Antichlamydial Drugs Sensitivity and Emergence of Resistance in *Chlamydia trachomatis* Isolated from Clinical Treatment Failures

THESIS

Submitted in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

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BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

PILANI (RAJASTHAN) INDIA

2011

BIRLA INSTITUTE OF TECHNOLOGY & SCIENCE
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CERTIFICATE

This is to certify that the thesis entitled **"Antichlamydial Drugs Sensitivity and Emergence of Resistance in *Chlamydia trachomatis* Isolated from Clinical Treatment Failures"** which is submitted by **Apurb Rashmi Bhengraj** (ID No. 2007PHXF441P) for award of Ph.D. Degree of the Institute embodies original work done by her under my supervision.

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Acknowledgment

In the first place I would like to record my gratitude to Dr. Aruna Singh for her supervision, advice, and guidance from the very early stage of this research as well as giving me extraordinary experiences throughout the work. Above all and the most needed, she provided me unflinching encouragement and support in various ways. Her involvement with her originality has triggered and nourished my intellectual maturity that I will benefit from, for a long time to come. I feel honoured for the opportunity to learn from her vast knowledge on chlamydial research, immunology and scientific writing. Her valuable criticism and friendly guidance certainly would lead me to become a better science person. I am indebted to her more than she knows.

I am deeply grateful to our Director, Dr. Sunita Saxena who let me join this exceptional institute and allowed me to pursue this research work. I sincerely thank you for this help.

Gratitude is accorded to B.I.T.S., Pilani for providing all the necessary facilities to complete the research work. I am immensely thankful to Prof. B. N. Jain, Vice-Chancellor, B.I.T.S, Pilani for providing me this opportunity to pursue the off-campus Ph.D. of the Institute. I express my gratitude to Prof A. K, Das, Dean, and Prof. R, Prakash (Ex-Dean), Research and Consultancy Division (RCD), BITS, Pilani for his constant official support, encouragement and making the organization of my research work through the past few years easy. I also thank my Doctoral Advisory Committee (DAC) members Dr. Uma Dubey and Dr. Rajesh Mehrotra. I thank Dr. Sharad Shrivastava, Mr. Dinesh Kumar, Dr. Hemanth Jadav, Ms. Monica Sharma, Mr. Gunjan Soni, Mr. Amit Kumar, Mr. Prabhat Nath Jha and Ms. Sunita Bansal, nucleus members of RCD and all office staff of RCD B.I.T.S, Pilani, for their cooperation and guidance in all official procedures associated with the doctoral program. Financial assistance in the form of research fellowship granted from University Grants Commission India during research work is greatly acknowledged.

I also take this opportunity to thank my labmates Harsh, Rishein, Rajneesh, Hem, Pragya, Vikas, Tanvi and Suraj. A mere thought of these people always brings a smile to my face and belief to tackle problem of any extent. All I can say to them is-thank you for standing by me always, and for everything that you all have done for me. I have been really fortunate to have great friends like you. I truly have enjoyed all our interesting discussions about scientific work, life and new "research" ideas during these years. I simply say thank you all.

I would also like to thank **Dr. Sudha Salhan** *for providing me clinical information and very precious specimen at gynecology and obstetrics department of Safdarjung hospital. I would like to thank* **Mrs Madhu Badhwar** *and* **Mrs Asha Rani** *for their excellent technical assistance and helping me in practical laboratory work. Rest of the laboratory staffs especially* **Mr Ajit** *and* **Mr Kamal***; thank you for making these years so pleasant and enjoyable. I wish to express my gratitude to the staff of the Administrative section, Library and store section at Institute of Pathology for all the support they provided me.*

I would like to throw out a special thanks to my friends **Dhiraj, Dhirendra, Abha, Regina, Subhra, Shashank, Varsha** *and* **Sonu** *from the bottom of my heart for their support, understanding and timely encouragement. Their support has been wonderful for me. I would also like to thank from the bottom of my heart to all my friends outside the scientific world.*

Finally, this thesis would not have been possible without the confidence, endurance and support of my family. My family has always been a source of inspiration and encouragement. I wish to thank **my parents** *whose love, teachings and support have brought me this far. During these long years of academic degree and with things that were not necessarily meant for my disposal; their patience and tolerance was really incredible. My family members* **Subani Barima, John Chachi, Guddi, Uppal, Priya, Anuradha, Amit, Jiju** *and* **Bhabhi** *I thank them for all the love, understanding, support and encouraging words. They have been my strongest source of strength as they supported me through all ups and downs. I sincerely thank my* **mother in-law** *and* **Nikhil, Lata, Mukund** *for their affection, understanding and patience. Special heartfelt thanks to my husband* **Randhir** *whose patient love enabled me to complete this work. He deserves all the ways of gratitude as he supported the best part of my marital life.*

Last but not least, **God Almighty***, thank you for the wonderful life that you have given me and the great set of human beings that you have made me friends with, I thank you for providing me the opportunity to step in the excellent world of science.*

Date: 20|12|2011

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Abstract

Sexually transmitted *Chlamydia trachomatis* infection is a widespread public health concern because of its high prevalence and potentially devastating reproductive consequences, including pelvic inflammatory disease (PID), infertility and ectopic pregnancy in females. Hence, the development of effective antimicrobial agents for use in the treatment of *C. trachomatis* infection is very important. Currently, the recommended first-line therapeutic regimens for chlamydial infections are the tetracyclines: doxycycline and the macrolide: azithromycin. Several studies have drawn the attention for the problem of recurrent genital *C. trachomatis* infections and its complex sequelae which is a major cause of concern for reproductive health of men and women. Reinfection through infected or untreated sexual partner and lack of awareness may be the main sources of recurrent *C. trachomatis* genital infections. However, bacterial atypical characteristics may results in treatment failure by bacterial resistance or persistence which can re-emerge when the antibiotic pressure is removed.

Studies related to determination of heterotypic resistance and its role in transmission and maintenance of *C. trachomatis* infections in treatment failures are needed. Therefore, the current study was undertaken with the aim of studying the heterotypic/ homotypic resistance in *C. trachomatis* isolates isolated from recurrent infected/ treatment failure patients.

Recurrent infections (23.5%) were detected in symptomatic *C. trachomatis* infected female patients attending Safdarjung hospital, New Delhi. *In vitro* antichlamydial drug susceptibility was evaluated and decreased susceptibility was observed in the clinical isolates of *C. trachomatis*. Heterotypic resistance was observed in the *in vitro* cell culture condition in the presence of antichlamydial drugs. This phenotypic characteristic of chlamydia is described as the presence of few resistant proportions out of the total population for the given antibiotic. Many other studies have also reported that treatment failures in clinical settings may be linked with the presence of heterotypically resistant bacteria. Further, genotypic characterization was performed to check for the presence of any mutational changes in the ribosomal rplD (L4), rplV (L22), 23SrRNA protein genes. No genetic mechanism was identified in the ribosomal L4, L22 and 23S rRNA genes for the phenotypic change in the *C. trachomatis* isolates. However a point mutation T to G at position 734318 in *C. trachomatis* genome has been detected in the *C. trachomatis* predicted efflux ygeD gene, suggesting that it may play a role in resistant characteristics of a bacteria.

Further, for phenotypic characterization of the resistant *C. trachomatis* isolate, efflux (*ygeD*) gene expression was checked at different time points of its life cycle in the presence of antichlamydial drugs. It was observed that, in the presence of doxycycline efflux gene was significantly expressed at 8 hour post infection (hpi) of life cycle while in the presence of

azithromycin it was significantly expressed at 24 hpi. Findings suggest that heterotypic resistant *C. trachomatis* isolate may have an active efflux strategy for its survival in the presence of antichlamydial drugs. In addition, by studying the host HeLa 229 cells harbouring resistant *C. trachomatis* isolate, no difference was observed in expression of plasma membrane and actin protein in the susceptible (serovar D) and heterotypic resistant (CT-244 isolate). Hence, this study suggest that *C. trachomatis* isolate with altered drug susceptibility profile may not affect its host cell plasma membrane or actin organization for its survival in order to resist the antichlamydial drugs.

In conclusion, this study support the emergence of clinical antibiotic resistance, not an impossible scenario for *C. trachomatis* despite their isolated niche which limits the opportunity for acquisition of antibiotic resistance genes from other organisms. Successful treatment is necessary for preventing sequelae of chlamydial infections; hence, treatment failures and *in vitro* antibiotic resistance characteristics of *C. trachomatis* is of great concern. The results of the study in characterizing resistance in clinical isolates obtained from recurrently infected treatment failure patients may enhance the understanding of chlamydial therapy and the nature or transmission of resistant *C. trachomatis*. In addition, as a prevention strategy for genital chlamydial infection, a polyherbal formulation is also suggested to have an effective inhibitory activity against *C. trachomatis*.

Contents

List of Tables

List of Figures

Abbreviations

α	Alpha
β	Beta
γ	Gamma
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
cm	Centimeter
CDC	Centre for Disease Control
CMI	Cell-mediated immunity
CT	*Chlamydia trachomatis*
DC	Dendritic cell
DEAE	Diethylaminoethyl
DFA	Direct Fluorescent Assay
ddH_2O	Double Distilled water
DNA/RNA	Deoxyribose/Ribose nucleic acid
DMEM	Delbecco's Minimum Essential Media
dNTP	Deoxyribose nucleotide triphosphate
EBs	Elementary Bodies
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
EMEM	Earle's Minimum Essential Media
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethgylpiperazine-N-2 ethane sulfonic acid
h	Hour
hpi	Hours post infection
IF	Immunofluorescence
IFU	Infection forming unit
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
Kb	Kilo base
LGV	Lymphogranuloma venereum

LPS	Lipopolysaccharide
M	Molarity
mg	Milligram
MBC	Minimum bactericidal concentration
MHC	Major histocompatibility complex
MIC	Minimum Inhibitory Concentration
MOI	Multiplicity of infection
MOMP	Major Outer membrane protein
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide
Min	Minute
ml	Mililitre
mm	Milimeter
mM	Milimolar
N	Normality
$NaHCO_3$	Sodium bicarbonate
NAATs	Nucleic Acid amplification tests
NAHTs	Nucleic Acid hybridization tests
ng	Nanogram
NO	Nitric oxide
°C	Degree Celsius
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
pg	Picogram
PID	Pelvic inflammatory disease
PVDF	Polyvinyl Difluoride
RBs	Reticulate Bodies
RFLP	Restriction fragment length polymorphism
RNase	Ribonuclease
rpm	Revolution per minute
RT-PCR	Reverse transcription- PCR
sec	Second
SD	Standard deviation
SE	Standard error
SEM	Standard Error Mean
SPG	Sucrose phosphate glutamate

STD	Sexually transmitted diseases
TAE	Tris acetate EDTA
Th	T-helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
U	Unit
UDL	Under detection Limit
UV	Ultra Violet
WHO	World Health Organization
μm/ μM	Micromole / Micromolar

Chapter 1

Introduction

Introduction

Chlamydia trachomatis infection is one of the most prevalent sexually transmitted bacterial diseases in the world (Paavonen 2010; Beagley and Timms 2000; Bebear and de Barbeyrac 2009). It is caused by a nonmotile, gram-negative, obligate intracellular bacterial parasite that infects and multiplies within a broad range of eukaryotic cells, including macrophages, smooth muscle, epithelial and endothelial cells. According to World Health Organization (WHO) report, 92 million new cases of genital *C. trachomatis* infections occur every year (Beagley and Timms 2000). In India, a high prevalence (>30%) of *C. trachomatis* infection in symptomatic female patients has been reported (Jalgaonkar, Pathak et al. 1990; Mittal, Kapur et al. 1993; Singh, Rastogi et al. 2002; Joyee, Thyagarajan et al. 2004). In addition, 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; (Singh, Salhan et al. 2003).

Chlamydial infections can be symptomatic or asymptomatic (50% - 80%) in men and women, causes urethritis, epididymitis in men and mucopurulent cervicitis, pelvic inflammatory disease, tubal factor infertility, salpingitis, endometritis and ectopic pregnancy in women (Morre, Rozendaal et al. 2000; Lyons, Ito et al. 2004). It may also cause further pregnancy complications and implantation failure after *in vitro* fertilization (Toth *et al.,* 1993; Sulak, 2003; Romero *et al.,* 2004). Asymptomatic or silent chronic *C.trachomatis* infection in women has been recognized as a significant cause of infertility (Geisler ; Lyons, Ito et al. 2004). 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 (Morre, Rozendaal et al. 2000) 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). Chlamydial genital tract infections are an important risk factor for Human immunodeficiency virus (HIV) transmission (Plummer et al. 1991; Laga *et al.*, 1993; Ho *et al.*, 1995). Studies have also implicated association of *C. trachomatis* infection with cervical and ovarian cancer (Koskela et al. 2000; Smith et al. 2009).

C. trachomatis has a unique developmental cycle of 48 to 72 hours which alternates between the infectious elementary bodies (EBs) and replicative bodies (RBs) that facilitates its efficient transmission. Developmental cycle is initiated by the extracellular elementary body (EB) which attaches to and invades epithelial cells at mucosal surfaces. Within 8 to 12 hours (h) after invasion, EBs develops into metabolically active reticulate bodies (RBs). RBs divide by binary fission inside a host-derived endosome that occupies much of the host cell cytoplasm which is termed as the inclusion body. 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 (Abdelrahman and Belland 2005). This unique and complex developmental cycle of *C. trachomatis* include metabolically inert forms such as elementary bodies (EBs) or intermediate and aberrant forms which are likely to be unaffected by antibiotics.

There is no vaccine against chlamydial infections and therefore antibiotics play a crucial role in the treatment. Doxycycline, azithromycin and erythromycin (for pregnant women) are considered by the centre for disease control as the first line drugs for the

treatment of chlamydial infections (Workowski and Berman; Wang, Papp et al. 2005). *C. trachomatis* has been historically sensitive to the tetracyclines, macrolides, and fluoroquinolones. However, there are recent reports of recurrent infections (10% - 15%) occur after appropriate antibiotic treatment which appeared to be a result of the persistent *C. trachomatis* infections (Dean, Suchland et al. 2000; Byrne 2001; Wang, Papp et al. 2005). The role played by antimicrobial resistance in *C. trachomatis* treatment failures or persistent infection is unclear.

Considering the drugs; economic aspects and possible emerging drug resistance which may results in recurrent infections there should be several approaches to control chlamydial infections. One of those approaches has been the development of topical microbicidal agents or microbicides. These are compounds when applied topically; protect the body's mucosal surfaces from infection by sexually transmitted disease-causing pathogens (Mauck and Doncel 2001). The need for microbicide is not only essential as a preventative measure; it is also necessary as a means of treatment. Topical microbicide could play a significant role in reducing the spread of sexually transmitted diseases including the HIV and HPV infections (Rowe 1995; Chirenje 2001; Keller, Klotman et al. 2003; Yamano, Yamazaki et al. 2005; Cutler and Justman 2008).

Earlier resistance in *C. trachomatis* was not a major concern, however; many studies suggest its emergence. Resistance has been recognized since 1980 but its clinical significance has been unclear. Somani et al described 2 patients with *C. trachomatis* infections that persisted after standard treatment and demonstrated multidrug resistance (Somani, Bhullar et al. 2000). Jones et al. reported 5 *C. trachomatis* isolates that exhibited resistance to tetracycline, doxycycline, erythromycin, and clindamycin but were sensitive to ofloxacin and

ciprofloxacin (Jones, Van der Pol et al. 1990). These were the first reported cases of clinically significant *C. trachomatis* infection resistant to ofloxacin, ciprofloxacin and azithromycin. Additionally, *in vitro C. trachomatis* resistance from female genital tract isolates has been described since the early 1990s. In France, a tetracycline-resistant isolate was recovered from a woman who had persistent infection after Doxycycline treatment (Lefevre, Lepargneur et al. 1997). *In vitro* antimicrobial resistance to tetracycline and erythromycin has also been described (Hsu, Knelsen et al. 1987; Jones, Van der Pol et al. 1990; Rice, Bhullar et al. 1995; Lefevre and Lepargneur 1998; Notomi, Ikeda et al. 1999; Samra, Rosenberg et al. 2001; Morrissey, Salman et al. 2002; Misyurina, Chipitsyna et al. 2004; Riska, Kutlin et al. 2004). These cases may signify an emerging problem with resistant *C. trachomatis* infections.

The resistance in *C.trachomatis* can be of two types heterotypic or homotypic. To date, there have been no reports of clinical *C. trachomatis* isolates displaying *in vitro* homotypic resistance to antimicrobials, but *in vitro* heterotypic resistance in *C. trachomatis* has been described (Lefevre and Lepargneur 1998; Suchland, Geisler et al. 2003; Wang, Papp et al. 2005). In particular, the potential for *C. trachomatis* to develop antimicrobial resistance has not been well studied, despite some published case reports that suggest resistance as a cause for clinical treatment failures (Jones, Van der Pol et al. 1990; Lefevre and Lepargneur 1998; Somani, Bhullar et al. 2000; Samra, Rosenberg et al. 2001; Stamm 2001). There have been no descriptions either of isolation of *C. trachomatis* strains that display stable resistance to antimicrobial agents recommended for therapy or of mechanisms of putative antimicrobial resistance for isolates obtained from patients with treatment failures. However, *in vitro* genetically mediated fluoroquinolone- and rifampin-resistant

variants have been described (Dessus-Babus, Bebear et al. 1998; Morrissey, Salman et al. 2002) and 4 clinical isolates that demonstrated *in vitro* resistance to macrolides were shown to carry mutations in the *23S rRNA gene* (Zhu, Wang et al. ; Tait-Kamradt, Davies et al. 2000; Misyurina, Chipitsyna et al. 2004). There has been no detection of or selection for homotypic resistance among *C. trachomatis* isolates, although homotypic resistance has been detected among *Chlamydia suis* isolated from pigs (Di Francesco, Donati et al. 2008).

The mechanism(s) responsible for heterotypic resistance in *C. trachomatis* is not known. It is possible that the multidrug resistance that was observed is phenotypic in nature rather than genotypic, because the molecular targets of azithromycin, doxycycline, and ofloxacin are quite different, and it is unlikely that a single or limited number of gene mutation(s) could be responsible for simultaneous resistance to these diverse agents. It is suspected that, rather than being direct resistance, heterotypic resistance may be a by-product of some undefined alteration of the growth rate or life cycle, resulting in a longer phase or intermediate stage that is more refractive to antimicrobial agents. Alternatively, heterotypic resistance may be mediated by some kind of mechanism(s) that excludes the drug from the chlamydial cell or inclusion (e.g., an efflux pump). Future studies are needed to test these hypotheses.

It is possible that the phenomenon of heterotypic resistance is not new but remains largely undetected, because test-of-cure is not routinely done for chlamydial infections and a clinician may not suspect persistence because the rate of "recurrent" infections due to reexposure is high. There are no data regarding management of clinically resistant *C. trachomatis* infection. Studies related to determination of heterotypic resistance and its role in transmission and maintenance of *C. trachomatis* infections in treatment failures are

needed. Although, re-infections are common in patients having chlamydial infection, yet there are no studies till date in India regarding antichlamydial drug susceptibility, drug resistance and type of resistance. Therefore, the current study was undertaken with the aim of characterizing the heterotypic/ homotypic resistance in *C. trachomatis* isolated from recurrent infected/ treatment failure patients. The study was planned with the following objectives:

(1) To determine the recurrent infections and to isolate *C. trachomatis* clinical isolates from female patients.

(2) To study drug susceptibility profiling of these isolates against first line antichlamydial drugs.

(3) To evaluate the *in vitro* inhibitory activity of a novel polyherbal cream against *C. trachomatis* as one of the alternate prevention approaches.

(4) To characterize the resistant isolate(s) towards antichlamydial drugs at genotypic level.

(5) To characterize the resistant isolate(s) towards antichlamydial drugs at phenotypic level.

