006, All India Institute of Medical Sciences, New Delhi, India.

6. Presented poster entitled "IS *CHLAMYDIA TRACHOMATIS*' PLASMID INDISPENSIBLE FOR ITS SURVIVAL?" **Gupta R.**, Jha R., Salhan S. and Mittal A. at International Conference on Chemistry Biology Interface: Synergistic New Frontiers : 21st-26th November 2004, New Delhi, India organized by Ambedkar Centre for Biomedial Research, University of Delhi, New Delhi, India.

NATIONAL

1. Received best oral presentation award on talk entitled "Mucosal and peripheral immune responses to chlamydial inclusion membrane proteins B and C in women infected with *Chlamydia trachomatis"* **Gupta R**., Salhan S. and Mittal A. at National Conference on Emerging Trends in Life Sciences Research: 6^{th}- 7^{th}, Birla Institute of Technology & Science, Pilani, Rajasthan, India, March, 2009.

2. Attended National Workshop on Bioinformatics Resources on miRNA and siRNA, 14^{th}-17^{th} November, 2006, Institute of Microbial Technology, Chandigarh, India.

3. Workshop "Genomics and Proteomics-SNP genotyping of human population for determining the differential susceptibility to diseases and 2D-gel electrophoresis based proteomic analysis for the identification of peripheral biomarkers" : 30^{th} January- 4^{th} February, 2006 at Industrial Toxicology Research Centre, Lucknow, India.

Bio-data of candidate

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DATE OF BIRTH	17th November, 1979
Postal Address	Lab. No.204/207, Molecular Immunology/ Tissue Culture, Institute of Pathology, Safdarjung Hospital Campus, Post Box 4909, New Delhi-110029, India.
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EDUCATIONAL QUALIFICATIONS

EXAMINATION	YEAR	BOARD/UNIVERSITY	PERCENTAGE OF MARKS	SUBJECTS STUDIED
ICSE-X	1996	Council for the I.S.C.E, New Delhi.	80.33	ENGLISH,HINDI,MATHEMATICS,HISTORY, CIVICS,GEOGRAPHY,SCIENCE,COMPUTERS
ISC-XII	1998	Council for the I.S.C.E, New Delhi .	75.00	ENGLISH,HINDI,MATHEMATICS,PHYSICS, CHEMISTRY, BIOLOGY, SUPW.

GRADUATION-B.Sc. ZOOLOGY (HONOURS)

EXAMINATION	YEAR	BOARD/UNIVERSITY	PERCENTAGE OF MARKS	SUBJECTS STUDIED
B.Sc. PART I (3 YEAR HONOURS) EXAMINATION	2000	UNIVERSITY OF CALCUTTA	54.25	ZOOLOGY(HON),CHEMISTRY,BOTANY ENGLISH,ALTERNATIVE ENGLISH
B.Sc. PART II (3 YEAR HONOURS) EXAMINATION	2001	UNIVERSITY OF CALCUTTA	63.00	ZOOLOGY(HON),ENVIRONMENTAL STUDIES

POST GRADUATION-M.Sc. BIOTECHNOLOGY

M.Sc. PART I EXAMINATION	2002	BANGALORE UNIVERSITY BANGALORE	71.20	BIOCHEMISTRY,BIOPHYSICS, BIOSTATISTICS,COMPUTERS, MICROBIOLGY,IMMUNOLOGY, GENETICS,CELL BIOLOGY
M.Sc. PART II EXAMINATION	2003	BANGALORE UNIVERSITY BANGALORE	69.69	IMMUNOTECHNOLOGY,MICROBIOL BT, ANIMAL & PLANT BT,MOLECULAR BIOLOGY,GENETIC ENGINEERING

AWARDS

- Awarded Junior Research Fellowship (JRF) in Life Sciences under University Grants Commission (UGC) Fellowship Scheme in Joint CSIR-UGC JRF & eligibility for Lectureship NET held on 28-12-2003. 10-2(5)/2003(II)-E.U II; 01.06.2004, Roll number 309802.

- Awarded Travel Grant to attend the 17 th Meeting of the International Society for Sexually Transmitted Diseases Research (ISSTDR) and the 10 th International Union against Sexually Transmitted Infections (IUSTI) World Congress held at Seattle ,Washington, USA from 29th July – 1st August, 2007.

- Awarded Travel Grant from Department of Biotechnology (DBT), Council for Scientific and Industrial Research (CSIR), Indian National Scientific Academy (INSA) and G.P.Talwar Immunology Foundation, New Delhi, India to attend the Sixth Meeting of the European Society for Chlamydia Research, University of Aarhus, Aarhus, Denmark, 1st -4th July, 2008.