Chapter 2

Review of Literature

Review of Literature

Chlamydiae

Phylum *Chlamydiae* comprises exclusively of gram negative obligate intracellular bacterial pathogens that shows a characteristic biphasic developmental cycle, including metabolically inert elementary bodies (EBs) and actively dividing reticulate bodies (RBs), which thrive within a host-derived vacuole termed inclusion (Hammerschlag 2002). This phylogenetically well-isolated group of closely related bacteria constituted the single family *Chlamydiaceae* of the order *Chlamydiales*, which form a separate phylum in the domain Bacteria, the *Chlamydiae.* Four families are recognized within the order *Chlamydiales* (Corsaro, Valassina et al. 2003), with a total of six genera and 13 species, namely *Chlamydiaceae* (*Chlamydia* and *Chlamydophila*), *Parachlamydiaceae* (*Parachlamydia acanthamoebae*, *Neochlamydia hartmannellae*, *Protochlamydia amoebophilia*), *Simkaniaceae* (*Simkania negevensis*, *Fritschea bemisiae*), and *Waddliaceae* (*Waddlia chrondrophila*) (Beagley, Huston et al. 2009).

History, taxonomy and evolution

1907 in Java, Indonesia, on a research expedition to find the causative agent of syphilis, the German radiologist Ludwig Halberst Adter and the Austrian zoologist Stanislaus

Von Prowazek discovered a conspicuous agent they considered responsible for trachoma, which was a global disease (Haferkamp, Schmitz-Esser et al. 2006). They found irregularly blue-stained inclusions with small, dense particles in giemsa-stained conjunctival epithelial cells of trachoma patients, which they called "Chlamydozoa" (from the Greek word χλαμυσ, meaning mantle or cloak) (Byrne 2003). It was originally considered neither protozoa nor bacteria and then regarded as viruses. In the 1960s they were recognized as bacteria later, these unique microorganisms were found to be among the most important bacterial pathogens of humankind (Moulder 1966). Halberst adter's and Prowazek's chlamydozoa are now called *CT*, and it is the most prominent representative of a small group of closely related bacteria, the chlamydiae (Horn, Collingro et al. 2004).

Chlamydial taxonomy evolves with the evolution of scientific techniques and computing ability. The availability of cell culture techniques and electron microscopy unequivocally demonstrated the bacterial nature of chlamydiae (Goldberg 1966; Moulder 1966). Subsequently the genus *Chlamydia* was established and divided into two species, *Chlamydia trachomatis* and *Chlamydia psittaci.* Earlier ocular isolates of *C. psittaci* taken from diseased human later proved to be common in human respiratory disease. Later, these organisms were designated as *Chlamydia pneumoniae (Grayston, Campbell et al. 1990).*

Similarly *Chlamydia pecorum* was established as a group of ruminant-infecting *C. psittaci* isolates that had been distinguished from other *C. psittaci* strains by bio-typing and immuno-typing (Perez-Martinez and Storz 1985).

The family *Chlamydiaceae* comprises the best known human and animal chlamydial pathogens (Table 2.1). For a long time, the single genus, *Chlamydia*, was the only genus within this family. In 1999, Everrett *et al.* proposed splitting of this family into two families, *Chlamydia* and *Chlamydophila*, based on a range of phenotypic, bio-ecological, and genotypic data (Everett, Bush et al. 1999). This subdivision into the two genera has not been widely accepted by the chlamydial community (Schachter 2001). A phylogenetic analysis based on 110 concatenated genes conserved in all *Chlamydia* genomes confirms and strengthens the close and linked evolutionary relationship among *Chlamydiae*. In this, species *trachomatis, muridarum, pneumoniae* and *pecorum* cluster away from species *felis, caviae, psittaci* and *abortus*, further depreciating the value of 16S rRNA gene-based trees for the purpose of taxonomic classification in the *Chlamydiaceae* (Stephens, Myers et al. 2009). Chlamydial genome analysis revealed the evolution of *Chlamydiae* as early as 60-100 million years ago (Stephens, Kalman et al. 1998). But, due to common constraints and intracellular niche, *Chlamydiae* genome reflected in the accumulation of mutations and gene loss while

Table 2.1: Taxonomy of *Chlamydiae* (Beagley, Huston et al. 2009)

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

retaining their unifying biology. Therefore, the spectrum of divergence among *Chlamydiae* is without genetic difference (Stephens, Myers et al. 2009). With the acceptance of single *Chlamydia* genus, there are now nine recognized species namely *C. trachomatis*, *C. muridarum*, *C. suis*, *C. psittaci*, *C. pneumoniae*, *C. caviae*, *C. felis*, *C. abortus*, and *C. pecorum* (Figure 2.1).

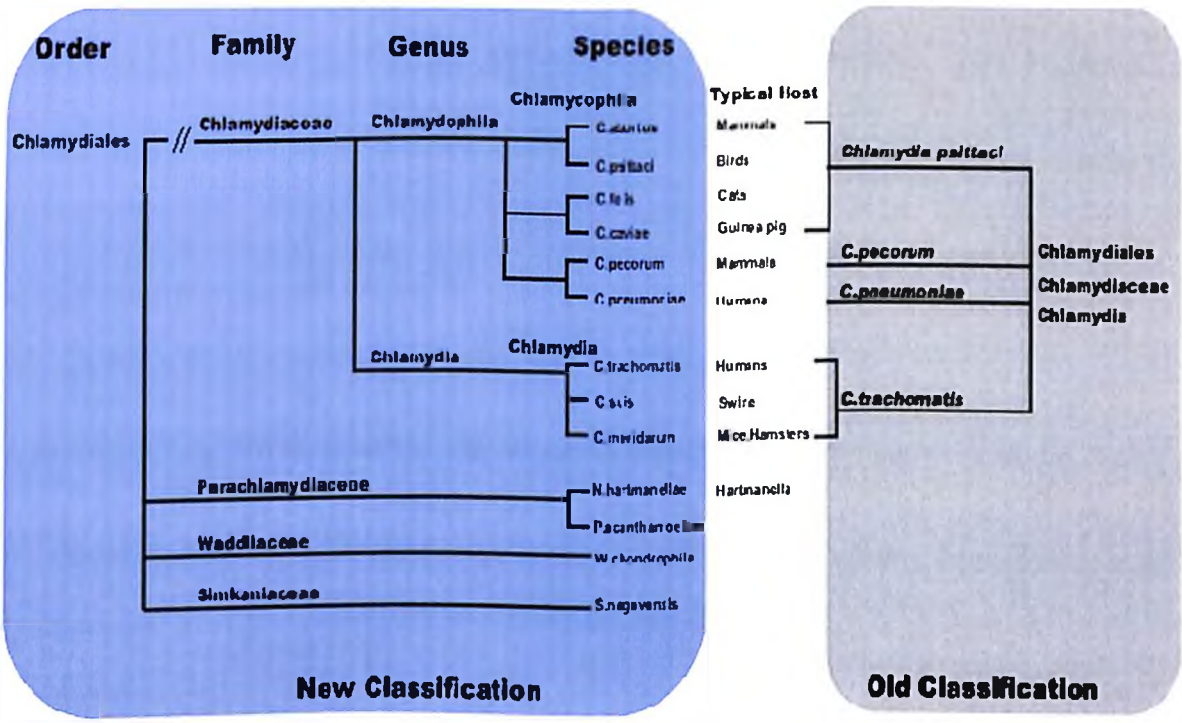


Figure 2.1: Old versus new classification of *Chlamydiales* (Bush and Everett 2001).

Chlamydial Genome Organization

Chlamydial genome comprises of closed circular double stranded DNA and all the strains of *C. trachomatis* and many of *C. psittaci* have 7-10 copies of plasmid. The chromosomal DNA is approximately 600-850 kb in size (Becker 1978) that provided a much

clearer picture on genome organization. The first *Chlamydia* genome sequences of *C. trachomatis* (Stephens, Kalman et al. 1998) and *C. pneumoniae* (Kalman, Mitchell et al. 1999) are available after the completion of *Chlamydia* genome project in 1998 (http://chlamydia-www.berkeley.edu:4231/ and GenBank accession no. AE001273). These sequences provide insight into the pathogenic and metabolic pathways of *Chlamydia* and form a basis for further research to understand *Chlamydia* biology. The sequenced *C. trachomatis* genome serovar D consists of circular chromosome of 1,042,519 base pairs (bp), which is about one quarter of the size of *Escherichia coli* DNA (Stephens, Kalman et al. 1998).

The whole genome is encoding about 894 protein coding genes with 58.7% A+T content. Plasmids of size 7,493 bp are detected initially in three strains from serovars B, C and L2 of *C. trachomatis.* In a later report, plasmids were detected in strains from serovars B, C, D, L1, L2, and L3 (Hyypia, Larsen et al. 1984). It has been suggested that plasmid is essential for the survival of *C. trachomatis*. However, some reports suggest that it may not be essential. A strain of serovar L2 that lacks the plasmid and does not contain plasmid integrated into the chlamydial chromosome was isolated from the patient with proctocolitis (Peterson, Markoff et al. 1990). *C. pneumoniae* does not have plasmid (Campbell, Kuo et al.

1987) and strains of *C. psittaci* showed considerable diversity. Most strains of plasmid range from 6.2-7.9 Kb, but some strains lack plasmids (Joseph, Nano et al. 1986). Some plasmid-free isolates of *C. trachomatis* have been described, but these are exceedingly rare and the only viable clinical isolates described that are plasmid free belong to serotypes L2, D and E (Peterson, Markoff etal.1990; Farencena, Comanducci etal.1997; Stothard, Williams etal. 1998).

Clustering by sequence similarity revealed that 256 chlamydial proteins (29%) belong to 58 families of similar genes within the genome (paralogs), a fraction similar to other bacteria with relatively small genomes such as the *mycoplasmas* and *Haemophilus influenzeae* (Brenner, Hubbard et al. 1995; Koonin, Mushegian et al. 1997; Tatusov, Koonin et al. 1997). The limited and obligate intracellular growth of *Chlamydiae* and lack of any direct and indirect genetic transfer method is also confirmed by absence of genes involved in DNA uptake and insertion sequences (Stephens 1992). Also, chlamydial genes encoding proteins to synthesize amino-acids is greatly reduced. A large number of genes encoding different ABC transporters which are primarily involved in smaller peptides and amino acids transport are found in chlamydial genome (Stephens, Kalman et al. 1998). Therefore, *Chlamydiae* have traditionally been described as energy parasites obtaining ATP from host

cells (Hatch, Al-Hossainy et al. 1982; Moulder 1991) and the genomes of *C. trachomatis* confirmed the presence of two genes CT065 and CT495 which is homologous to genes encoding ATP transporting proteins from *Rickettsia prowazekii* (Andersson, Zomorodipour et al. 1998). This ability enables *Chlamydia* to obtain ATP from the host cells in the early and late stages of developmental cycle (Hatch, Al-Hossainy et al. 1982).

Chlamydial developmental cycle

Chlamydiae a group of obligatory intracellular bacteria undergo a distinct developmental cycle, converting between two morphologically and functionally discrete forms, the infectious but metabolically inactive elementary body (EB) and the noninfectious but metabolically active reticulate body (RB). The basic cycle follows this sequence: I) attachment and internalization, II) EB to RB differentiation, III) remodeling of the parasitophorous vacuole ("inclusion") and bacterial replication, IV) inclusion expansion and transition of RB into EB, and V) release of bacteria from the host cell and infection of new target cells by EBs (Abdelrahman and Belland 2005)(Figure 2.2). However, the classical hypothesis of a lytic biphasic cycle reflect only optimized growth conditions and needs to be extended by persistence as a third state (Beatty, Morrison et al. 1994). Chlamydial persistence is known as a state of infection during which the pathogen remains viable but

noncultivable, while the host immune system is incapable of eliminating it (Beatty, Morrison et al. 1994; Bragina, Gomberg et al. 2001). Morphologically, this reversible state is characterized by aberrant bodies, i.e., enlarged pleomorphic RBs, and reduced inclusion size (Figure 2.3). The complete developmental cycle takes between 36 to 72 hours, depending on the chlamydial species.

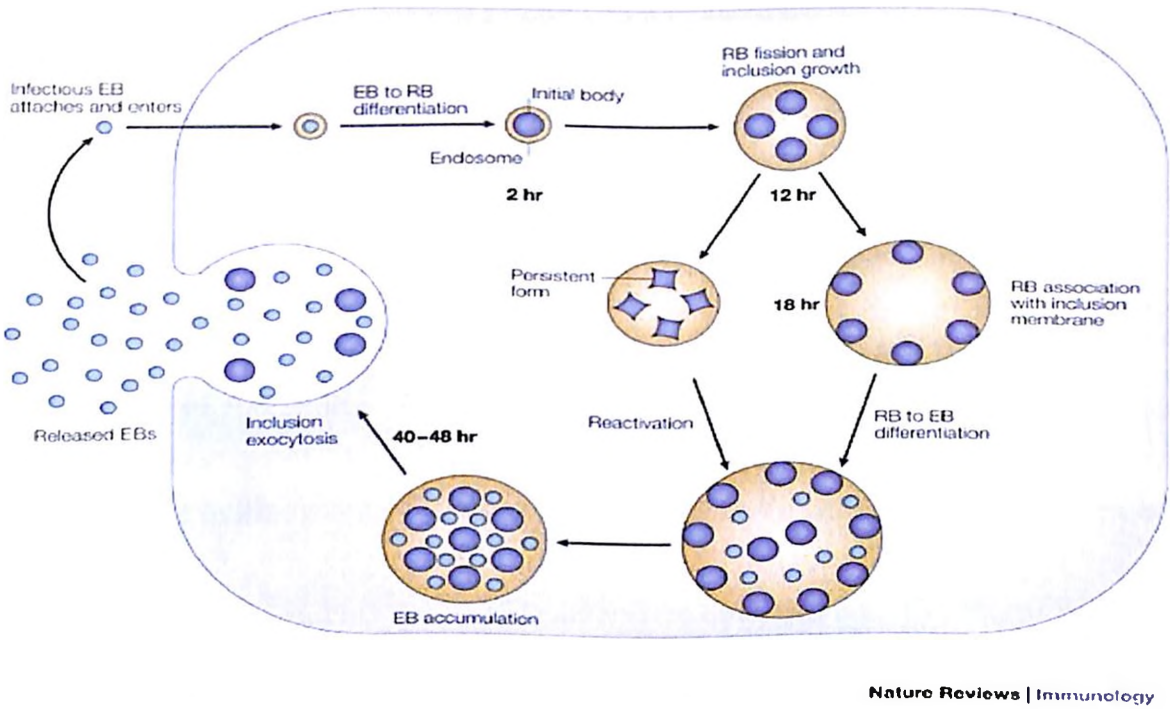


Figure 2.2: Developmental cycle of *Chlamydia trachomatis* (Brunham and Rey-Ladino 2005)

History

In 1932, Bedson and Bland in a light microscopic study described the developmental cycle of *C. psittaci* (Barwell and May 1971) had put the base for the future studies employing light and electron microscopy revealing similar developmental changes for *CT* (Higashi

1964; Kramer and Gordon 1971; Kramer and Gordon 1971). In latter studies using fast growing LGV and TRIC from the infected human conjunctiva and genital tract has been described in various cell culture systems (Gordon, Harper et al. 1969; Richmond 1976).

I) Entry: Attachment and internalization

Complex infectious cycle of Chlamydiae starts with attachment of a metabolically inactive 'spore-like' form [elementary body (EB)] of the bacteria, to the surface of epithelial cells (Kumar and Valdivia 2008). An initial electrostatic-mediated cell association induces the localized activation of the Rho-GTPase Rac1 and is followed by irreversible secondary binding, during which actin is rapidly recruited to the site of EB resulting in filamentous actin reorganization (Carabeo, Grieshaber et al. 2004). These signaling cascades translate in to transient microvillar reorganization and the formation of pedestal-like structures beneath the attached EB and culminate in EBs internalization (Kumar, Cocchiaro et al. 2006). RacGTPase facilitate the activation of Arp2/3 complex by recruiting WAVE2 and Abi-1 for actin reorganization. However this process is augmented by at least one chlamydial effector protein translocated actin recruiting phosphoprotein Tarp (Jewett, Fischer et al. 2006). Tarp is present in all pathogenic strains of Chlamydia, is translocated early in the invasion process, and plays a key role in actin recruitment and remodeling via modulation of cytoskeletal

signaling (Dautry-Varsat, Balana et al. 2004). In spite of modern development it is likely that Tarp represents only stepping stone towards unraveling the chlamydial entry conundrum and that additional bacterial and host factors are involved in modulation of the host cytoskeleton to facilitate chlamydial entry. Indeed, it is likely that multiple pathways are equally capable of promoting chlamydial entry. For example, host receptor-mediated initiation of actin recruitment subsequent to chlamydial attachment represents one alternative (Derre, Pypaert et al. 2007). Given the absolute dependence of chlamydial development on gaining entry it is not surprising that redundant mechanisms may exist.

Establishment of Niche: II) EB to RB differentiation, **III)** Remodeling of the parasitophorous vacuole ("inclusion") and bacterial replication, **IV)** Inclusion expansion and transition of RB into EB

As the process is termed as 'parasite-specific phagocytosis' completed, intracellular growth of Chlamydia spp. is initiated by differentiation of EBs into the metabolically active reticulate bodies (RB) (Byrne and Moulder 1978). Phagocytic vacuole containing RBs bypasses normal endosomal maturation pathways and generates a membrane-bound parasitophorous vacuole termed an 'inclusion'. The plasma membrane markers are shed from the nascent inclusion within 30 min after entry, however inclusion intimately associates with

recycling endosomes and recruits the minus-end-directed motor dynein to migrate along microtubules to the Microtubule Organizing Center (MTOC) (Grieshaber, Grieshaber et al. 2003).

Chlamydial infective units EBs are approximately 300 nm in diameter and its surface is ornamented with hexagonally arranged projections. These ''supramolecular structures'' extend approximately 30 nm from the EB surface and have a rotational symmetry corresponding to a 9-subunit composition (Abdelrahman and Belland 2005). Earlier it has speculated that these spike-like projections correspond to Type III secretion system (TTSS) ''needle'' structures, similar to those seen in *Salmonella enterica* serovar typhimurium. This may play a role in invasion of the host cell (Bavoil and Hsia 1998). The chlamydial outer membrane complex (OMC) consists of dominant structural molecule major outer membrane protein (MOMP) (Caldwell, Kromhout et al. 1981). Further, In EBs, OMC strengthens by highly cysteine-rich proteins (CRPs) that are tightly locked together by disulfide bonds (Hatch, Allan et al. 1984). The DNA within EB is supercoiled and it is maintained by histone-like DNA-binding proteins, Hc1 and Hc2, thereby inhibiting transcription (Hackstadt, Baehr et al. 1991). Within 2 h following internalization, EB begin to convert to larger (1000nm), metabolically active reticulate bodies (RB). The highly expressed early

upstream open reading frame (EUO) gene product help in chromosome dispersal by degrading Hc1 and contributes to OMC relaxation by repressing transcription of the genes encoding CRPs (Kaul, Hoang et al. 1997). Chlamydial histone–DNA interactions are disrupted upon germination by a small metabolite in the non-mevalonate pathway (MEP) pathway of isoprenoid biosynthesis. The metabolite is thought to be 2-C-methylerythritol 2,4-cyclodiphosphate and is involved in functional antagonism of HctA (Belland, Zhong et al. 2003). The RB multiply by binary fission, localizing to the inclusion membrane, which itself expands. The predicted TTS projections protrude through the inclusion membrane and are therefore likely to inject virulence-related effector proteins into the host cell cytoplasm (Hueck 1998). After 8 to 12 rounds of multiplication, RB reverts asynchronously to EB, disengaging from the inclusion membrane. Expression of Hc1, Hc2 and the CRPs is specific to these late stages of the developmental cycle. At 30 to 84 h post infection (PI), depending primarily on the infecting species, infectious EB progeny are released from the host cell to initiate another cycle (Wolf, Fischer et al. 2000).

V) Exit: Release of bacteria from the host cell and infection of new target cells by EBs

In spite of significant advances that have been made for understanding the unique strategies used by bacteria to invade cells, however little is known for exit mechanisms. The

specific pathways of pathogen exit are of immense importance to understand microbial pathogenesis because of its intimate association with dissemination, transmission and inflammation (Hybiske and Stephens 2007). Exit of pathogens is an organized and directed process mediated by both bacterial and cellular factors. This is best exemplified by *Shigella* and *Listeria*, which promote their escape from phagosomes through the action of pore-forming cytolysins (High, Mounier et al. 1992; Lianou, Geornaras et al. 2007). Cellular release then occurs as these bacteria use actin polymerization to protrude out of the cells, although additional unknown mechanisms are likely involved. In contrast, it has been found that phospholipase activity mediates the release of *R. prowazekii* from phagosomes (Winkler and Daugherty 1989), and a hemolysin is responsible for the phagosomal exit of *T. cruzi* (Rosenthal 2004). However, little information exists concerning the mechanisms that mediate the release of chlamydia from either the inclusion or cell. As for most pathogens, it is assumed that chlamydia are released by lysing their host cell , although it has also been proposed that chlamydia may exit by exocytosis or apoptotic pathways (Todd and Storz 1975; Hackstadt, Rockey et al. 1996). It have been shown that chlamydia release occurred by two mutually exclusive pathways. The first is lysis pathway consisted of an ordered sequence of membrane permeabilisation: inclusion, nucleus and plasma membrane rupture.

The second release pathway- packaged release mechanism, called as extrusion (Hybiske and Stephens 2007). Chlamydia release is a consequence of a biological interaction between the inclusion and cell established late in the developmental cycle and is not due to physical stress imposed on the cell simply by a massive inclusion (Clifton, Fields et al. 2004). In addition, very late (30 h after infection) gene expression patterns have been reported, thus supporting the view that a late-expressed chlamydial protein could induce release (Nicholson, Olinger et al. 2003). The dual strategies used by intracellular bacteria to escape cells are poorly understood. However, Chlamydia induced lysis and its regulation by cysteine proteases are analogous to what has been described for the exit of *P. falciparum* from erythrocytes (Rosenthal 2004). Given the disparity between organisms, this important similarity suggests that protease mediated membrane lysis might be a fundamental strategy used by intracellular pathogens. In contrast, the extrusion phenomenon characterized for *Chlamydia* represents an unusual escape mechanism for intracellular bacteria.

Persistent body:

Morphologically aberrant forms of chlamydiae have been identified during chronic infections (Bragina, Gomberg et al. 2001), as well as in the laboratory in response to adverse environmental conditions such as nutrient depletion (Beatty, Belanger et al. 1994;

Beatty, Morrison et al. 1994; Bragina, Gomberg et al. 2001; Stamm 2001), inflammatory cytokines, and even antibiotic treatment. Indeed, abnormal chlamydial forms have been observed in infected cultures exposed to penicillin, ampicillin, D-cycloserine, sulfonamides, fluoroquinolones, doxycycline, erythromycin, and azithromycin. The specific inhibition of RB-to-EB differentiation leads to the formation of enlarged, non-dividing, but viable chlamydial forms that can potentially revert back to typical RBs with maturation to infectious EBs after removal of the inducer. These so-called persistent bodies are characterized by an abnormal metabolic activity and consequently appear more resistant to conventional antibiotics that target the transcription, translation, or cell division of metabolically active organisms (Beatty, Morrison et al. 1994; Gerard, Kohler et al. 1998; Bragina, Gomberg et al. 2001; Byrne 2001; Stamm 2001; Gerard, Whittum-Hudson et al. 2004).

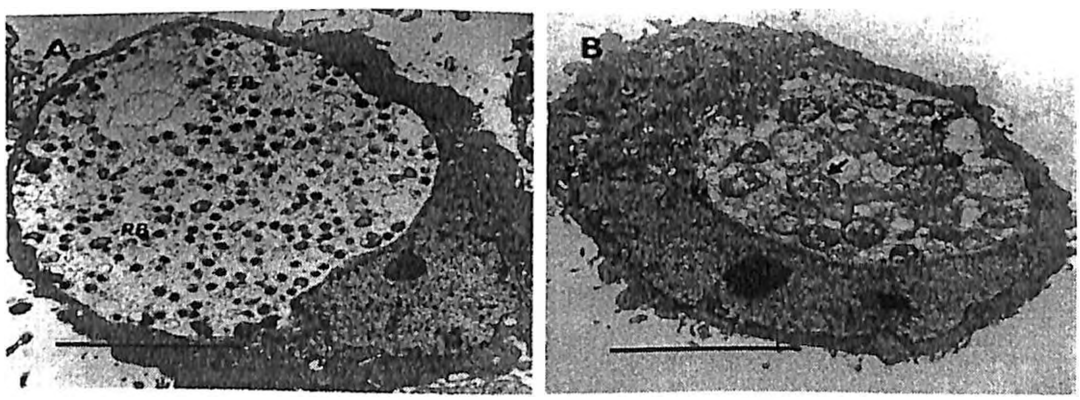


Figure 2.3: Electron micrographs of Chlamydia-infected cells. (A) A typical chlamydial inclusion containing reticulate body (RB) and elementary body (EB) forms, 48 h after infection. (B) Persistent body (PB): Treatment of infected cells with gamma-interferon

results in smaller inclusions containing enlarged, atypical RB forms (Beatty, Morrison et al. 1994).

***Chlamydia trachomatis* Associated Clinical Complications**

Chlamydiae have a broad host spectrum. They are clinically and epidemiologically important throughout the world, both in human and in veterinary medicine. Human pathogenic chlamydiae typically cause infections of the eye, or of the urogenital or respiratory tracts. These infections are often initially not recognized or misinterpreted, as they are difficult to diagnose and the symptoms are mild. Chronic and repeated infections may lead to irreversible damage, including blindness (trachoma) after infection of the eye and tubal infertility after infection of the female genital tract. Clinical manifestations of *C. trachomatis* infection are given in Table 2.2. Complications after infection, most importantly reactive arthritis are presumably linked to the immunological response. Moreover, chlamydiae have been linked etiologically to a series of chronic inflammatory processes, particularly artherosclerosis (Beagley and Timms 2000; Beagley, Huston et al. 2009).

C. trachomatis has been divided into three biovars: trachoma, lymphogranuloma venereum (LGV), and murine (mouse pneumonitis [MoPn] agent). DNA homology studies of genomic DNA and comparison of DNA sequences of specific genes have shown that the trachoma and LGV biovars appear to be essentially identical and the murine biovar is more

distantly related. The trachoma and LGV biovars are distinguished by significantly different clinical features. Serovars of *C. trachomatis* are determined by serological assays, and not by DNA sequencing and are currently divided into 19 serovars, according to the specificity of major outer membrane protein (MOMP) epitopes (Schachter 1999; Morre, Rozendaal et al. 2000; Singh, Salhan et al. 2003). Serovars A, B, Ba and C are the agents of trachoma, a major cause of blindness in Africa, the Middle East, Asia and South America. Serovars D–K, including D, Da, E, F, G, Ga, H, I, Ia, J and K, are the most common sexually transmitted bacteria, and serovars L1, L2, L2a and L3 are the agents of transmission of lymphogranuloma venereum (LGV). However, most of these infections produce few or no symptoms in approximately 70% of women and 50% of men and thus remain undetected (Morre, Rozendaal et al. 2000; van Valkengoed, Morre et al. 2002).

Trachoma

Trachoma is the world's leading cause of preventable blindness. The ocular infection manifests from mild conjunctival lesions (follicular conjunctivitis) to severe inflammatory forms that eventually lead to scarring and blindness (Skwor, Kandel et al.). Severe forms develop through repeated or persistent infections by *C. trachomatis* serovars A, B, Ba and C (Taylor, Johnson et al. 1982; Abu el-Asrar, Geboes et al. 2001). Trachoma is endemic mainly

in tropical and subtropical countries. Main reservoir of the organism is eye of the infected person, usually a child, and transmission may be potentiated by flies that carry infected secretions from person to person (Jones 1974; Emerson, Lindsay et al. 1999).

Table 2.2 Clinical manifestations of *C. trachomatis* infection

Serovars	Clinical Manifestations	Complications	Distribution
A, B, Ba, C	Trachoma	Blindness	Asia and Africa
D to K	Disease of eye and genitals Urethritis, proctitis mucopurulent cervicitis Respiratory System: Infant pneumonia	Epidymitis Endometriosis, salpingitis Pelvic pain, ectopic pregnancy Infertility Reactive arthritis	Worldwide
LGV1 LGV2 LGV3	Lymphogranuloma venerum (LGV)	Fibrosis, rectal stricture	Worldwide

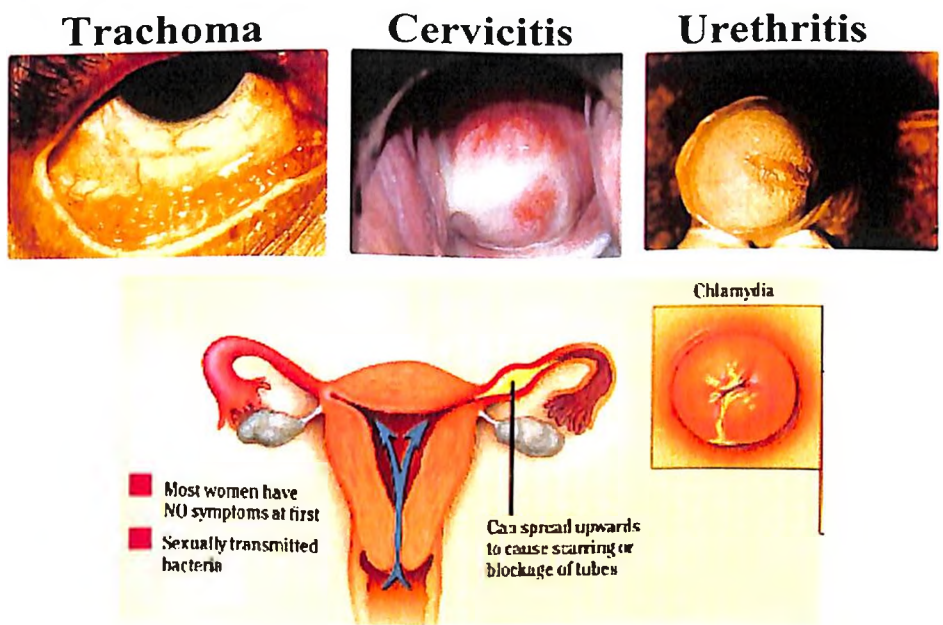


Figure 2.4: Representative symptoms of a few clinical manifestations of *C. trachomatis* infection.

Sexually Transmitted Infection

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

transmitted to the infant during delivery causing conjunctivitis and nasopharyngeal infection (Jain 1999).

In men, the most common clinical manifestation of *C. trachomatis* infection is nongonococcal urethritis. Other clinical syndromes in men include acute epididymitis, acute proctitis, acute proctocolitis, conjunctivitis, and Reiter's syndrome (Berger, Alexander et al. 1978; Stamm, Koutsky et al. 1984; Paavonen and Eggert-Kruse 1999). In fact, *C. trachomatis* causes approximately 35 to 50 percent of all cases of nongonococcal urethritis in heterosexual men. Symptoms of nongonococcal urethritis may develop after an incubation period of 7 to 21 days and include dysuria and mild to moderate whitish or clear urethral discharge. In most cases, physical examination reveals no abnormalities other than the discharge. Male infertility, chronic prostatitis, and urethral strictures are possible results of infection. Both Reiter's syndrome (urethritis, conjunctivitis, arthritis, and mucocutaneous lesions) and reactive tenosynovitis or arthritis (without the other components of Reiter's syndrome) has been associated with genital *C. trachomatis* infection (Stamm 1999).

LGV is a systemic disease caused by invasive serovars (L1, L2 and L3) of *C. trachomatis*. LGV infects lymphatic and sub-epithelial tissues which spread exclusively through sexual contact. Although, it is an important pathogen in some developing countries

and relatively uncommon in industrialized countries (Stamm 1999). However, all the reported cases of LGV from Netherlands, France, Sweden, Canada and United States since 2003 have been caused by the L2 serovars, although there is some evidence that a number of genetically distinct strains of *C. trachomatis* L2 are responsible for these outbreaks (Blank, Schillinger et al. 2005; French, Ison et al. 2005).

Numerous studies have also shown a higher risk of acquiring HIV infection in the presence of various sexually transmitted diseases, such as *C. trachomatis* infection. It can facilitate acquisition and transmission of HIV in a number of ways. On the susceptibility side, it can reduce physical and mechanical barriers of the virus (eg, by causing lesions in the mucosa), increase the numbers of receptor cells or density of their receptors (eg, by causing persistent inflammation), and produce a vaginal environment that is more conducive to transmission (eg, via presence of bacterial vaginosis and increased levels of anaerobes or amines). It has also been reported that, on the infectiousness side, various STDs might evoke a more infectious HIV variant (Ping, Cohen et al. 2000) and can increase HIV concentrations in genital lesions, semen, or both (Cates and Wasserheit 1991; Cohen, Hoffman et al. 1997).

Laboratory Diagnosis for *C. trachomatis* Infection

Diagnosis of chlamydial infections include cell culture, enzyme-immuno assays (EIA), direct fluorescence assays (DFA), nucleic acid hybridization and amplification tests (NAHTs and NAATs, respectively), microimmunofluorescence (MIF) and enzyme-linked immuno assays (ELISA) (Battle, Golden et al. 2001).

Cell culture:

As *C. trachomatis* is an obligate intracellular bacterium, cell culture remains a method of choice for diagnosis. Although, cell culture has near 100% specificity, it is not recommended for routine use because of its lack of sensitivity and technical complexicity (Black 1997). Since, cell culture detects only viable organisms, it is useful for antibiotic susceptibility testing thereby suggesting clinicians to accurate therapy (Chernesky 2005; Jespersen, Flatten et al. 2005; Domeika, Savicheva et al. 2009).

Antigen detection:

Antigen based detection methods DFA and EIA is very specific and rapid to perform but not suitable for large number of specimens (Black 1997). EIA is more reproducible than DFA and its sensitivity is comparable to culture but lower than that of NAATs (Bebear and de Barbeyrac 2009). Recently, a rapid or 'point of care' test was developed by the wellcome

trust based on signal amplification test EIA for chlamydial LPS in a dipstick-type format (Michel, Solomon et al. 2006; Mahilum-Tapay, Laitila et al. 2007). NAHTs are based on DNA probing (with Pace 2, Gen Probe, Digene Hybrid Capture II) which is comparable to cell culture methods. The sensitivity of Digene Hybrid Capture II test is substantially higher than that of Pace 2 test and is comparable to that of PCR (Schachter, Chow et al. 2006). These tests can be used with endocervical or urethral swabs but is not recommended for use with noninvasive specimens. It is also noteworthy that multiple site sampling for the diagnosis of *C. trachomatis* does not increase the sensitivity of diagnostic test (Dietrich, Rath et al.), 2010).

Table 2.3: Methods for diagnosis of *C. trachomatis* (Bebear and de Barbeyrac 2009)

Method	Turn-around time	Advantages	Limits
Cell culture	72 h	Specificity, strain	Sensitivity 80–85%
Antigen detection			
DFA	45 min	Simple, unit test	Sensitivity 75–80% Subjective reading
EIA	4 h	Automation	Sensitivity 75–80%
Point of care	30 min	Low cost, unit test	Low specificity (confirmatory test)
Molecular methods			
DNA probing	2 h	Easy to perform	Sensitivity 75–80%
Hybrid capture	4 h	Sensitivity 95% Specificity 99%	Only for cervical specimens (FDA)
NAAT (real-time PCR, SDA, TMA, NASBA)	2–4 h	Sensitivity >95% Specificity 99%	Contamination, costly processing of specimen

Molecular methods:

Because of high sensitivity, specificity and their possible use of large range of sample types, NAATs can be considered as tests of choice for diagnosing *C. trachomatis* infection (Puolakkainen, Hiltunen-Back et al. 1998; Paavonen and Eggert-Kruse 1999). The major targets for amplification-based tests are generally multiple copy genes, e.g. those carried by the cryptic plasmid of *C. trachomatis*, or gene products such as rRNAs. Several commercial NAATs, using different technologies: PCR and real-time PCR (Roche Diagnostics, Abbott, IL, USA); strand displacement amplification (Becton Dickinson, NJ, USA); transcription-mediated amplification (Gen Probe); and nucleic acid sequence-based amplification (bioMerieux, Nancy L'Etoile, France) are available for diagnosis of *C. trachomatis* (Kellogg, Baillargeon et al. 2004). The goal for the future is to improve the laboratory tests; in particular DNA microarray technology seems to be promising for the diagnosis of *C. trachomatis*.

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

Host defense system against *C. trachomatis* infection

Although *C. trachomatis* has evolved to survive intracellularly within the host cell, the host has in turn evolved an elaborate system to detect as well as to control infection. The host triggers off its own immunological defense mechanisms to combat infection (Rank and Whittum-Hudson). All components of host immunity include innate immune cells, B cells, and T cells which 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 (Batteiger, Xu et al.).

During genital infection with *C. trachomatis*, the mucosal barrier of the genital tract provides the first line of host defense. The ability of the *Chlamydia* organisms to enter this physical barrier is influenced by the stage of the estrus cycle. Shedding of the endometrial epithelium during certain stages of this cycle can limit the ability of *C. trachomatis* to establish a robust infection (Tuffrey, Falder et al. 1986; Ramsey, Cotter et al. 1999). Urethral samples of infected individuals also contain defensins that have been shown to inhibit *C. trachomatis* infection *in vitro* (Porter, Yang et al. 2005). Although epithelial cells are not classically considered critical players within the innate immune system, they are capable of initiating and propagating innate immune responses. When *Chlamydia* organisms are able to

enter the mucosal lining and establish productive infection, immune systems provide the next line of defense against the bacteria by producing various types of pro-inflammatory cytokines and chemokines.

Antibiotic activity and Intracellular bacteria

The intracellular localisation of some bacteria remains a critical point explaining the failure of some antibiotic treatments in infected hosts (Hammerschlag 2002). Increased attention is required on the intracellular pharmacokinetics and pharmacodynamics of antibiotics, as a complement to the evaluation of their properties in acellular systems. Figure 2.5 shows a schematic representation of the main cellular pharmacokinetic and pharmacodynamic properties which are of importance for the activity of an antibiotic against intracellular bacteria (Tulkens 1991; Beringer, Huynh et al. 2005).

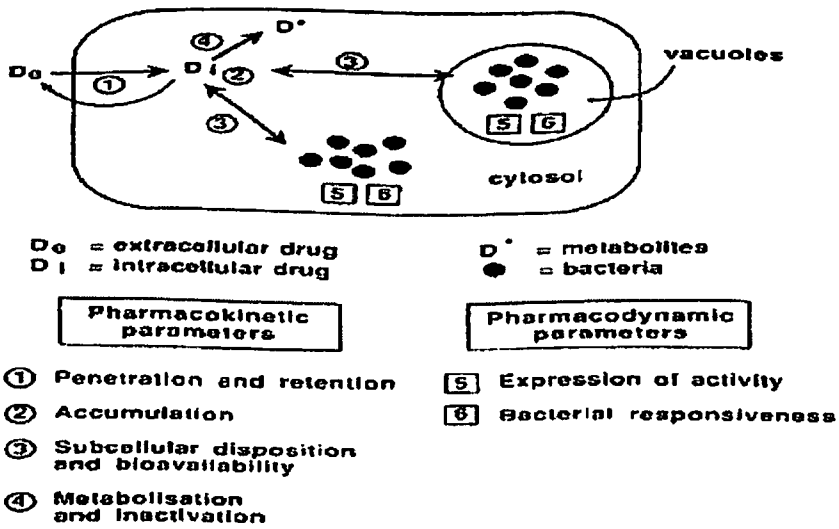


Figure 2.5: Pharmacokinetic and pharmacodynamic parameters involved in the activity of antimicrobial drugs against intracellular microorganisms.

Intracellular penetration, accumulation and disposition are important parameters governing the activity of antibiotics against intracellular bacteria. It depends on several factors, including pharmacodynamic and pharmacokinetic properties of antibiotics. First, in order to be active, antibiotics must reach the infected cells in their tissue compartments via the systemic route. Second, antibiotics need to reach and concentrate within intracellular compartments. Third, antibiotics should remain active within the targeted intracellular compartment, without inactivation by cellular metabolism and/or deleterious effect of pH (Shima, Szaszak et al. ; Tulkens 1991; McOrist 2000; Beringer, Huynh et al. 2005). Some antibiotics are more effective at neutral or basic pH values (e.g. fluoroquinolone compounds) but others (e.g. rifampicin) are more effective at acidic pH values.