- Best oral presentation award at National Conference on Emerging Trends in Life Sciences Research held at Birla Institute of Technology & Science, Pilani, Rajasthan, India, 6th- 7th March, 2009.

RESEARCH EXPERIENCE

1. PROJECT: "***STUDIES ON THE BIOTRANSFORMATION OF WASTE BIOMASS INTO BY-PRODUCTS BY ANAEROBIC DIGESTION"***
DURATION: 8th July-21st August, 2002.
SCIENTIST : Dr.Vipin Chandra Kalia
DEPARTMENT: Environmental Biotechnology Unit,
Centre for Biochemical Technology, (C.S.I.R), [Presently I.G.I.B]
Delhi University Campus, New Delhi, India.

2. PROJECT: "***BASIC TECHNIQUES IN MOLECULAR BIOLOGY WITH EMPHASIS ON RIBOTYPING FOR IDENTIFICATION OF MICRO-ORGANISMS AND GENOMIC LIBRARY CONSTRUCTION"***
DURATION: 5th November-31st December, 2002.
SCIENTIST : Dr.Kedarnath Sastry
DEPARTMENT: Molecular Biology Division,
Syngene International Pvt.Ltd,
Biocon Group of Companies, Bangalore, India.

3. PROJECT: "***MOLECULAR BIOLOGY TECHNIQUES USING YEAST (Saccharomyces cerevisiae) AS A MODEL SYSTEM"***

DURATION: 12th August -12th October, 2003
SCIENTIST :Dr.Beena Pillai
DEPARTMENT: Functional Genomics Unit,
Institute for Genomics and Integrative Biology,
Delhi University Campus, New Delhi, India.

4. PROJECT: ***"ROLE OF CHLAMYDIAL HEAT SHOCK PROTEINS IN THE PATHOGENESIS OF GENITAL TRACT INFECTION IN WOMEN"***
DURATION: 14th February -29th August, 2004.
POSITION: Junior Research Fellow.
SCIENTIST: Dr.Aruna Mittal
DEPARTMENT: Chlamydia Biology,
Institute of Pathology (ICMR), Safdarjung Hospital Campus,
Post Box 4909, New Delhi-110029, India.

5. SEPTEMBER 2004 ONWARDS: Pursuing **Doctoral Program** as UGC-JRF.
SEPTEMBER 2006 ONWARDS: Pursuing **Doctoral Program** as UGC-Senior Research Fellow.

COURSE ATTENDED

SEQUENCE DETECTION SYSTEM COURSE (ABI PRISM 7000)-Lab India, New Delhi, India 6TH—7TH JUNE, 2005.

PRESENTATIONS

- Best oral presentation award on talk entitled "Mucosal and peripheral immune responses to chlamydial inclusion membrane proteins B and C in women infected with *Chlamydia trachomatis"* **Gupta R**., Salhan S. and Mittal A. at National Conference on Emerging Trends in Life Sciences Research held at Birla Institute of Technology & Science, Pilani, Rajasthan, India, 6th- 7th March, 2009.

- Poster entitled "Host immune responses against Chlamydial inclusion proteins in infected women may provide protection against establishment of infection" **Gupta R**., Salhan S. and Mittal A. at International Congress on Bio-immunoregulatory Mechanisms Associated with Reproductive Organs: Relevance in Fertility and in Sexually Transmitted Infections, 9th-13th February, 2009, National Institute of Immunology, New Delhi, India.

- Poster entitled "Chlamydia trachomatis heat shock protein 60-specific antibody and cell-mediated responses can predict tubal factor infertility in infected women" **Gupta R**., Srivastava P., Bhengraj A.R., Jha R.,. Jha H. C, Vardhan H., Salhan S. and Mittal A. at Sixth Meeting of the European Society for Chlamydia Research, 1st -4th July, 2008, University of Aarhus, Aarhus, Denmark.

- Poster entitled "Three-dimensional Structure of *Chlamydia trachomatis* Heat Shock Protein 60 Reveals Cross Presentation of Epitopes in Infected Women

Causing Infertility" Jha R., Jha H. C, Vardhan H., **Gupta R.**, Srivastava P., Bhengraj A. R. and Mittal A. at International Conference in Structural Biology, 19th -22nd November, 2007, The Chinese University of Hong Kong, Hong Kong.

- Poster entitled "Differential cytokine responses to *Chlamydia trachomatis* infection in cervical lavages of women may indicate extent of disease progression" **Gupta R.**, Dutta R., Srivastava P., Bhengraj A.R., Salhan S. and Mittal A. at 17th Meeting of the ISSTDR and the 10th IUSTI World Congress, 29th July – 1st August, 2007, Seattle (WA), USA.