Treatment of chlamydial infection

The treatment of *C. trachomatis* infection depends on the site of the infection, the age of the patient, and whether the infection is complicated or uncomplicated. Treatment also differs during pregnancy (Gottlieb, Berman et al.). For uncomplicated genitourinary chlamydial infection, the Centre for Disease Control (CDC) recommends 1 gm azithromycin orally in a single dose, or 100 mg doxycycline orally twice per day for seven days. Further, a meta-analysis of 12 randomized clinical trials of azithromycin versus doxycycline for the

treatment of genital chlamydial infection demonstrated that the treatments were equally efficacious, with microbial cure rates of 97% and 98%, respectively (Black-Payne, Ahrabi et al. 1990; Mahilum-Tapay, Laitila et al. 2007). Erythromycin might be less efficacious than either azithromycin or doxycycline, mainly because of the frequent occurrence of gastrointestinal side effects that can lead to noncompliance. Levofloxacin and ofloxacin are effective treatment alternatives but are more expensive and offer no advantage in the dosage regimen. Other quinolones either are not reliably effective against chlamydial infection or have not been evaluated adequately. Doxycycline, ofloxacin, and levofloxacin are contraindicated in pregnant women. However, clinical experience and published studies suggest that azithromycin is safe and effective (Fry, Jha et al. 2002). Repeat testing to document chlamydial eradication (preferably by NAAT) 3 weeks after completion of therapy with the following regimens is recommended for all pregnant women to ensure therapeutic cure, considering the severe sequelae that might occur in mothers and neonates if the infection persists (Black-Payne, Ahrabi et al. 1990).

If symptoms suggest recurrent or persistent cervicitis or urethritis, the CDC recommends treatment with 2 gm metronidazole orally in a single dose plus 500 mg erythromycin base orally four times per day for seven days, or 800 mg erythromycin

ethylsuccinate orally four times per day for seven days . The CDC does not recommend repeat testing for *Chlamydia* after completion of the antibiotic course unless the patient has persistent symptoms or is pregnant. Because reinfection is a common problem during chlamydial infection, the CDC recommends that women with chlamydial infection should be rescreened three to four months after antibiotic completion (Workowski and Berman).

Table 2.4: Treatment regimens for Chlamydial infections. (CDC MMWR 2010- (Workowski and Berman)

Recommended Regimens
Azithromycin 1 g orally in a single dose
OR
Doxycycline 100 mg orally twice a day for 7 days

Alternative Regimens
Erythromycin base 500 mg orally four times a day for 7 days
OR
Erythromycin ethylsuccinate 800 mg orally four times a day for 7 days
OR
Levofloxacin 500 mg orally once daily for 7 days
OR
Ofloxacin 300 mg orally twice a day for 7 days

Complication with an effective antibiotic regimen has nevertheless reportedly been associated with an increase frequency of recurrent infection (Dean, Suchland et al. 2000; Stamm 2001), tubal infertility (Patton, Askienazy-Elbhar et al. 1994). An *in vitro* study of latent genital chlamydial infections using polarized endometrial epithelial cells reported that a persistent form of *C. trachomatis* did not have the same susceptibility to antibiotics as compared with actively growing *Chlamydiae*, with persistent *Chlamydiae* phenotypically

resistant to azithromycin (Wyrick and Knight 2004). It has been hypothesized that women with high chlamydial load may be increased the risk of antibiotic treatment failure (Horner 2006). Complications of chlamydial infections need longer treatment. Patients need to be followed-up and undergo repeat bimanual examination to ensure resolution of their physical signs. Because of clinical diagnosis of PID, sexual partners of patients with PID need to be treated for chlamydial infection irrespective of their female partners' STI screening result.

Antichlamydial Agents

Macrolides

Macrolides are natural polyketide products of secondary metabolism in many actinomycete species (Laureti, Song et al.). Clinically useful macrolides consist of a 14-membered (clarithromycin, dirithromycin, erythromycin, and roxithromycin), 15-membered (azithromycin), or 16-member lactone ring that is generally substituted with two or more neutral and/or amino sugars (Bulkley, Innis et al. ; Ungureanu). The structure of the 15 member-ring azithromycin a macrolides derivative is shown in figure 2.6. Azithromycin is a bacterial protein synthesis inhibitor and front-line drug for the treatment of chlamydia infections. Clinical studies demonstrate that a single dose is sufficient to eradicate genital

chlamydial and gonorrheal infections (Christmas, Wendel et al. 1989; Vasil'ev and Gazarian 1996; Steedman and McMillan 2009).

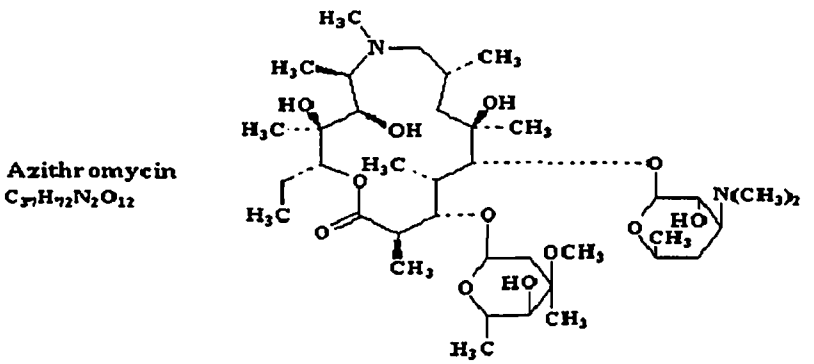


Figure 2.6: Chemical structure of the 15 member-ring azithromycin.

The exact mechanism by which AZI inhibits chlamydial growth has not been rigorously tested. Like its close relative erythromycin (ERY), AZI may block chlamydial protein synthesis. In fact, studies suggest that AZI competes effectively for [14C] erythromycin ribosome binding sites (Bulkley, Innis et al.). Such a general inhibition of bacterial protein synthesis may be sufficient to inhibit chlamydial growth and replication. Alternatively or in addition, AZI might block a specific stage in the chlamydial intracellular life cycle, such as the differentiation of EBs to RBs or vice versa. Lastly, AZI might block the chlamydia specified inhibition of phagolysosomal fusion that is critical to its intracellular survival. Very little is known about this process other than that it requires active chlamydial protein synthesis. AZI is not directly toxic to EBs; EBs can be washed free of the drug after a

brief exposure, and their subsequent infection proceeds normally. AZI does inhibit chlamydial protein synthesis. This inhibition appears quite general in nature; there is no selective inhibition of a particular subclass of chlamydial proteins. Moreover, the onset of the inhibition of protein synthesis is quite rapid and can be seen as early as 5 min after exposure to the drug. That the inhibition of chlamydial protein synthesis by AZI appears to be a direct effect on translation is based on two criteria. First, AZI does not inhibit mRNA synthesis of chlamydial mRNAs. Second, AZI blocks chlamydial protein synthesis in host-free RBs in a manner indistinguishable from its inhibition in infected cells, although it requires a slightly higher concentration of drug (Ossewaarde, Plantema et al. 1992; Narcio Reyes, Casanova Roman et al. 1993; Lea and Lamb 1997; Skerk, Schonwald et al. 2003; Skerk, Krhen et al. 2004; Smelov, Krylova et al. 2004).

Erythromycin

Erythromycin is a macrolide antibiotic that has an antimicrobial spectrum similar to or slightly wider than that of penicillin, and is often used for people who have an allergy to penicillins. Erythromycin displays bacteriocidal activity, in particular, at higher concentrations,(Robbel, Knappe et al.) but the mechanism is not fully understood. By binding to the 50s subunit of the bacterial 70s rRNA complex. Erythromycin interferes with

aminoacyl translocation, preventing the transfer of the tRNA bound at the A site of the rRNA complex to the P site of the rRNA complex. Without this translocation, the A site remains occupied and, thus, the addition of an incoming tRNA and its attached amino acid to the nascent polypeptide chain is inhibited. This interferes with the production of functionally useful proteins, which is the basis of this antimicrobial action. Erythromycin is easily inactivated by gastric acid; therefore, all orally-administered formulations are given as either enteric-coated or more-stable salts or esters, such as erythromycin ethylsuccinate. Erythromycin is very rapidly absorbed, and diffuses into most tissues and phagocytes. Due to the high concentration in phagocytes, erythromycin is actively transported to the site of infection, where, during active phagocytosis, large concentrations of erythromycin are released.

Tetracycline

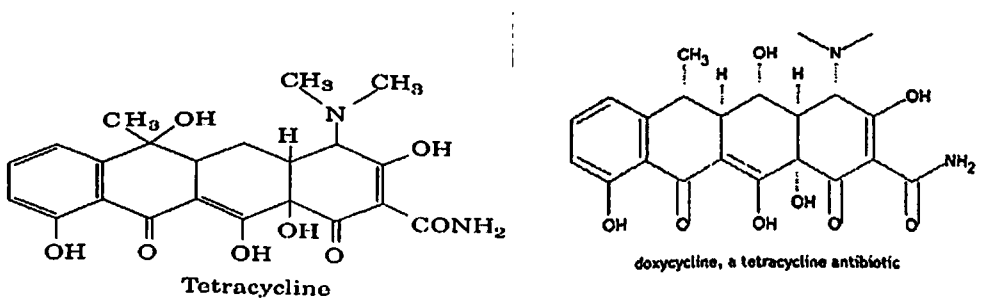


Figure 2.7: Chemical structure of Tetracycline and its derivative Doxycyline.

Tetracyclines are broad-spectrum antimicrobial agents with activity against a broad range of pathogenic bacteria, including intracellular bacteria (Chopra and Roberts 2001). Tetracycline is thought to inhibit the growth of bacteria by entering the bacterial cell, binding to ribosomes and inhibiting protein synthesis. Several studies have found a single, high affinity binding site for tetracyclines in the ribosomal 30S subunit (Craven, Gavin et al. 1969; Calame, Nakada et al. 1978; Hasan, Goldman et al. 1985). TETs are widely used in both human and veterinary medicine because of their broad spectrum of activity and excellent tissue distribution. TET and its derivatives are often well-absorbed, have low toxicity and are relatively inexpensive (O'Reilly and Nelson 1961; Fabre, Milek et al. 1971).

In most species, resistance to tetracycline is conferred by genes with two main modes of action. The first group of genes encodes efflux systems that transport the drug from the inside to the outside of the bacterial cell; the second group encodes ribosomal protection proteins, which remove tetracycline from the ribosome. All of the *tet* efflux genes encode membrane-associated proteins that export tetracycline from the cell. These tetracycline resistance determinants are often associated with transmissible genetic elements including plasmids, transposons and integrons (Paulsen, Chen et al. 2001; Tauch, Gotker et al. 2002; Kumar and Schweizer 2005; Piddock 2006; Kazimierczak, Rincon et al. 2008).

Quinolones

The quinolone antibiotics act by interfering with the folding of bacterial nucleic acid (DNA) by DNA gyrase and topoisomerase IV. DNA gyrase, encoded by *gyrA* and *gyrB*, is the enzyme responsible for inducing negative supercoils in DNA, whereas topoisomerase IV, encoded by *parC* and *parE*, is involved in DNA relaxation and separation. In general, inhibition of both enzymes is necessary for bactericidal activity and the *in vitro* activity of a fluoroquinolone is dictated by the relative affinity for both target enzymes (Gordon, Pfaller et al. 2002). In general, the older quinolone antibiotics, e.g. ciprofloxacin or perfloxacin, have not proved clinically useful against chlamydiae despite their reasonable *in vitro* performance (Ridgway 1997; Ridgway, Salman et al. 1997). However ofloxacin, which is only twice as effective as ciprofloxacin *in vitro*, is very effective in eradicating *C trachomatis* in cases of urethritis or cervicitis (Blomer, Bruch et al. 1988; Kitchen, Donegan et al. 1990).

Levofloxacin

Levofloxacin, the L- optical isomer (structurally a mirror image) of ofloxacin, has similar activity to ofloxacin in the laboratory and had similar efficacy to the macrolides against respiratory infection with *C. pneumoniae* (Hammerschlag and Roblin 2000).

Microbicides

The word microbicide defines an agent that kills microbes, when applied topically to protect the body's mucosal surfaces from infection. The use of topical microbicides is an alternative emerging strategy for the prevention of various sexually transmitted diseases (STDs) and human immunodeficiency virus (HIV). Microbicides act by disrupting or disabling organisms or block their entry into host cells by interfering with cell surface receptors (Chirenje 2001; Harrison, Rosenberg et al. 2003; Van Damme 2004; Cutler and Justman 2008; Nath and Garg 2009). A list of potential microbicides is shown in the table 2.5 which are under human trial.

Characteristics of an ideal microbicide

Laboratory evaluation of an ideal microbicidal activity must take account of the physiological changes such as: compounds must retain activity in the presence of semen over a broad pH range and for several hours. It would be advantageous to also have activity against other sexually transmitted infections, but products must not disrupt the normal vaginal flora, and non-spermicidal microbicides should not be teratogenic. Topical microbicides should show negligible systemic absorption and be free from local toxic effects

Table 2.5: List of potential microbicides (McCormack, Hayes et al. 2001)

Product groups and active agents	Activity		Human trials
	Anti-STI	Spermicidal	
Broad spectrum activity			
Non-ionic surfactants:			
Nonoxinol 9*	++	+	Efficacy
Chlorhexidine*	++	+	Efficacy (vertical transmission)
Octoxinol 9*	++	+	Safety
Anionic surfactants:			
Docusate sodium*	++	NA	Safety
Cationic surfactants:			
Glyminox (C31G)*	++	+	Safety
Benzalkonium chloride*	++	NA	Safety
Geda plus*	++	+	Safety
Acid buffers:			
Buffer gel	++	+/–	Safety
Plant extracts:			
Praneem polyherbal	++	+	Safety
Gossypol	NA	+	Safety
Bacteria:			
Lactobacilli	–	–	Safety
Peptides:			
IB367 (protegrin)	+	NA	Safety
Inhibitors of viral entry			
Sulphated polysaccharides:			
Dextrin sulphate	+	–	Safety
Carrageenan	++	NA	Safety
Cellulose sulphate	++	–	Safety
Sulphonated polymers:			
PRO 2000	++	–	Safety
Inhibitors of viral replication			
Reverse transcriptase inhibitors:			
Tenofovir (PMPA)	–	–	Safety (oral administration)

STI=sexually transmitted infection. NA=not assessed. *Known to be cytotoxic to HIV.

(Rowe 1995; Keller, Klotman et al. 2003; Keller, Klotman et al. 2003; Van Damme 2004; McGowan 2008; Talwar, Dar et al. 2008). The use of surfactants has been associated with disruption of genital epithelia, which may enhance HIV transmission.

Mechanism(s) of Action

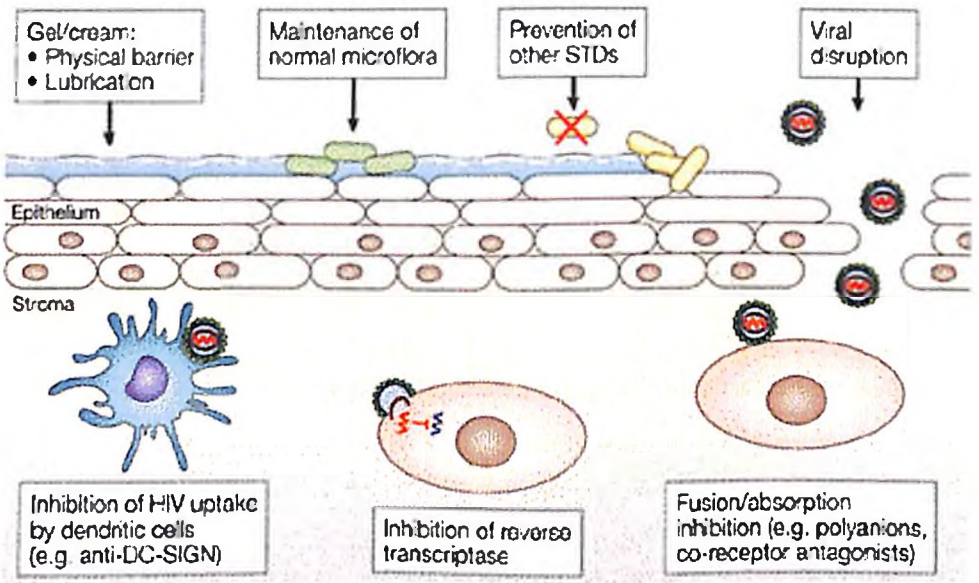


Figure 2.8: Mechanism(s) of action of microbicides

Most microbicides under development act through one or more of five mechanisms: 1) Killing or inactivating pathogens by breaking down the surface or envelope of the pathogen, 2) Inhibiting adsorption of pathogens by creating a physical barrier, 3) Inhibiting entry and fusion of pathogens, 4) Boosting the vagina's natural defenses, 5) Inhibiting viral replication (Harrison, Rosenberg et al. 2003; Joshi and Mehendale 2006; Joshi, Kulkarni et al. 2009).

C. trachomatis antimicrobial susceptibility test methodologies

Antimicrobial susceptibility testing is crucial to guide effective treatment against obligate intracellular bacteria and to understand therapeutic successes and failures. To be

efficient against intracellular pathogens, an antibiotic compound must reach the infected tissue, enter the infected cell and be active within the pathogen's specific intracellular niche, that is, not be inactivated by pH or anaerobic conditions. For these reasons, the laboratory testing of antibiotic susceptibility of intracellular bacteria is difficult and lacks standardization.

The antimicrobial activity of antibiotics against chlamydia or any other organism is usually verified by determination of the minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). All antimicrobial susceptibility involve isolating and expanding clinical isolates and then culturing chlamydial progeny in cells with media containing different dilutions of antibiotics. The methods currently in use vary considerably from laboratory to laboratory(Lycke 1982) (Suchland, Geisler et al. 2003; Siewert, Rupp et al. 2005). Many variables such as; the inoculum size, the cell type used, the interval between establishment of infection and addition of antibiotic, the effects of different media, and the timing of antibiotic removal have effects on the susceptibility determination .

Cell culture

Chlamydiae are obligate intracellular bacteria that have a strict requirement for ATP produced by the host eukaryotic cell (Weiss 1967). Established epithelial cell lines have long

been used to cultivate chlamydiae *in vitro* and are the most likely candidates for cell culture when determining the effects of antimicrobials on the growth of the organism. A number of epithelial cell lines are permissive to *C. trachomatis* infection and support intracellular growth of the organism. McCoy, HeLa, BGMK, HEp-2, HL, and Vero cell lines can be used for *in vitro* antimicrobial susceptibility testing of *C. trachomatis* against antichlamydial drugs (Suchland, Geisler et al. 2003). In cell culture chlamydial inclusions are identified by microscopic examination after reaction with fluorescence-conjugated monoclonal antibodies directed against cell-wall components of the organism. Monoclonal antibodies against the major outer membrane protein are specific for *C. trachomatis,* whereas those against the lipopolysaccharide react with all chlamydial species. The appearance of characteristic inclusions after the addition of fluorescence-conjugated monoclonal antibodies is used in determining antimicrobial susceptibility.

Molecular methods

A flow-cytometric antibiotic susceptibility assay using genus-specific fluorescence-conjugated monoclonal antibodies compared favorably to microscopic reading of MICs (Dessus-Babus, Belloc et al. 1998). However, no data were presented on the sensitivity of the flow-cytometric method in detecting aberrant inclusions. Molecular techniques for

identifying infected cells (e.g., real-time polymerase chain reaction or detection of messenger RNA) can be used besides the method of inclusion staining (Cross, Kellock et al. 1999).

Animal models

Animal models of human chlamydial infections have contributed to understanding the pathogenesis of reproductive tract disease and related immunobiological factors. Surrogate mouse models for *C. trachomatis* infection of the reproductive tract are difficult to adapt to the study of antimicrobial susceptibility, because such infections are of shorter duration than *C. trachomatis* infections in humans (Ramsey, Miranpuri et al. 2001). The monkey model for *C. trachomatis* infection may be more relevant but is expensive (Peeling, Patton et al. 1999). Therefore, more useful information on the potential for chlamydiae to become resistant to antimicrobials would require the study of animals such as birds, ruminants, and pigs, in which chlamydiae are natural pathogens. *C. suis* infection in pigs is widespread and not often associated with clinical disease. Investigation of homotypically resistant *C. suis* isolates could provide a model for the examination of mechanisms of antimicrobial resistance (Dugan, Rockey et al. 2004), which may provide insight into the potential for the development of resistance in human chlamydiae isolates.