- Poster entitled "Similarity in domains of HSPs in humans and *Chlamydia trachomatis* are suggestive of their role in causation of damaging sequalae in infertile women" Jha R., Jha H. C., **Gupta R.**, Vardhan H., Srivastava P., Bhengraj A. R. and Mittal A. at 11th ADNAT Convention, 26th February-8th March 2007, Centre for Cellular and Molecular Biology, Hyderabad, India.

- Second Prize: Poster entitled "Role of *Chlamydia pneumoniae* and its association with other risk factors in coronary artery disease" Jha H. C., Srivastava P., Jha R., **Gupta R.,** Vardhan H., Bhengraj A. R., Prasad J. and Mittal A. at Biosparks'07, 5th Annual Research Festival, School of Life Sciences, Jawaharlal Nehru University, 23rd -24th February 2007, New Delhi, India.

- Best Poster prize: Poster entitled "*Chlamydia pneumoniae* and associated risk factors in coronary artery disease patients" Jha H. C., **Gupta R.,** Srivastava P., Vardhan H., Jha R., Bhengraj A. R., Prasad J., Mittal A. at International symposium on recent advances in cardiovascular sciences (RACS). "Global conference on heart disease, 14th -15th February, 2007, Delhi Institute of Pharmaceutical Sciences and Research, New Delhi, India.

- Poster entitled "Role of IFN-γ and IL-10 in persistence of genital chlamydial infection in women." Dutta R., **Gupta R.**, Salhan S., Mittal A. at Indian Association of Pathologists and Microbiologist, 14th April, 2006, Institute of Pathology, New Delhi, India.

- Poster entitled "Cytokine gene expression profiles in cervical lavages of *Chlamydia trachomatis* positive women" **Gupta R.**, Dutta R., Salhan S. and Mittal A. at 8th FIMSA/IIS Advanced Immunology Course :Focus on Clinical Immunology, 1st -5th March 2006, All India Institute of Medical Sciences, New Delhi, India.

- Best Poster Prize: Poster entitled "Detection of anti cHSP60 in the serum of *Chlamydia* infected women,a serological marker: An association with severity of disease" Dutta R., Jha R., Gupta S., **Gupta R.**, Salhan S. and Mittal A. at 32nd Annual Conference of Indian Immunology Society - IMMCON 2005, 24th-27th November 2005, Post Graduate Institute of Medical Education and Research, Chandigarh, India

- Poster entitled "IS *CHLAMYDIA TRACHOMATIS*' PLASMID INDISPENSIBLE FOR ITS SURVIVAL?" **Gupta R.**, Jha R., Salhan S. and Mittal A. at International Conference on Chemistry Biology Interface: Synergistic New Frontiers 21st-26th November 2004, New Delhi, India organized by Ambedkar Centre for Biomedial Research, University of Delhi, New Delhi, India.

PARTICIPATIONS

- National Conference on Emerging Trends in Life Sciences Research, 6th- 7th March, 2009 held at Birla Institute of Technology & Science, Pilani, Rajasthan, India.

- International Congress on Bio-immunoregulatory Mechanisms Associated with Reproductive Organs: Relevance in Fertility and in Sexually Transmitted Infections, 9th-13th February, 2009, National Institute of Immunology, New Delhi, India.

- Fourth Winter School of Immunology, Cochin, India from 30th July - 5th August, 2008 conducted by Dr. Vijay Khuchroo (Harvard Medical School, Boston, MA, USA) and Dr. Abul K. Abbas (University of California, San Francisco, USA).

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- Sixth Meeting of the European Society for Chlamydia Research , University of Aarhus, Aarhus, Denmark, 1st -4th July, 2008.

- 17th Meeting of the ISSTDR and the 10th IUSTI World Congress, 29th July – 1st August, 2007, Seattle (WA), USA.

- National Workshop on Bioinformatics Resources on miRNA and siRNA ,14th – 17thNovember, 2006, Institute of Microbial Technology, Chandigarh, India.

- 8th FIMSA/IIS Advanced Immunology Course: Focus on Clinical Immunology, 1st -5th March, 2006. All India Institute of Medical Sciences, New Delhi, India.

- Workshop "Genomics and Proteomics-SNP genotyping of human population for determining the differential susceptibility to diseases and 2D-gel electrophoresis based proteomic analysis for the identification of peripheral biomarkers" : 30th January-04th February, 2006 at Industrial Toxicology Research Centre, Lucknow, India.

- Talk on "National Biotechnology Development Strategy":27th May 2005, Gurgaon, India organized by LabIndia.

- Seminar on "Critical Factors for successful PCR": 5th October 2004, New Delhi, India organized by Eppendorf India.

- "50 years of Collagen Triple Helix: A celebration of Science":7th August 2004, Vigyan Bhawan, New Delhi,India.

- Workshop on Biotechnology Awareness: 16th November, 2002 organized by FBAE, Foundation for Biotechnology Awareness and Education, Bangalore, India.
- Conference on "Mosquito Day Celebration and Management of Mosquito borne diseases" organized by Society for Applied Genetics, Bangalore University,Bangalore,2002 ,India.

Biography of the Supervisor

NAME	:	**Dr. Aruna Mittal**
DESIGNATION	:	**Scientist F/** ***Deputy Director(Senior Grade)***
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ACADEMIC QUALIFICATIONS : *M.Sc., Ph.D.*

FELLOWSHIPS / RECOGNITIONS / AWARDS:

- 'Fellowship of Indian College of Allergy and Applied Immunology' in recognition of original contribution to subject of allergy, applied immunology, 1976.
- Was awarded 'Shakuntala Amir Chand Prize for Young Scientists' for miniaturizing radiometric assay for *M.Leprae* viability and drug resistance by ICMR, 1984.
- Was selected for advance training at Rockfeller University, New York, USA under 'Indo-US SSP Program', 1984.
- Was awarded 'Biotechnology Overseas *long-term* Associateship' by Department of Biotechnology, Ministry of Science & Technology to work at Center for Disease Control, Atlanta, USA, 1988-89.
- Was awarded title of '*Chlamydia* Farmer' by Center for Disease Control, Atlanta, USA, 1989.
- Won 'D.N. Ranganathan Award' for the best scientific paper presentation in the Annual Conference of Sexually Transmitted Diseases, 1990.
- Was awarded research sabattical by I.C.M.R. to work on 'Role of antigen-presenting cells in expression of T-helper cell phenotypes in leprosy by RT-PCR & genotyping of *C.trachomatis* isolates by PCR and RFLP' with Dr. Indira Nath, Prof. of Biotechnology at Department of Biotechnology, All India Institute of Medical Science, October, 1996-October 1997.
- Awarded certificate of achievement by BD Biosciences, San Jose, California, on successful completion of FACS (Flow cytometry) calibur course, May 2001.
- Won ICMR Kshanika award for her research work on chlamydia for the year 2003

ADDITIONAL TRAININGS ACQUIRED ABROAD / INDIA:

1. Under 'Indo-US SSP Program', undertaken training at Rockfeller University, New York, USA from September 1984 - November 1984.

2. Availed 'Biotechnology Overseas *long-term* Associateship' at Centre for Disease Control, Atlanta, USA awarded by Department of Biotechnology, Ministry of Science and Technology. Attended Immunology course of six-weeks duration conducted by Center for Disease Control, Atlanta, USA, 1989.

3. Availed research sabattical granted by I.C.M.R. to work on the project 'Genotyping of *C.trachomatis* isolates collected from cervix by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP)' at Department of Biotechnology, All India Institute of Medical Sciences, New Delhi, October 1996-97.
4. Attended advanced training course on Flow cytometry at BD Biosciences, San Jose California, May 2001.

5. Attended meeting of International Mucosal Immunology society at Boston, USA from 24-28th June 2005.

6. Worked at Roswell Park cancer Institute, Buffalo, USA from 29th May to 19th June and Dartmouth medical center, Lebanon, Hanover USA from 20th -23th June 2005 under Indo-US (Contraceptive research and Reproductive health) programme.

ACADEMIC DEGREES:

B.Sc(Hons)	1969	Delhi University
M.Sc.	1971	Delhi University
Ph.D.	1977	V.P. Chest Institute, Delhi University

PROFESSIONAL CAREER:

Research Assistant	1971	V.P. Chest Institute
Sr. Research Fellow	1977-78	Lady Harding Medical College
Research Officer	1978-79	National Institute of Allergy
Research Associate	1979-80	R.M.R. Institute of Medicine
Research Associate	1980-81	All India Institute of Medical Sciences
Sr. Research Officer	1981-86	All India Institute of Medical Sciences
Sr. Research Officer	1986-91	Institute of Pathology (ICMR)
Assistant Director	1992-97	Institute of Pathology (ICMR)
Deputy Director	1997-02	Institute of Pathology (ICMR)
Deputy Director (Sr.Grade)	2002- contd	Institute of Pathology-(ICMR)

NUMBER OF PUBICATIONS: 115

NUMBER OF Ph. D students supervised 2