In natural infections, chlamydiae are usually only exposed to antimicrobials long after an intracellular infection has been well established, an inflammatory response induced, and often after chronicity and infection of several cell types has occurred. In contrast, the *in vitro* systems used for testing antimicrobial resistance in chlamydiae could be characterized as models of "hyperacute" infections in that the antimicrobials are added soon (sometimes simultaneously with) the infectious agent rather than after a chronic, persistent infection is present (Whittington, Kent et al. 2001).

Treatment failures and Resistance

Various studies have found that higher percentage of women treated for C. trachomatis were infected when retested 3 to 4 months after treatment, possibly due to treatment failure, reinfection from an untreated partner, or infection from a new partner. Untreated C. trachomatis infections can persist for years and put infected women at risk for complications of asymptomatic pelvic inflammatory disease (Taylor, Johnson et al. 1982; Richey, Macaluso et al. 1999; van Valkengoed, Morre et al. 2002; Wang, Papp et al. 2005). In addition, transmission from asymptomatic persons may be responsible for most new infections in a community. A multicenter cohort study of determinants of persistent and

recurrent C. trachomatis infection among young women and a multicenter randomized trial of patient delivered partner treatment with active followup of women with C. trachomatis infection who were treated demonstrated that 10%–15% of women in these studies were infected with C. trachomatis when retested □4 months after C. trachomatis treatment (Whittington, Kent et al. 2001; van Valkengoed, Morre et al. 2002). Post treatment persistence or recurrence of C. trachomatis infection after CDC-recommended antimicrobial therapy may represent (1) reexposure to an untreated, infected sex partner and, thus, reinfection; (2) treatment failure due to noncompliance with treatment, antimicrobial resistance, or poor absorption of the drug; or (3) persistence due to host factors such as immune response or other undefined host factors (Richey, Macaluso et al. 1999).

There are only a few reports describing the isolation of antibiotic-resistant C. trachomatis strains from patients (Mourad, Sweet et al. 1980; Jones, Van der Pol et al. 1990; Lefevre, Lepargneur et al. 1997; Samra, Rosenberg et al. 2001; Vester and Douthwaite 2001). Although 11 of the 15 reportedly resistant isolates were associated with clinical treatment failure, all of the isolates screened displayed characteristics of 'heterotypic resistance', a form of phenotypic resistance in which a small proportion of an infecting microbial species is capable of expressing resistance at any one time. The term "heterotypic

resistance" refers to the replication of a heterogeneous population of resistant and susceptible bacteria from a subculture of a single resistant organism propagated on antimicrobial-containing medium. In contrast, "homotypic resistance" refers to replication of a homogeneous, clonal population of resistant organisms from a subculture of a single resistant organism (Sandoz and Rockey ; Wang, Papp et al. 2005). There has been no detection of or selection for homotypic resistance among *C. trachomatis* isolates, although homotypic resistance has been detected among *Chlamydia suis* isolated from pigs (Lenart, Andersen et al. 2001; Di Francesco, Donati et al. 2008). Results of one study of isolates from women with recurrent *C. trachomatis* infection suggested that treatment failures were more common in women infected with heterotypic resistant strains, as determined in McCoy cells infected with 5000–20,000 IFUs/culture well, than in women infected with susceptible isolates (Suchland, Geisler et al. 2003). These data highlight the difficulty of interpreting *in vitro* susceptibility test results for *C. trachomatis* and the need for clinical correlation with *in vitro* findings.

Chlamydial resistance to individual antibiotic classes

Chlamydiae are known to acquire resistance through mutations to six major classes of antibiotics. Both naturally acquired and laboratory-generated resistance have been found and

studied (Fan, Brunham et al. 1992; McCoy, Sandlin et al. 2003; Binet and Maurelli 2009). Resistance phenotypes that are stably expressed by Chlamydiae in cell culture systems are described as follows.

Tetracyclines

Tetracyclines block bacterial protein synthesis by preventing aminoacyl tRNAs from interacting with ribosome. TETs are widely used in both human and veterinary medicine because of their relatively low cost, broad-spectrum of activity and excellent tissue distribution. TET and its derivatives are often well-absorbed, have low toxicity and are relatively inexpensive. In many bacterial systems, TET resistance is quite common (Piddock 2006), 38 genes that encode TET efflux pumps, ribosomal protection proteins or inactivating enzymes are known (Roberts 2005). The first stably resistant *Chlamydia suis* strains were isolated from diseased and normal pigs in the Midwestern USA (Di Francesco, Donati et al. 2008). Further, eight independent strains were identified, and each exhibited high level resistance to TET. These strains were passaged up to 15 times in antibiotic-free media, and survived in media containing antibiotics without showing signs of morphological abnormalities (Lenart, Andersen et al. 2001). Genetic characterization of the isolates revealed the presence of foreign genomic islands (ranging in size from 6 to 13.5kb) that had integrated

into the chlamydial chromosome (Dugan, Rockey et al. 2004). Each island carries genes encoding a TET efflux pump and a regulatory repressor (*tet*[C] and *tetR*, respectively), a unique insertion sequence (IScs605) plus three to ten additional genes involved in plasmid replication and mobilization. This TET resistance allele is identical to the *tet*(C) gene in the cloning vector pSC101 and a wide range of other vectors. In 2008, a report identified 14 additional *C. suis* strains collected in Italy that shared 100% nucleotide identity with the *tet*(C) gene from the original US strains (Di Francesco, Donati et al. 2008). The discovery of the *tet*(C) islands represents the first identification of antibiotic resistance acquired through horizontal gene transfer in any obligate intracellular bacteria.

Rifamycins

Rifamycins or rifampin (RIF), are bactericidal antibiotics that specifically interact with the b-subunit of RNA polymerase to inhibit bacterial transcription. These are not primary drugs of choice for treating chlamydial infections, although they do possess strong *in vitro* activity and are a therapeutic option in the treatment of clinical infections. Rapid emergence of resistance *in vitro* has been demonstrated in *C. trachomatis*, *C. pneumoniae*, *C. caviae*, *C. psittaci*, *C. suis* and *C. muridarum* after exposure to subinhibitory concentrations of drug (Dreses-Werringloer, Padubrin et al. 2003; Binet and Maurelli 2005; Kutlin,

Kohlhoff et al. 2005; Suchland, Bourillon et al. 2005; Demars, Weinfurter et al. 2007; DeMars and Weinfurter 2008; Suchland, Sandoz et al. 2009). Amino acid substitutions in the RNA polymerase (RNAP) b-subunit decrease the binding capacity of RNAP to RIF, which allows bacterial survival even under high concentrations of drug. Many bacterial species develop resistance through nucleotide changes in the RNAP b-subunit gene, *rpoB*. Similar to these bacteria, RIF-resistant Chlamydiae carry a variety of conserved and unique nucleotide changes in the central region of *rpoB*. A singular amino acid substitution leads to low-level resistance, but the acquisition of an additional substitution increases the MIC several fold. Single mutations increased the MIC from 0.008 μg/ml to between 0.5 and 64 μg/ml in *C. trachomatis* serovar D, and to between 4 and 64 μg/ml in serovar K. The nucleotide at position 471 of *rpoB* (*Escherichia coli* position 526) was the most common site mutated in resistant clones of *C. trachomatis* serovars D and K. When this nucleotide change was found in combination with one additional mutation, the MIC increased from 64 to 512 μg/ml for a serovar D isolate, and from 64 to 256 μg/ml for a serovar K isolate (Dreses-Werringloer, Padubrin et al. 2003; Suchland, Bourillon et al. 2005). In most cases, resistance was associated with mutations in *rpoB*; however, of the two *C. pneumoniae* strains evaluated, only one strain (TW-183) developed resistance and carried the *rpoB* mutations (Kutlin,

Kohlhoff et al. 2005). Rifalazil (RZL), a semisynthetic rifamycin derivative, has high efficacy against *C. trachomatis* infections in clinical trials and is effective *in vitro* against *C. pneumoniae*. Both *C. trachomatis* and *C. pneumoniae* strain TW-183 develop resistance to RZL when passaged in subinhibitory concentrations of the drug and acquire mutations in *rpoB*; however, *C. pneumoniae* strain CWL-029 did not develop such resistance (Kutlin, Kohlhoff et al. 2005). Although clinical resistance to rifamycins in chlamydia has not been documented, the ability of these organisms to quickly accumulate mutations *in vitro* raises concern about the use of these drugs in treating infections.

Fluoroquinolones

Fluoroquinolones are bactericidal antibiotics that inhibit DNA gyrase and DNA topoisomerase IV (Jacoby 2005). *C. trachomatis*, *C. muridarum* and *C. suis* can each develop quinolone resistance *in vitro* when exposed to subinhibitory concentrations of antibiotic (Dessus-Babus, Bebear et al. 1998; Yokoi, Yasuda et al. 2004; Kutlin, Kohlhoff et al. 2005; Demars, Weinfurter et al. 2007; DeMars and Weinfurter 2008; Suchland, Sandoz et al. 2009). After only four passages in 0.5 μg/ml of ofloxacin, the *C. trachomatis* MIC increased from 1 to 64 μg/ml. A similar result was achieved after four passages in the presence of 0.015 μg/ml of sparfloxacin. Two additional studies identified similar mutations associated with passage

of *C. trachomatis* in the presence of quinolones, but the number of passages required to select for resistant mutants varied between four and 24 (Dessus-Babus, Bebear et al. 1998; Morrissey, Salman et al. 2002). Quinolone-resistant strains were resistant to multiple derivatives and carried the same point mutation in the quinolone-resistance determining region of *gyrA*. There is evidence for natural quinolone resistance, via mutations in the quinolone-resistance determining region of *gyrA*, in *C. muridarum* and the distantly related Chlamydiae-like bacteria, including *Parachlamydia acanthamoebae*, *Neochlamydia hartmannellae*, *Simkania negevensis* and *Waddlia chondrophila.*

Aminoglycosides

Aminoglycosides interfere with translation initiation by interacting with the 30S ribosome. These antibiotics have poor penetration into mammalian cells, leading to MIC values for Chlamydiae that are extremely high (~1 mg/ml). Kasugamycin (KSM) and spectinomycin (SPC) are antibiotics used to generate aminoglycoside-resistant chlamydial strains in the laboratory. Resistant strains carried mutations in the 16S rRNA gene at the KSM binding site (Binet and Maurelli 2009; Binet and Maurelli 2009). Strains of *C. trachomatis* that were resistant to KSM did not have a mutation in the 16S rRNA, but did carry a two-nucleotide insertion in *ksgA*, which encodes a protein (KsgA) that is responsible

for post-transcriptional methylation of ribosomal adenosine residues in other bacteria. The resistant *C. psittaci* strain was stable and grew comparable to wild-type strains. By contrast, the *C. trachomatis* KSM mutant was severely impaired for growth and was sensitive to high concentrations of antibiotic (Binet and Maurelli 2009; Binet and Maurelli 2009). Similar to KSM, *in vitro* generated and naturally occurring resistance to SPC is associated with mutations in the 16S rRNA gene. Spectinomycin-resistant *C. trachomatis* L2 mutants have not yet been generated. The inability to produce these mutants is likely due to the duplicity of rRNA operons and drug target sites (Binet and Maurelli 2005; Binet and Maurelli 2005; Binet and Maurelli 2009). For antibiotics that target ribosomal machinery, a single mutation in an organism encoding more than one rRNA operon is typically recessive, and the frequency at which resistant mutants can be recovered correlates with the number of ribosomal operons encoded in the genome. *C. trachomatis* encodes two nearly identical copies of the operon, whereas *C. psittaci* 6BC only encodes one. Simultaneous complementary mutations in two rRNA operons would arise at very low frequencies *in vitro*, possibly explaining why aminoglycoside-resistant strains containing mutations in 16S rRNA genes were not recovered in *C. trachomatis*.

Macrolides

Azithromycin is a bacterial protein synthesis inhibitor and front-line drug for the treatment of chlamydia infections. High-level resistance to AZM was selected for in *C. psittaci* 6BC and *C. caviae* GPIC, while a *C. trachomatis* L2 strain was selected for in lower concentrations of AZM (Binet, Bowlin et al. ; Binet and Maurelli 2007). AZM-resistant *C. psittaci* strains were also resistant to other macrolides as well as a lincosamide, which share similar 23S rRNA target sites. Resistant strains were stable and survived passage in the presence and absence of these drugs. The AZM-tolerant *C. trachomatis* strain harbored a mutation in *rplD* that encodes the ribosomal protein L4. Although some antibiotic-resistant mutations resulted in no overall effect on the physiology of the bacteria, *in vitro* AZM resistance imposes a competitive defect. The resistant *C. psittaci* strains were delayed in their differentiation from EB to RB compared with wild-type strains, and also had a slower doubling rate, produced significantly smaller plaques and were outcompeted in the absence of selection by the wild-type parent strain. The drug-tolerant *C. trachomatis* strain did not grow well in the absence of antibiotics, formed smaller plaques and produced fewer infectious particles than wild-type parent strains. The *C. caviae* AZM resistant strains carried

mutations in the 23S rRNA of their single rRNA operon, produced fewer infectious particles *in vitro* and were less fit *in vivo*, compared with the wild-type strain (Binet, Bowlin et al.).

Lincosamides

Lincomycin is a bacteriostatic protein synthesis inhibitor that causes premature dissociation of peptidyl-tRNA from the ribosome (Tenson, Lovmar et al. 2003). There is a single report of *in vitro*-generated lincomycin-resistant *C. trachomatis* mutants. These mutants were recovered at very low frequencies ($<5 \times 10^{10}$) by growing and passaging infected cells in subinhibitory concentrations of antibiotic. The resistant mutants carried mutations in both 23S rRNA genes, corresponding to sites in *E. coli* that conferred similar resistance (Demars, Weinfurter et al. 2007).

Although there is no genetic evidence of antibiotic resistance leading to treatment failures in humans, the possibility of emergence of resistance in future can not be neglected. Little is known about the heterotypic-resistant phenotype observed in the MIC assays; hence its biological relevance to *in vivo* conditions or correlation with cases of treatment failure is needed to explore. Although there is currently no evidence for heritable antibiotic resistance in human clinical settings, however studies indicate that several antibiotic-resistance genotypes can be generated and transferred to most *C. trachomatis*, *C. suis* or *C. muridarum*

isolates (Gomes, Bruno et al. 2004; Demars, Weinfurter et al. 2007; DeMars and Weinfurter 2008).

Induction of antimicrobial resistance in the laboratory

While antimicrobial resistance in chlamydiae is infrequently reported in humans, it is clearly possible to induce resistance in the laboratory by serial passage of organisms in subinhibitory concentrations of antimicrobials. With *C. trachomatis,* this has been demonstrated with sulfonamides, penicillins, rifampin, and fluoroquinolones (Michea-Hamzehpour, Kahr et al. 1994; Morrissey, Salman et al. 2002). When Dessus-Babus et al. exposed *C. trachomatis* L2 strains to subinhibitory levels of ofloxacin and sparfloxacin, the originally susceptible strains (MICs of ofloxacin and sparfloxacin of 1.0 and 0.03 mg/mL, respectively) had markedly increased MICs (16–64 mg/mL) for these drugs and for all other fluoroquinolones tested (Dessus-Babus, Bebear et al. 1998; Dessus-Babus, Belloc et al. 1998). While similar studies have not been reported for *C. pneumoniae,* it is clear that antimicrobial resistance to chlamydiae can be induced in the laboratory.

Clinical *Chlamydia* Isolates with Antimicrobial Resistance

Relatively few clinical *Chlamydia* isolates with antimicrobial resistance have been described. In 1990, Jones et al. (Jones, Van der Pol et al. 1990) identified *C. trachomatis* isolates described as resistant to tetracycline, doxycycline, erythromycin, and clindamycin but susceptible to ofloxacin and ciprofloxacin. Resistance was only apparent when a high inoculum was used in the cell culture system and only about 1% of the inoculum appeared to be resistant. Further, the strains did not demonstrate a stable resistance phenotype upon serial passage, and there was no apparent clinical correlation between resistance and treatment failure. More recently, Lefevre et al. (Lefevre, Lepargneur et al. 1997; Lefevre and Lepargneur 1998) reported a single *C.trachomatis* strain as tetracycline resistant. This isolate was from a patient who was persistently symptomatic after doxycycline therapy. The isolate's MIC and MBC for tetracycline were 164 μg/mL and, as reported by Jones et al. (Jones, Van der Pol et al. 1990), fewer than 1% of the organisms were resistant. Most recently, Black et al. (Black 1997; Somani, Bhullar et al. 2000) reported 3 patients who appeared to have strains of *C. trachomatis* that exhibited multiple antimicrobial resistance in the setting of persistent clinically apparent infection and persistent positive LCRs or cultures. One pregnant women had persistent positive LCRs despite treatment with several

antimicrobials, including erythromycin, amoxicillin, and azithromycin. The strain exhibited an MCC of 14.0 μg/mL to azithromycin, ofloxacin, and doxycycline. Two other patients (a husband and wife) also had infection with a strain shown to be identical by OMP-1 genotype with a resistance profile similar to that described for the first patient.

Gap in existing Research:

Genital chlamydial infection is one of the most prevalent sexually transmitted bacterial diseases in the world, have a major impact on reproductive health. Antibiotics play a crucial role in treating chlamydial infections with high treatment efficacy of first-line drugs. Despite this effective antibiotic treatment profile several reports suggests the problem of high rate of recurrent infections, which may be the risk for developing reproductive complexities such as tubal scarring, ectopic pregnancy, PID and infertility. There is a gap in understanding the role played by antimicrobial resistance in *C. trachomatis* treatment failures or persistent infections. Further its role in transmission and maintenance of *C. trachomatis* infections is unclear. Besides these first line drugs another approaches for prevention is also needed which may help in controlling the infection. In India, a high prevalence of genital *C. trachomatis* infection in women has been reported, but studies regarding recurrent chlamydial infections

and treatment failures among symptomatic women are lacking. Therefore, understanding the recurrent infections, antichlamydial drug susceptibility profiles and emerging resistance is important for the development of prevention and control strategies for reducing the burden of genital *C. trachomatis* infections.

Chapter 3

Aims and Objectives

Aims and Objectives

The aim of the study is to evaluate the heterotypic/ homotypic resistance in *C. trachomatis* recurrent infected/ treatment failure patients. Objectives include the determination of *C. trachomatis* recurrent infections in symptomatic female patients. Evaluation of *in vitro* susceptibility profiling of first line antichlamydial drugs and a novel potential microbicide against clinical *C. trachomatis* isolates. Further to characterize the heterotypic resistant *C. trachomatis* clinical isolate(s) at genotypic and phenotypic level. The specific objectives of the present study are defined as under.

(1) To determine the recurrent infections and to evaluate the antichlamydial first line drugs susceptibility profiles of clinical *C. trachomatis* isolate(s) obtained from symptomatic female patients.

Recurrent infections were determined in *C. trachomatis* infected female patients and clinical isolate(s) of *C. trachomatis* were obtained by cell culture method. *In vitro* drug susceptibility profiling of these isolate(s) were performed by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against first line antichlamydial drugs (azithromycin and doxycycline).

(2) To evaluate the *in vitro* inhibitory activity of a novel polyherbal cream against *C. trachomatis* as one of the alternate prevention approaches.

A novel polyherbal cream "BASANT" which has shown a good antimicrobial activity against various pathogens was chosen to evaluate the antimicrobial activity against *C. trachomatis*. Further, *in vitro* inhibitory activity was determined by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

(3) To characterize the resistant isolate(s) towards antichlamydial drugs at genotypic level.

Resistance in *C. trachomatis* has been primarily associated with the mutation(s); hence the resistance markers were selected for genotypic studies for the heterotypic resistant *C. trachomatis* clinical isolate(s). Target gene(s) were amplified from the *C. trachomatis* isolate(s) which have shown decreased susceptibility and sequencing was done to test them for presence of possible mutation(s).

(4) To characterize the resistant isolate(s) towards antichlamydial drugs at phenotypic level.

Heterotypic resistant *C. trachomatis* clinical isolate(s) were studied for predicted efflux (ygeD) gene expression at various time points of its life cycle in the presence of antichlamydial drugs. Further, host HeLa 229 cells harbouring these resistant *C. trachomatis* isolate(s) were studied for any phenotypic changes at the cellular level.

Chapter 4

Determination of In vitro susceptibility profile of clinical isolates of Chlamydia trachomatis towards first line antichlamydial drugs

Determination of in vitro susceptibility profile of clinical isolates of Chlamydia trachomatis towards first line antichlamydial drugs

Introduction

Chlamydia (C.) trachomatis infection is one of the most prevalent bacterial sexually transmitted disease. The World Health Organization (WHO) estimates that 92 million new infections occur each year worldwide (Gerbase et al. 1998). It is an important preventable cause of reproductive morbidity and can lead to serious sequelae such as; pelvic inflammatory disease (PID), chronic pelvic pain, ectopic pregnancy, reduced fertility and infertility. Vertical transmission from mother to infant can lead to conjunctivitis and pneumonia. Recent studies have also implicated association of *C. trachomatis* infection with cervical and ovarian cancer and increase in HIV infectivity (Luostarinen et al. 2004). Approximately 70% of the infections in women and 50% in men are asymptomatic or subclinical and can be persistent in nature for prolonged periods (van Valkengoed et al. 2002; Morre et al. 2000).

Antibiotics have the major role in treating chlamydial infections; azithromycin and doxycycline are considered as first line drugs by the Centers for Disease Control and Prevention (CDC) (Workowski and Berman 2010). Although the treatment efficacy of first line drugs for chlamydia is high, but the problem of recurrent infection remains (Horner 2006). The immunity following a chlamydia infection is short, hence high rate of recurrence is well documented among sexually active populations and suggest the risk for developing complex sequelae which increases with repeat chlamydial infections. These sequelae are responsible for most of the reproductive damages associated with genital *C. trachomatis*

infection (i.e., tubal scarring, ectopic pregnancy, PID and infertility) (Hillis et al. 1994; Hillis et al. 1997). Further, the emergence of resistance cannot be neglected, as persistent infection can be the consequence of increasing resistance to standard antimicrobial therapy (Wang et al. 2005; Dreses-Werringloer et al. 2000). The unique biphasic life cycle of *C. trachomatis* facilitates its efficient transmission and also challenges the effectiveness of antibiotic treatment. Its infectious, metabolically inactive form known as elementary bodies (EBs) infects the columnar epithelium of male and female lower genital tract and replicate within host cells in membrane-bound inclusions as reticulate bodies (RBs) which are metabolically active form. After completion of its life cycle, RBs convert back to EBs and get released from the cells. These EBs infects adjacent cells or transmit to uninfected individuals through sexual contact (Abdelrahman and Belland 2005; Siewert et al. 2005). Another aberrant form in its life cycle is known as persistent body (PB), which can be generated in the pressure of antibiotics and persists for a long time presenting subclinical symptoms. These PBs can revert back to active form if the antibiotic pressure is removed and result in recurrent infections with complex sequelae (Beatty et al. 1994b; Hogan et al. 2004).

In India, a high prevalence of genital *C. trachomatis* infection in women has been reported earlier (Singh et al. 2002; Singh et al. 2003; Joyee et al. 2004), but studies regarding recurrent chlamydial infections and treatment failures among symptomatic women are lacking which has impact on reproductive health. Therefore, understanding the recurrent infections and antichlamydial drug susceptibility profile of clinical isolates from these patients are important for the development of prevention and control strategies. This would help in reducing the reproductive complexity and morbidity associated with genital *C. trachomatis* infections. Hence, in this study the pattern of recurrent infections were

determined among the symptomatic female patients and evaluated the antichlamydial drug susceptibility of clinical isolates of *C. trachomatis* obtained from chlamydia infected patients including recurrently infected females.

Materials

Unless otherwise noted, all fine chemicals used in the study were purchased from Sigma Aldrich (St. Louis, USA). Plasticware and glassware used in cell culture was purchased from Greiner, Germany.

Experimental methods

Patient's Enrollment

During a period of November 2006 to December 2007, symptomatic females (n = 163) with abnormal cervical discharge, lower abdominal pain and infertility attending the gynecology outpatient department, Safdarjung Hospital, New Delhi were enrolled in this study. The study received approval from the hospital's ethical committee. Informed written consent was obtained from all the patients. Patients who had visited twice/thrice within a period of 1-6 months to 1year and found positive for *C. trachomatis* infection after appropriate antibiotic treatment are categorized as recurrent infected patients. The standard regimens for treatment are doxycycline 100 mg twice a day for 7 days or azithromycin 1 g single dose. These patients were asked if they had discontinued the prescribed treatment regimens and the associated problem and symptoms each time they visited.

Collection of Samples

The vulva was examined for lesions and the cervix for warts, ulcers, ectopy, erythema and discharge, if any. After cleaning the endocervix with cotton swab (Hi Media, Mumbai, India), endocervical swabs were collected from patients for diagnosis of *C. trachomatis* and other sexually transmitted disease (STD) pathogens. The cotton swab was then transferred to

a sterile tube containing sterile phosphate-buffered saline (PBS) (pH 7·0) supplemented with 100 U penicillin/ml, 100 mg streptomycin/ml and 100 mg glutamine/ml for direct fluorescent assay (DFA) and DNA isolation. Another cotton swab was transferred in to a sterile vial containing sucrose phosphate glutamate (SPG) media (pH 7.0) for isolation and propagation of *C. trachomatis*. Samples were kept at ice and transported to the laboratory for further processing.

Detection of C. trachomatis and other STD pathogens

Five-millimetre spots were made on clean glass slides using endocervical swabs. Samples were stained with flourescein isothiocyanate (FITC) - conjugated monoclonal antibodies to cMOMP using *C. trachomatis* Direct Specimen Test kit (Microtrak, Syva Corporation, Palo Alto, CA, USA) according to the manufacturer's instructions. In principle, this kit involves the use of FITC labeled monoclonal antibodies directed against cMOMP which is a common protein antigen located in the outer membrane of the cell wall in all *C. trachomatis* serotypes. This FITC labeled antibody binds specifically to *C. trachomatis* present in the methanol-fixed smears previously applied to a well on a microscope slide. When viewed under a fluorescent microscope, *C. trachomatis* exhibits a bright apple-green fluorescence which is either EBs or RBs and contrast with the reddish-brown colour or counter-stained material. A sample was considered to be positive when at least 10 EBs were detected by DFA (Singh et al. 2003; Vats et al. 2004). Samples with greater than one and less than 10 EBs were confirmed for positivity by polymerase chain reaction (PCR) analysis using *C. trachomatis* specific 200 base pair (bp) primers of cryptic plasmid.

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

Polymerase Chain Reaction for Detection of C. trachomatis

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

DNA from endocervical samples were tested for chlamydial positivity by PCR analysis using *C. trachomatis* plasmid (pCT) specific primers as described earlier (George et al. 2003). The sequences for primers were: forward primer 5'- CTA GGC GTT TGT ACT CCG TCA-3' and reverse primer 5'- TCC TCA GGA GTT TAT GCA CT -3'; Each reaction contained, in a total of 25 µl, 5 µl DNA, 2.5 µl 10X PCR buffer (200mM Tris-HCL [pH 8.4], 500 mM KCl), 0.5 µl 10 mM dNTP mixture, 0.8 µl 50 mM $MgCl_2$, 3 µl of each primer (5pM/ µl), 0.1 µl Taq Polymerase (5U/ µl) (Invitrogen). The thermal conditions were initial denaturation at 95° C for 1 min followed by 40 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1.5 min, extension at 74°C for 2 min, then a final extension at 72° C for 10 min. 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 stained with ethidium bromide (EtBr) and were visualized on Alpha Imager gel documentation system (AlphaInnotech, San Leandro, USA).

Culture

HeLa cell culture

The human cervical epithelial adenocarcinoma cell line HeLa 229 was procured from National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained and cultured in Eagle's Minimum Essential Medium (EMEM; HiMedia, India) supplemented with 10% heat inactivated fetal bovine serum (FBS; PAA, Germany), 2mM L-glutamine (Sigma-Aldrich, USA), 20µg/ml gentamicin (Sigma-Aldrich), 2µg/ml amphotericin B (Sigma-Aldrich), HEPES buffer (SRL, India), and 25mM sodium bicarbonate, incubated in a

humidified incubator at 37°C with 5% CO_2. Cells were grown in antibiotic free medium prior to *in vitro* susceptibility assay.

Isolation and propagation of C. trachomatis

Isolation: Isolation of *C. trachomatis* was done from endocervical swabs collected in sucrose phosphate glutamate (SPG) media. Vials containing swab material were vortexed vigorously with glass beads to release intracellular EBs. Centrifugation was done at 3000 rpm for 5 minutes to settle down the cellular debris. Supernatant was collected and overlaid on to HeLa 229 cells monolayer for infection. These cells were incubated at 35°C for 30 min followed by centrifugation at 1000 rpm for 30 min subsequently after centrifugation supernatant was replaced by Dulbecco's Modified Eagle's Medium supplemented with 5% FBS, 10µg/ml gentamicin, 1µg/ml amphotericin B, 1µg/ml cycloheximide and incubated for 72hours at 35°C with 5%CO_2.

Propagation: *C. trachomatis* isolates were propagated for at least 10 passages in DEAE- dextran (30 µg/ml) treated HeLa 229 cells in Dulbecco's Modified Eagle's Medium supplemented with 5% FBS, 10 µg/ml gentamicin, 1 µg/ml amphotericin B, 1 µg/ml cycloheximide at 35°C with 5%CO_2, purified and stored at -80°C in sucrose phosphate glutamate medium (SPG, pH 7.0). Infectious titers were determined by titration on HeLa 229 cell monolayers by staining with fluorescein isothiocyanate (FITC) labeled monoclonal antibody against chlamydial major outer membrane protein (MOMP; MicroTrack, San Jose, CA) and are expressed as inclusion forming units (IFU). Immediately prior to use, the purified organisms were thawed and diluted in SPG.

Antibiotic susceptibility assay

Antimicrobial Agents

Azithromycin and doxycycline (Sigma-Aldrich) were dissolved according to the instructions of the manufacturer. Two-fold serial dilutions were prepared before each testing in DMEM cell culture medium without antibiotics. Susceptibility assay was done by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using cell culture technique as described in earlier studies (Notomi et al. 1999; Suchland et al. 2003).

Cytotoxicity Assay

The cytotoxic effects of azithromycin and doxycycline on host HeLa 229 cells were determined by methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. This method is based on the reduction of the tetrazolium salt, MTT into a crystalline blue formazan product by the cellular oxidoreductases of viable cells. The resultant formazan crystal formation is proportional to the number of viable cells (Mosmann 1983; Han et al. 2008; Yang et al. 2009). HeLa 229 cells were grown to confluency ($2x10^4$ cells/well) in 96-well plate in EMEM containing 10% FBS at 37°C with 5%CO_2. Drug dilutions (0.015 µg/ml to 60 µg/ml) were prepared in EMEM, added in to each well and incubated for 24hrs. After incubation 25 µg/ml MTT was added and incubated at 37°C for 2 hrs. Formazan crystal was solubilized in acidic isopropanol (0.04M HCl) and reading was taken at 570 nm with 630 nm reference wavelength using Biotech plate reader (µquant, Bio-Tek instrument Inc., Germany).

Minimum Inhibitory Concentration (MIC) determination

To determine the minimum inhibitory concentration for each antibiotic, experiment was carried out in triplicate with *C. trachomatis* laboratory reference Serovar D (UW-3/CX)

previously characterized as susceptible. Clinical isolates (n=21) isolated from *C. trachomatis* infected female patients were tested for evaluating MIC. Fresh monolayers were prepared by seeding 1 ml of $2x10^5$ cells/well on 12 mm glass coverslips contained in 24 well tissue culture plate and incubated for 24 hrs at 37°C in a humidified atmosphere with 5% CO_2 to obtain sub-confluent monolayers. Prior to infection, cell monolayers were washed once with phosphate buffered saline (PBS, pH7.0) and treated with DEAE- dextran (30µg/ml) at 35°C for 30min. Chlamydial inoculum was added on to the cell monolayers at a multiplicity of infection (MOI) of 1 and incubated for 1hr at 35°C. Cells were centrifuged at room temperature for 30min. at 1000 rpm and incubated at 35°C for 2 hrs. After incubation, inoculum was replaced with two fold serially diluted (0.015 µg/ml to 10µg/ml) antibiotics prepared in DMEM supplemented with 5% FBS, 1 µg/ml cycloheximide and incubated for 48 hrs in a humidified incubator at 35°C with 5% CO_2. As control, infected cells were incubated in the absence of respective drug.

The MIC (minimum inhibitory concentration) was defined as the lowest drug concentration required for complete inhibition of inclusion forming units of *C. trachomatis* compared with controls.

Minimum Bactericidal Concentration (MBC) determination

For determination of MBC, HeLa 229 cell monolayer ($2x10^5$cells/well) grown in 24 well plate was infected with inoculum of MOI 1 as described in the previous section. Antibiotic dilutions prepared in DMEM was added at 2 hours post infection (h.p.i.) and incubated at 35°C in a humidified chamber for 48 hours. Chlamydia were then harvested by using pipette tips, disrupted cells were collected in a tube and sonicated by using

ultrasonicator (Hielscher, Germany). Centrifugation was done at 3000 rpm for 10 min. at 4°C. Supernatant was collected and used for reinfecting the fresh confluent monolayer of HeLa 229 cells grown on the glass coverslips placed in 24 well plate. These infected cells were incubated for further 48 hours in the absence of antibiotics at 35°C in a humidified incubator with 5% CO_2.

The MBC (minimum bactericidal concentration) was defined as the lowest drug concentration required for complete removal of *C. trachomatis* inclusions after one passage in the absence of antibiotics as compared with controls.

Immunofluorescence assay

Detection of *C. trachomatis* inclusion bodies was done by immunofluorescence assay after staining with fluorescein conjugated monoclonal antibody directed against *C. trachomatis* specific major outer membrane protein (MOMP). For this, cell monolayers on the coverslips were washed with PBS, fixed with cooled methanol for 10 minutes and stained with FITC labeled monoclonal antibody. Inclusions were appeared as apple green in colour while host HeLa 229 cells appeared red as visualized by fluorescence microscope (Olympus BX51) at a magnification of ×100. Ten fields per well each consisting of 10 to 15 cells were counted and the average inclusion counts calculated. Percent of inhibition was calculated using the following formula:

Percent inhibition = [(mean total number of IFU in control - mean IFU in test)/ (mean total number of IFU in control)] ×100.

Results

Chlamydia trachomatis infection

Fifty one (31%) out of 163 symptomatic female patients who were enrolled for the study was *C. trachomatis* positive diagnosed by both DFA and PCR methods (Figure: 4.1A, 4.1B, 4.1C). Out of these patients, 12 (23.5%) were categorized as recurrently infected patients. *C. trachomatis*-infected patients with other STD pathogens were excluded.

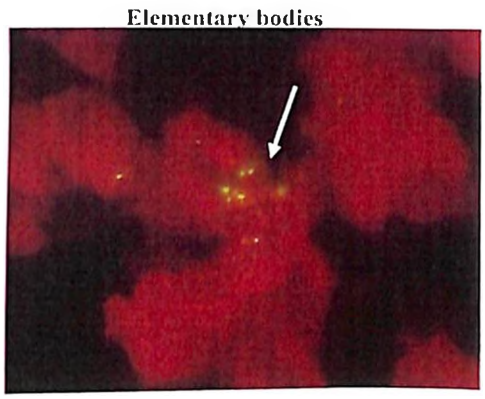


Fig. 1A- DFA: Clinical sample

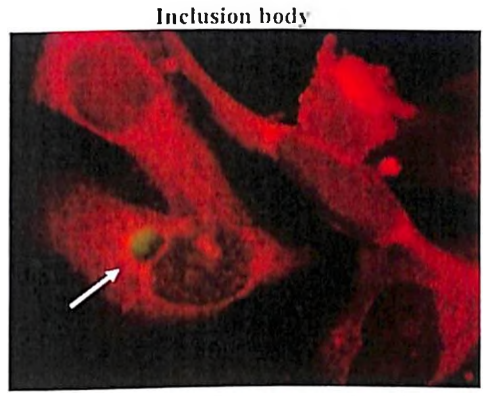


Fig. 1B- DFA: Culture confirmation

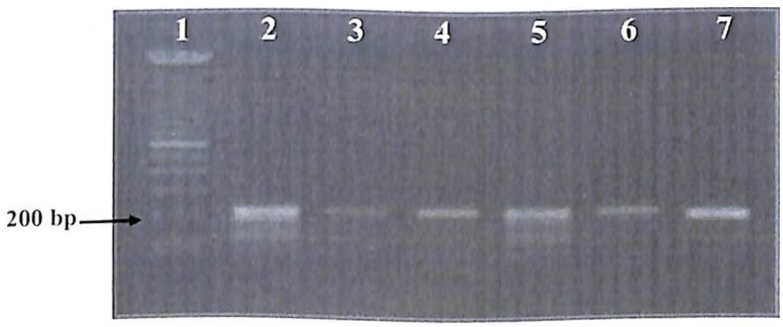


Fig. 1C Diagnostic PCR

Figure 4.1: Representative figures showing presence of chlamydial (A) elementary bodies in clinical sample and (B) Inclusion body in culture confirmation test by DFA (100X magnification. (C) 1.2 % agarose gel electrophoresis of 200 bp plasmid amplicon showing PCR positivity for detection of *C. trachomatis* in endocervical swabs collected from patients. Lane 1 is 100 bp DNA Ladder, Lane 2 is positive control, Lane 3-7 are *C. trachomatis* detected in patient samples.

Clinical isolates

Twenty one *C. trachomatis* isolates were successfully isolated from positive patients. Among these isolates 9 were originated from recurrently infected patients while rests 12 were from non-recurrently infected patients. These isolates were cultured atleast for 10 passages (≥ 10) for the enrichment of inoculum for antibiotic susceptibility assay.

Cell cytotoxicity assay

Host cell health is crucial in chlamydial sensitivity testing therefore, cytotoxic effects of azithromycin and doxycycline was tested on Hela 229 cells by the MTT assay. At concentrations range (0.015 to 60μg/ml) no cytotoxic effect on these cells were observed.

Antibiotic susceptibility

Azithromycin and doxycycline were used for the susceptibility assay using cell culture technique for the 21 *C. trachomatis* isolates. Thirteen isolates (61.9%) were found to be susceptible to these drugs and have shown their MIC values equal to the laboratory standard isolate (serovar D) ≤0.125μg/ml, ≤0.25μg/ml for azithromycin and doxycycline respectively. However, eight isolates (38%) showed modified antibiotic susceptibility profile. The susceptibility profile of these isolates is presented in table 1. The MIC values for these eight isolates vary from 0.12 to 8μg/ml for azithromycin and 0.025 to 8.0μg/ml for doxycycline. Six isolates obtained from the recurrently infected patients showed decreased susceptibility towards both the drugs (Table 4.1), while three isolates which were also obtained from recurrently infected patients had shown the susceptibility comparable to the control.

Table 4.1: Antibiotic susceptibility profile of clinical *C. trachomatis* isolates showing varying levels of sensitivity towards azithromycin and doxycycline

No.	Isolate	Age	Diagnosis	Previous treatment	MIC, µg/ml		MBC, µg/ml			
					azithro-mycin	doxy-cycline	azithromycin		doxycycline	
							4 µg/ml	8 µg/ml	4 µg/ml	8 µg/ml
1	Control	–	–	–	0.12	0.25	–	–	–	–
2	CT222	38	chronic cervicitis	doxycycline	0.12	8.0	–	–	–	–
3	CT227	30	chronic cervicitis	azithromycin	8.0	8.0	+	+	+	+
4	CT231	40	PID	doxycycline	1.0	2.0	–	–	–	–
5	CT232	29	cervicitis	doxycycline	4.0	4.0	–	–	–	–
6	CT233	32	cervicitis	n.c.	0.5	0.025	–	–	–	–
7	CT235	24	infertility	doxycycline	2.0	4.0	–	–	+	+
8	CT244	20	PID	doxycycline	8.0	8.0	+	+	+	+
9	CT247	32	infertility	n.c.	2.0	2.0	+	–	–	–

+ = Inclusion bodies were detected at the given concentration of antibiotic; – = inclusion bodies were not detected at the given concentration of antibiotic; n.c. = status of previous treatment not clear from the history.

Two isolates (CT227, CT244) which have shown high MIC values (8µg/ml) for azithromycin and doxycycline were isolated from treatment failure patients. One of the patients had symptoms of chronic cervicitis while another had PID. Out of these one (CT227) was treated with azithromycin and another (CT244) with doxycycline. In addition there was one more isolate (CT222) which had 8.0µg/ml MIC value for doxycycline diagnosed with chronic cervicitis. The inclusion bodies which were found in higher concentrations of drugs were less in number and appeared small as compared to the control. MBC was determined for isolates at higher drug concentrations i.e. 4 and 8µg/ml to check whether theses isolates were killed by the tested drugs. No inclusion bodies were detected in four of the isolates at the given concentrations of both the drugs, while for one isolate (CT235) inclusion bodies were observed at 4 and 8µgml of doxycycline. Of major interest were isolate no. CT227 and CT244 which had shown higher MIC values; inclusion counts were represented in the Figure

4.2.,4.3. The number and size of the inclusion bodies decreased with the increasing concentrations of the drugs.

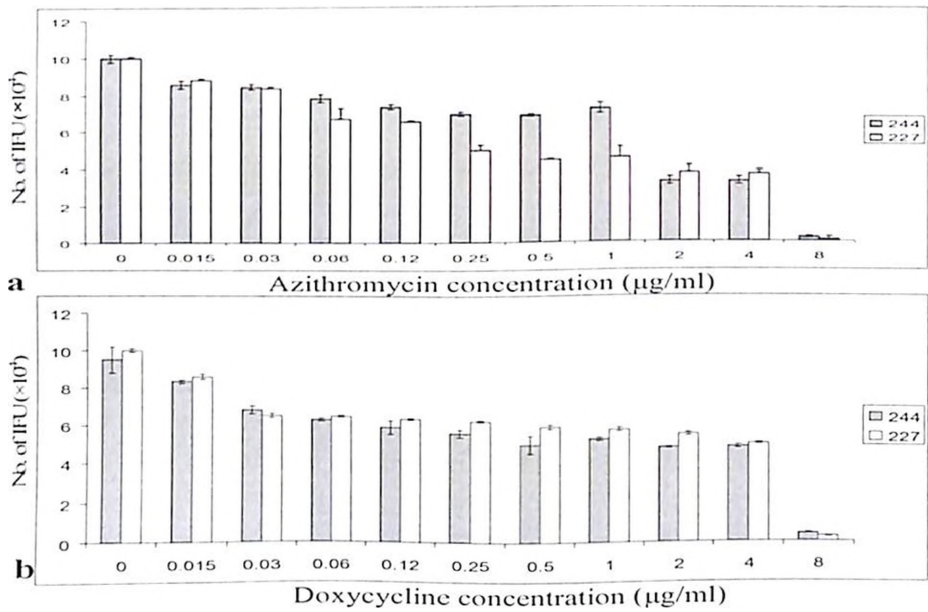


Figure 4.2: Graphical representation of *C. trachomatis* inclusion-forming unit counts in the presence of different concentrations of azithromycin (a) and doxycycline (b) in HeLa 229 cells.

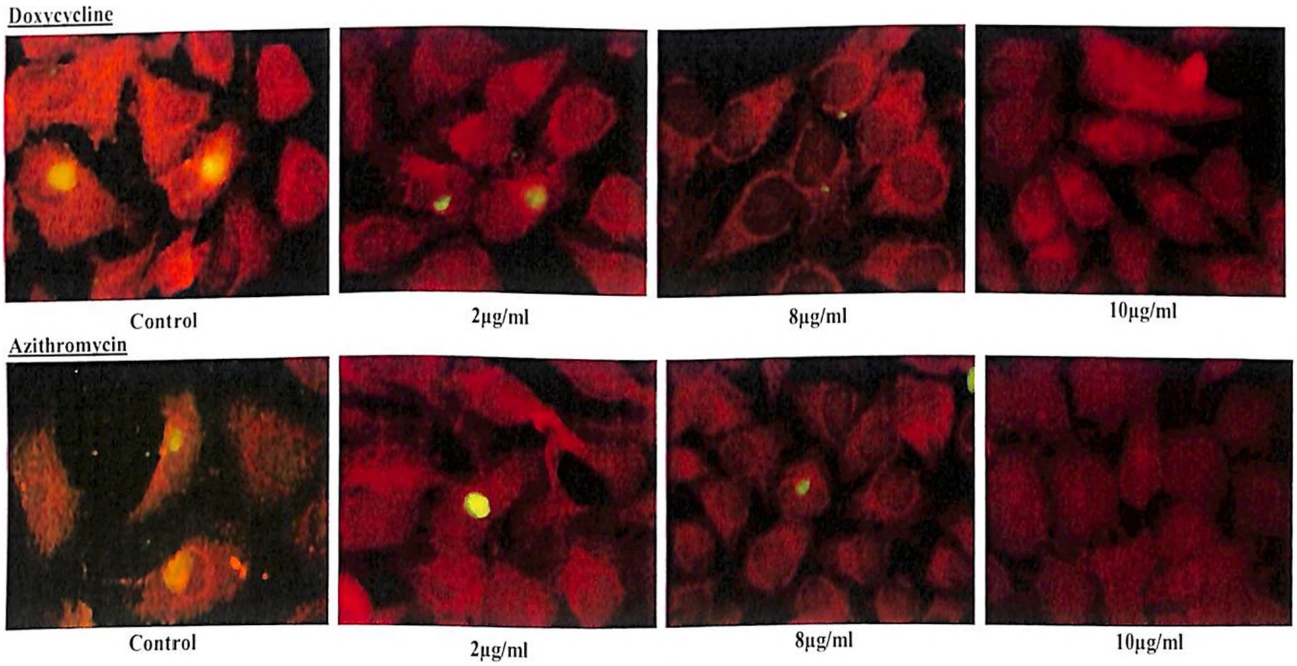


Figure 4.3: Representative images showing the clinical *C. trachomatis* isolates' inclusions in the presence of different concentrations of drugs (azithromycin and doxycycline) in HeLa 229 cells.

Discussion

C. trachomatis genital infection and its associated complex sequelae cause a major concern for reproductive health. The prevalence of recurrent chlamydial infection is well reported which ranges between 6% - 30% within 6 months to 1 year of treatment and responsible for most of the reproductive damages (Wang et al. 2005; Beatty et al. 1994a; Stamm 1999). In India *C. trachomatis* infected female patients are large in number i.e. 30-40% (Joyee et al. 2004; Singh et al. 2002; Singh et al. 2003). Despite this high number of infected patients, there is a lacking scenario for understanding recurrent infections and it's relation to clinically treatment failures. Current antibiotic regimens here are mainly doxycycline and azithromycin; however doxycycline is preferably prescribed due to its lower cost with same efficacy as of azithromycin.

In this study we have determined the recurrent infections in symptomatic female patients with cervicitis, PID and infertility. Recurrent chlamydial infections were found in 23.5% of chlamydia positive patients within the study period of 1 year. Further, *C. trachomatis* isolates (n = 21) were obtained from the positive patients to know the *in vitro* drug susceptibility profile towards the current regimen (doxycycline and azithromycin). Out of 21 *C. trachomatis* isolates, 13 isolates were susceptible towards the azithromycin and doxycycline, of which 3 were obtained from recurrently infected patients. However, 8 isolates had shown the modified antibiotic susceptibility profiles, were obtained from recurrently infected patients and 1 isolate from non-recurrently infected patient. The MIC values of all these isolates were significantly higher than the standard isolate. Azithromycin was more active as compared to the doxycycline. The MIC and MBC values of two isolates was 8μg/ml and it was seen that its complete eradication could not occur at this higher

concentration. However, the number and size of inclusion bodies were found to be decreased with increasing concentration of antibiotics as also observed in other studies (Jones et al. 1990; Lefevre and Lepargneur 1998; Somani et al. 2000; Samra et al. 2001). This small percentage of organisms may reflect the presence of heterotypic resistance.

Clinical isolates tested in our study had varying level of susceptibility pattern for these two antibiotics. Other studies have also shown that several antibiotics vary in level of activity against *C. trachomatis* and may differ in their specific effects on different stages of the chlamydial life cycle. Being an obligate intracellular parasite its two forms EBs and RBs develop diverse strategies to survive within this compartment. The barrier to its antibiotic treatment is the difference between the localization of antibiotics within the cellular compartment of infected cells and the localization of bacteria, hence antibiotics need to reach and concentrate within intracellular compartments (McOrist 2000; Tulkens 1991; Biswas et al. 2008). Azithromycin is known to have high penetration and accumulation capacity intracellularly as compare to other antibiotics found more effective against intracellular pathogens (Niki et al. 1994). Other reasons for therapeutic failure which results in recurrent infection may be reexposure to the infected partner, resistance to the antibiotic used or resurgence of persistent infection into active infection (van Valkengoed et al. 2002). Previous studies report increase in antibiotic resistance, however heterotypic resistance phenomenon is common in chlamydia and its relation to clinical treatment failures is suggested (Wang et al. 2005; Somani et al. 2000). From our study we suggest that the complex clinical symptoms were correlated with the higher MIC and MBC values. Wherein, most of the isolates which have shown higher values obtained from recurrent infected patients and were diagnosed with

chronic cervicitis, PID and infertility. However, studies including large number of patients are needed to affirm the findings.

In summary, this study suggests emerging problem of decrease in efficacy of first line drugs for chlamydial infection which can hamper the treatment effectiveness. This suggest that all women treated for chlamydial infection should be retested after cure for preventing long term complications that would help in controlling chlamydial infection.

Chapter 5

Determination of In vitro inhibitory effects of a novel polyherbal formulation BASANT on Chlamydia trachomatis

Determination of in vitro inhibitory effects of a novel polyherbal formulation BASANT on Chlamydia trachomatis

Introduction

Chlamydia trachomatis infection is the most prevalent sexually transmitted bacterial disease worldwide (Gaydos et al. 1998; Beagley and Timms 2000). Up to 80% of women with genital chlamydial infection are asymptomatic (Gaydos et al. 1998; Morre et al. 2000). Chronic chlamydial infection may affect upper genital tract leading to pelvic inflammatory disease, chronic pelvic pain, salpingitis, secondary infertility, and ectopic pregnancies (Cohen and Brunham 1999). Azithromycin and doxycycline are recommended as first-line drugs for the treatment of chlamydial infections by the US Centers for Disease Control and Prevention (CDC) and have a >95% microbiological cure rate (Workowski and Berman). There are, however recent reports world wide of recurrent chlamydial infections (10% - 15%) occurring after treatment with various antibiotics (Dean et al. 2000; Katz et al. 1991; Lefevre et al. 1997; Samra et al. 2001; Somani et al. 2000; Wang et al. 2005). It has been reported that *C. trachomatis* infection persists due to many of the reasons such as treatment failures, reinfection through infected partner or bacterial persistence and it may lead to the complex sequelae resulting in poor reproductive health (Hillis et al. 1997).

Besides first line oral antichlamydial drugs; azithromycin and doxycycline, other options such as a topical microbicide would be useful to control chlamydial infections. An effective microbicide can be used as one of the most promising prevention option for various sexually transmitted diseases. It can be used topically for direct killing of the pathogens at the site thus, preventing the pathogen's entry into the host (Van Damme 2004). Development

and testing of an effective microbicide is needed as a prevention measure for various sexually transmitted infections.

A polyherbal cream, Basant, has been developed by Talwar et al. for topical use, which has been formulated employing a combination of active compounds such as Aloe vera (2.5% w/v), Amla (2.5% w/v), curcumin (0.36% w/v) and reetha saponins (1% w/v). Aloe vera has been reported to have wound healing properties (Davis et al. 1989) and has an inhibitory effect on human immunodeficiency virus (HIV) and human papillomavirus (HPV), whilst Amla has antioxidant, anti-inflammatory and antimutagenic properties (Levine 1986). Curcumin (diferuloylmethane), the active component of *Curcumin longa*, is used traditionally for application on wounds where it is believed to act as an antiseptic with wound healing properties along with anti-inflammatory and antitumour properties (Chandra and Gupta 1972; Qin et al.). The combination of these ingredients has not only widened the spectrum of infections inhibited by Basant, but has also resulted in synergism, thus enhancing their action. Basant is free from local and systemic side effects and has wide-spectrum inhibitory action against genital pathogens such as *Neisseria gonorrhoeae and Candida* spp. It has a potent virucidal action against HIV-1 and prevents the transduction of HPV-16 in HeLa cells (Talwar et al, 2008). With the permission of the Drugs Controller General of India and the Institutional Ethics Committee, it is in Phase II clinical trials in three major centres in India for the early stage of cervical dysplasia in women positive for HPV 16 & 18.

Thus in this study *in vitro* inhibitory activity was determined for a novel polyherbal microbicide candidate "Basant" against *C. trachomatis*. The inhibitory efficacy was assayed firstly on standard isolate of *C. trachomatis*, in both forms of its life cycle i.e. on infectious

but metabolically inactive elementary bodies (EBs) and non-infectious metabolically active reticulate bodies (RBs). Subsequently its inhibitory activity was also determined on clinical isolates of *C. trachomatis.*

Materials

Eagle's Minimum Essential Medium (EMEM) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from HiMedia; Mumbai, India. Fetal bovine serum (FBS) was from PAA Laboratories GmbH; Colbe, Germany. L-glutamine, gentamicin, amphotericin B and methylthiazolyldiphenyl-tetrazolium bromide MTT were purchased from Sigma-Aldrich; St Louis, MO, USA. HEPES buffer and sodium bicarbonate were from SRL; Mumbai, India. Glass coverslips (12mm) were purchased from Blue Star; Mumbai, India and tissue culture plates from Greiner, Germany. Trizol, DNase I, Taq polymerase, dNTPs, $MgCl_2$, DNA ladder was from Invitrogen, USA. Primers were synthesized by MWG-Biotech, Germany.

Experimental Methods

Cell culture

The human cervical epithelial adenocarcinoma cell line HeLa 229 was procured from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained and cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 20 µg/mL gentamicin, 2 µg/mL amphotericin B, HEPES buffer and 25 mM sodium bicarbonate, incubated in a humidified incubator at 37 °C with 5% CO_2. Cells were grown in antibiotic-free medium prior to the *in vitro* sensitivity assay.

Chlamydia

Chlamydia trachomatis laboratory reference serovar D (D/UW-3/Cx) was maintained and propagated in the laboratory. Six clinical isolates were also used in this study, which were previously isolated from endocervical swab obtained from women with suspected chlamydial infection attending the gynaecology outpatient department at Safdarjung Hospital, New Delhi, India. These isolates were confirmed to be positive by Giemsa, culture confirmation test, direct fluorescence assay and polymerase chain reaction (PCR) analysis using a *C. trachomatis*-specific 200-bp amplicon of cryptic plasmid.

Chlamydia trachomatis isolates were propagated in HeLa 229 cells as described in chapter 4, purified and stored at –80°C in sucrose–phosphate–glutamate medium (SPG) (pH 7.0). Infectious titres were determined by titration on HeLa 229 cell monolayers and staining with fluorescein isothiocyanate (FITC)-labelled monoclonal antibodies against chlamydial major outer membrane protein (MOMP) (MicroTrack, San Jose, CA) and are expressed as inclusion-forming units (IFU).

Basant

Basant cream was formulated using purified diferuloylmethane (curcumin), purified extracts of *Emblica officinalis* (designated Amla), purified saponins from *Sapindus mukorosii*, purified Aloe vera powder and rose extract water. Quality control criteria were developed for each ingredient to assure reproducibility from batch to batch. The ingredients were formulated in pharmacopoeially-approved excipients: sodium alginate as gelling agent, xanthan gum as bioadhesive agent, lactic acid, citric acid and potassium sodium tartrate as buffering agents, benzoic acid as preservative and glycerol as humectant. A batch of Basant

was prepared under GMP (Good Manufacturing Practice) conditions by M/s Gufic Biosciences Ltd., a company licensed by the Drugs Controller for the manufacture of herbal creams.

Cytotoxicity assay

The cytotoxic potential of Basant was determined on HeLa 229 cells using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay as described elsewhere (Mosmann 1983) and in chapter 4. HeLa 229 cells were grown to confluency (2×10^4 cells/well) in a 96-well plate in EMEM containing 10% FBS at 37 °C with 5% CO_2. Basant dilutions (0.015–60 µg/mL) were prepared in EMEM and added to each well. After 24 h of incubation, 25 µg/mL MTT was added and incubated at 37 °C for 2 h. Formazan crystals were solubilized in acidic isopropanol (0.04 M HCl) and a reading was taken at 570 nm with 630 nm reference wavelength using a Bio-Tek microplate reader (Quant; Bio-Tek Instruments Inc.; Winooski, Vermont, USA).

In vitro sensitivity determination

Two methods were employed to determine the *in vitro* sensitivity of *C. trachomatis* towards Basant:

(i) Pre-infection incubation and

(ii) Post-infection incubation

Pre-infection incubation with Basant

The pre-infection incubation method was performed three times to evaluate the direct effect of Basant on *C. trachomatis*. Basant dilutions (0.06–60 µg/mL) were prepared in SPG and a chlamydial inoculum of 2×10^4 IFU was added to each dilution, mixed by vortexing and incubated at 35 °C for 15, 30 and 60 min. After completion of incubation, the chlamydial inoculum was infected into HeLa 229 cells on 12 mm glass coverslips contained in 24-well tissue culture plates and incubated for 48 h at 35 °C with 5% CO_2. A monolayer of cells infected with inoculum incubated in the absence of Basant was taken as a control.

Post-infection incubation with Basant

To evaluate the minimum inhibitory concentration (MIC), the experiment was performed in triplicate with laboratory reference serovar D (D/UW-3/Cx) and six clinical isolates of *C. trachomatis*. One of the isolates was obtained from a patient who did not respond to the standard treatment course of doxycycline. Infection was carried out in 2×10^5 HeLa 229 cells/well with a chlamydial inoculum of multiplicity of infection (MoI) of 1 as described previously (Ridgway, 1976; Suchland, 2003; Cross, 1999; Yamazaki, 2003; Yamano, 2005). At 2 h post infection, the inoculum was replaced with two-fold serially diluted Basant (0.03–10 µg/mL) prepared in complete Dulbecco's Modified Eagle's Medium (DMEM) and incubated for 48 h at 35 °C with 5% CO_2. As a control, infected cells were incubated in the absence of Basant.

The MIC was defined in both assays as the lowest Basant concentration required for complete inhibition of *C. trachomatis* IFU compared with controls.

Immunofluorescence assay

An immunofluorescence assay was performed for the determination of the MIC. This assay is based on FITC-labelled monoclonal antibodies, which detect chlamydial MOMP with high sensitivity and specificity (Cles et al. 1988). Inclusions were visualised after 48 h of incubation by staining with FITC-labelled monoclonal antibodies using a fluorescence microscope (Olympus BX 51) at a magnification of 100X. All assays were performed in triplicate and the inclusion counts were calculated by counting three fields per well. The lowest concentration of Basant that completely inhibited the formation of visible chlamydial inclusions was taken as the MIC. Percent inhibition was calculated using the following formula:

Percent inhibition = [(mean total number of IFU in the control – mean IFU in the test)/ (mean total number of IFU in the control)] × 100.

RNA isolation

HeLa 229 cells (3×10^5 cells/well) were grown in six-well tissue culture plates and infected with chlamydial inoculum at a MoI of 1. Serial dilutions of Basant were added at 2 h post infection and incubated for 48 h at 35°C with 5% CO_2. Infected cells incubated in the absence of Basant were taken as a control. Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions and quantified using a UV-VIS spectrophotometer. RNA was treated with DNase I to prevent DNA carryover. The isolated RNA was tested by PCR to check any carryover DNA contamination. No amplification of product was detected and the RNA was considered as DNA free.

Reverse transcriptase (RT)-PCR

The 5 µg of RNA was reverse transcribed into cDNA using RETROscript™ Kit (Ambion; Austin, Texas, USA), according to the manufacturer's instructions. Amplification of cDNA was carried out in a total volume of 25 µL using the primer sequences of 16S rRNA gene as described elsewhere (Mpiga and Ravaoarinoro 2006). The thermal conditions were initial denaturation at 92 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 45 s, primer annealing at 60 °C for 45 s and synthesis at 72 °C for 1 min, then a final extension at 72°C for 10 min in a thermal cycler (Applied Biosystems; Foster City, CA, USA). The PCR product was visualised by electrophoresis on a 2% agarose gel stained with ethidium bromide.

Results

Cell cytotoxicity assay

No cytotoxic effect and morphological changes by Basant on HeLa 229 cells were observed at the tested concentrations.

Direct effect of Basant on C. trachomatis

To mimic closely the topical action of an ideal microbicide, the pre-infection incubation assay was performed as described in previous section. It was observed that Basant caused a decline in the number of inclusion bodies as a function of its concentration and incubation time, with complete block at 12 μg/mL of Basant after 60 min of incubation. For 30 min and 15 min of incubation, complete block was observed at 30 μg/mL and 60 μg/mL Basant, respectively as shown in figure 5.1.

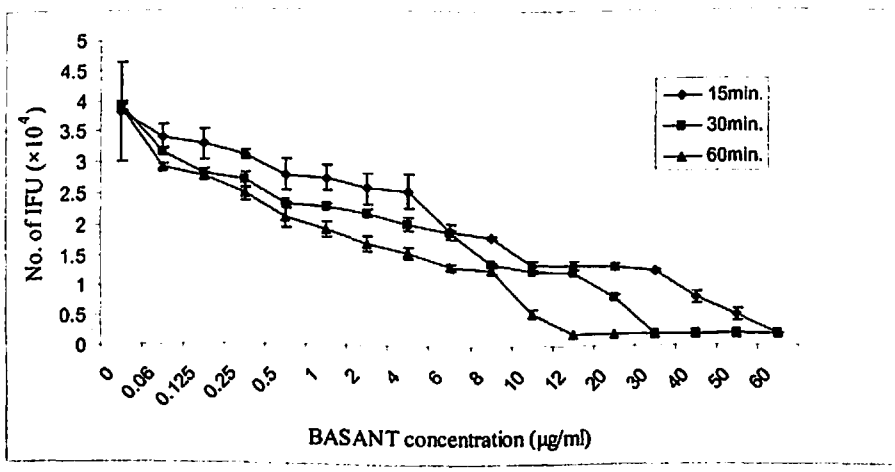


Figure 5.1: Inhibitory effect of Basant on *Chlamydia trachomatis* serovar D (D/UW-3/Cx) using the pre-infection incubation method. Standard deviations from triplicate tests are indicated by error bars. The number of inclusion-forming units (IFU) decreased with the incubation time and concentration of Basant used.

Effect of Basant on C. trachomatis within HeLa cells

HeLa cells infected with *C. trachomatis* serovar D were incubated with increasing concentrations of Basant. A progressive decline was observed in the number of inclusion bodies in previously infected cells, with complete demise of replicative *C. trachomatis* at a concentration of 8 μg/mL Basant (Figure 5.2).

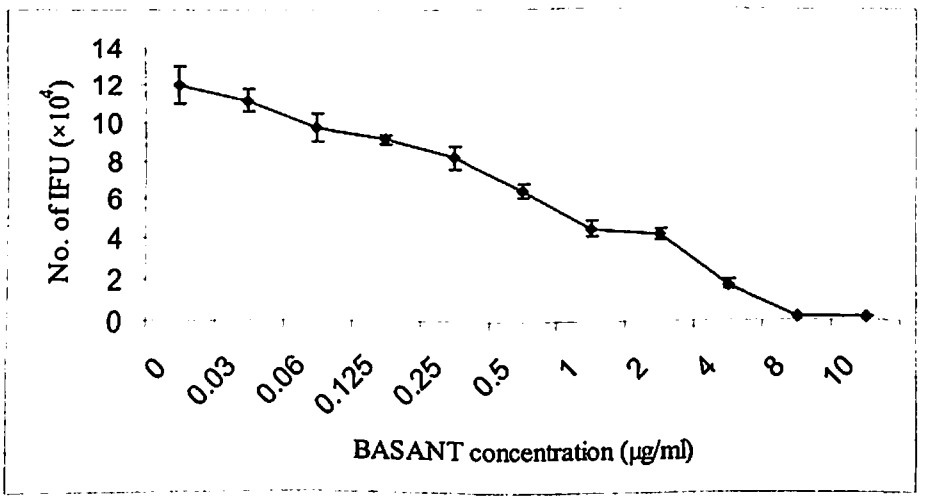


Figure 5.2: Inhibitory effects of Basant on *Chlamydia trachomatis* serovar D (D/UW-3/Cx) using the post-infection incubation method. Standard deviations from triplicate tests are indicated by error bars. The minimum inhibitory concentration (MIC) was determined at 8 μg/mL Basant. IFU, inclusion-forming units.

Effect of Basant on clinical isolates of C. trachomatis

From the above mentioned experiments it was evident that *C. trachomatis* within and outside host cells was sensitive to Basant. In the next experiment, the susceptibility of clinical isolates was determined as described in previous section. It was observed that clinical isolates CT231 and CT272 were inhibited completely at 8 μg/mL Basant. Complete inhibition of other isolates was observed at 10 μg/mL Basant (Table 5.1). However, complete

inhibition of a treatment failure clinical isolate was observed at a higher concentration of Basant (30 μg/mL), as detected by immunofluorescence assay.

Table 5.1: *In vitro* minimum inhibitory concentration (MICs) of Basant for clinical isolates of *Chlamydia trachomatis* using the post-incubation method.

Clinical isolates	*MIC (μg/ml)*[a]
CT231	**8.0**
CT233	**10.0**
CT239	**10.0**
CT272	**8.0**
CT279	**10.0**
CT244[b]	**30.0**

[a] Basant concentrations for complete inhibition (100%)

[b] *Chlamydia trachomatis* isolate obtained from doxycycline treatment failure patients

The inhibitory effect of Basant was clearly detected in the treatment failure isolate (CT244) by immunofluorescence assay in the infected HeLa cells. No inclusion bodies were observed at 30 μg/mL concentration while the size of the inclusion bodies found to be decreased at the higher concentration (10 μg/mL) of Basant (Figure 5.3). In addition to the immunofluorescence assay, the MIC for treatment failure clinical isolate (CT244) was also determined by RT-PCR method for the transcription of 16S rRNA gene. It was observed that while the RNA transcript was still evident at 10 μg/mL of Basant, at 20 μg/mL Basant partial inhibition was evident and complete inhibition of *C. trachomatis* was observed at 30 μg/mL Basant (Figure 5.4).

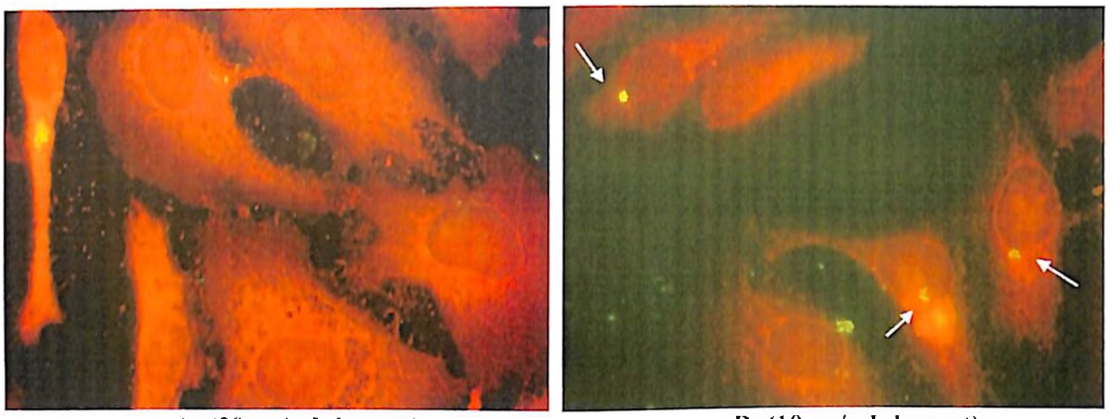


Figure 5.3: Images showing the decreased size of inclusion bodies of *C. trachomatis* isolate obtained from treatment failure patient in the presence of different concentrations of basant in HeLa 229 cells.

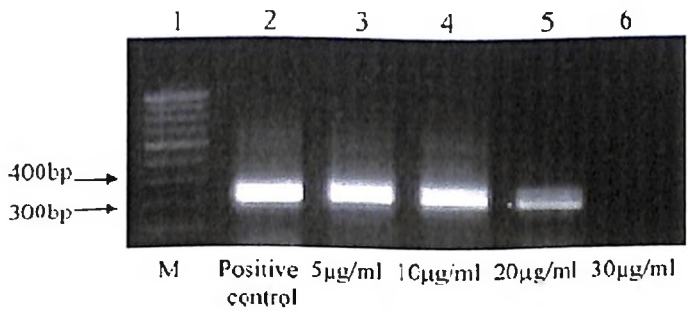


Figure 5.4: Reverse transcriptase polymerase chain reaction (RT-PCR) amplification of 16S rRNA gene transcripts from *Chlamydia trachomatis* (D/UW-3/Cx) isolate obtained from a doxycycline treatment failure patient in the presence and absence of Basant. The minimum inhibitory concentration was 30 µg/mL. Lane 1, marker; lane 2, positive control; lanes 3 - 6, Basant at 5, 10, 20 and 30 µg/mL, respectively.

Discussion

Both antibiotic resistance and persistence of *C. trachomatis* infection after treatment have been reported in several clinical studies from different countries (Wang et al. 2005). To develop new microbicides with the ability to prevent sexual transmission of *C. trachomatis*, this study was undertaken to evaluate the potential clinical utility of a novel polyherbal formulation, Basant, on *C. trachomatis*. We observed that Basant has no cytotoxic effect on the epithelial adenocarcinoma cells (HeLa 229), indicating the safety of Basant on epithelial cells, which are the primary target of various pathogens.

The infectious elementary bodies (EBs) of *C. trachomatis* were decreased as seen by inclusion counts with respect to the time of incubation and the concentration of Basant. Since EBs have a dense and thick envelope and are metabolically inactive, the desired compound may take longer to reach the target molecule. In our study, pre-infection incubation for 60 min with 12 μg/mL Basant was sufficient for complete inhibition of *C. trachomatis*. However, in the case of the post-infection incubation assay where Basant was added after establishment of *C. trachomatis* infection, inhibition was observed at 8 μg/mL. Basant also completely inhibited the clinical isolates of *C. trachomatis* at the concentration of 8–10 μg/mL, as well as one doxycycline treatment failure isolate at 30 μg/mL.

As an antichlamydial topical microbicide, Basant has shown its inhibitory activity on *C. trachomatis,* suggesting it has the potential ability for clinical use, as there would be a mixture of developmental forms of *C. trachomatis* in vivo. Intravaginal application of Basant prior to sexual contact may kill the infectious EBs before they infect the target epithelial cells, thus preventing chlamydial infection. Basant has been formulated as a viscous cream

and tablet form that would stay in the vagina for enough time to provide protection against the infectious pathogens.

In conclusion, this study suggests that Basant has antimicrobial activity against *C. trachomatis* with standard as well as clinical isolates. This implies the potential clinical utility of Basant for the prevention of *C. trachomatis* infection. The mechanism by which Basant inhibits *C. trachomatis* is not known and requires further investigation.

Chapter 6

Characterization of the heterotypic resistant Chlamydia trachomatis clinical isolate(s) towards antichlamydial drug at genotypic level

Characterization of the heterotypic resistant Chlamydia trachomatis clinical isolate(s) towards antichlamydial drug at genotypic level

Introduction

Chlamydia trachomatis is an obligate intracellular parasite that causes a wide range of inflammations of the urogenital tract resulting in reproductive complexities and is one of the most prevalent sexually transmitted bacterial diseases in the world (Bebear and de Barbeyrac 2009; Beagley and Timms 2000; Belland et al. 2003). Antibiotics play a major role in treating chlamydial infections and efficacy of these drugs for treatment of chlamydial infections are high, however many researchers report the problem of recurrent infections and treatment failures (Whittington et al. 2001; Workowski and Berman ; Katz et al. 1991; Horner 2006; Hillis et al. 1997). It has also been reported that 50% of chlamydial infection resolve spontaneously in infected women within one year of testing, but in those with persistent or repeated infections, the infection can spread upwards from the endocervix to the fallopian tubes and may result in infertility or ectopic pregnancy (Belland et al. 2003; Beagley and Timms 2000; Beatty et al. 1994).

Currently, the recommended first-line therapeutic regimens for chlamydial infections are the tetracyclines: doxycycline and the macrolide: azithromycin (Workowski and Berman ; Binet and Maurelli 2007). Both the drugs have shown effective coverage against most pathogenic bacteria however, studies suggest the emerging resistance to these drugs. Tetracycline resistance in chlamydia was firstly identified in *Chlamydia suis* which were isolated from diseased and normal pigs in the Midwestern USA (Lenart et al. 2001; Di Francesco et al. 2008) and then followed by identification of eight independent strains of *Chlamydia suis* each exhibited high level resistance to tetracycline. Genetic characterization of the isolates revealed the presence of foreign genomic islands (ranging in size from 6 to 13.5kb) that had integrated into the chlamydial chromosome (Dugan et al. 2007). Each island carries genes encoding a tetracycline efflux pump [*tet*(C)], a regulatory repressor [*tet(R)*] and a unique insertion sequence (IScs605) plus three to ten additional genes involved in plasmid replication and mobilization (Dugan et al. 2004). Further, in 2008, a report identified 14 additional *C. suis* strains collected in Italy that shared 100% nucleotide identity with the *tet*(C) gene from the original US strains (Di Francesco et al. 2008; Lenart et al. 2001). The discovery of the *tet*(C) islands represents the first identification of antibiotic resistance acquired through horizontal gene transfer in any obligate intracellular bacteria. Although there is currently no evidence for heritable antibiotic resistance in human clinical settings

however, in France a tetracycline-resistant isolate was recovered from a woman who had persistent infection after doxycycline treatment (Lefevre et al. 1997).

The resistance of various microorganisms to macrolides is often associated with mutation(s) in ribosomal protein genes, particularly in L4 and L22, as well as with mutations in the peptidyl transferase region of the 23S rRNA gene (Canu et al. 2002; Vester and Douthwaite 2001). There are few documented reports of antibiotic resistance against macrolides in *Chlamydia* but no examples of natural and stable antibiotic resistance in strains collected from humans (Wang et al. 2005). Recently, 4 clinical isolates that demonstrated *in vitro* resistance to macrolides were shown to carry mutations in the 23S rRNA gene (Misyurina et al. 2004). However, there has been no detection of or selection for homotypic resistance among *C. trachomatis* isolates was observed, although homotypic resistance has been detected among *Chlamydia suis* isolated from pigs (Lenart et al. 2001; Di Francesco et al. 2008). *In vitro* studies suggest that antibiotic-resistant genotypes of *C. trachomatis* can be generated and transferred to *C. trachomatis*, *C. suis* or *C. muridarum* isolates with capability of expressing significant resistant phenotypes (Suchland et al. 2009). Hence, emerging heterotypic bacterial resistance against antichlamydial drugs resulting in treatment failures in clinical settings can not be neglected. Studies are needed for characterization of *C. trachomatis* clinical isolates showing decreased susceptibility towards the antichlamydial

drugs which may results in resistance characteristics of the bacteria and can be concluded with respect to the patient's treatment failure(s) or reinfection(s).

In India, a high prevalence (>30%) of *C. trachomatis* infections in symptomatic female patients have been reported (Joyee et al. 2004; Singh et al. 2002; Singh et al. 2003). The antibiotics which are majorly prescribed here are azithromycin and doxycycline. In the previous study (chapter 4) the antibiotic susceptibility profile was studied towards the first line antichlamydial drugs; azithromycin, doxycycline and found that eight isolates majorly from recurrently infected patients had decreased *in vitro* susceptibility. Few of them appeared as of heterotypic resistant isolates in cell culture as observed by decrease i) in size of inclusion body and ii) number of inclusion bodies as compared to control in the presence of antichlamydial drugs. Thus the aim of this study is to explore if the *in vitro* heterotypically resistant characteristic was associated with mutational changes in the resistant bacteria. Hence, amplification of the ribosomal L4, L22, 23SrRNA and efflux gene(s) was done to study them for the presence of possible mutation(s).

Materials

Unless otherwise noted, all fine chemicals used in the study were purchased from Sigma Aldrich (St. Louis, USA). Eagle's Minimum Essential Medium (EMEM) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from HiMedia; Mumbai, India. Fetal bovine serum (FBS) was from PAA Laboratories GmbH; Colbe, Germany. Plasticware and glassware used in cell culture was purchased from Greiner, Germany. Trizol, DNase I, Taq polymerase, dNTPs, $MgCl_2$, DNA ladder was from Invitrogen, USA. Primers were synthesized by MWG-Biotech, Germany. The antimicrobial agents tested in this study were azithromycin and doxycycline purchased from Sigma - Aldrich, St. Louis, Missouri, USA). Azithromycin and doxycycline were supplied as powder form and solubilized according to the manufacturer's instructions.

Experimental methods

Cell culture and Propagation of C. trachomatis clinical isolates

The human cervical epithelial adenocarcinoma cell line HeLa 229 was procured from National Centre for Cell Sciences (NCCS), Pune, India. These cells were maintained and cultured as described in chapter 4. *C. trachomatis* clinical isolates (n = 8) which showed decreased susceptibility profile against doxycycline and azithromycin were propagated in

host HeLa 229 cells as described in chapter 4 to get enough number of *C. trachomatis* inclusion bodies.

DNA Isolation and Polymerase chain reaction (PCR)

HeLa 229 cells infected with *C. trachomatis* isolate(s) were subjected to DNA extraction using QIAamp Viral RNA mini Kit (Qiagen, CA, USA) according to manufacturer's instructions. Briefly infected Hela 229 cells were harvested at 48 hour post infection (hpi) by using a cell scrapper (Greiner, Germany) and the harvested cell suspension was centrifuged at 3000 rpm for 10 min at 4°C. Supernatants were collected and centrifuged at 16000 rpm for 1hr at 4°C; pellets were collected and processed for DNA isolation. Concentration of DNA was quantified spectrophotometrically at 260 nm (Biometra, USA). The amplification of each gene was carried out by polymerase chain reaction (PCR) in a DNA Eppendorf Mastercycler personal Thermal Cycler (Eppendorf GmbH, Hamburg, Germany). The primer sequences are given in table 3.1 and as described elsewhere (Hsu et al. 2006; Ngandjio et al. 2004; Stothard et al. 1998; Binet and Maurelli 2007).

RNA isolation

Total RNA was isolated using Trizol reagent (Invitrogen, Foster City, CA). Briefly, the cells were lysed in Trizol, at room temperature and to the homogenized samples, 0.2

volumes of chloroform was added followed by centrifugation at 12,000 rpm for 15 min. The aqueous phase was collected and 0.5 volumes of isopropyl alcohol were added to precipitate total RNA. The total RNA was pelleted by centrifugation and washed in 70% ethanol and stored at –70 ° C till further use.

RNA was treated with DNase I (Fermentas Inc., Glen Burnie, MD) to prevent DNA carryover. The isolated RNA was tested by PCR to check any carryover DNA contamination. No amplification of product was detected and the RNA was considered as DNA free.

Reverse transcription (RT)-PCR

RNA (2 μg) was reverse transcribed into cDNA using SuperScript™ First-Strand Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Amplification of cDNA was carried out in a total volume of 25μl using the primer sequences of 23S rRNA gene given in Table 3.1 and as described elsewhere (Misyurina et al. 2004).

Table 6.1: PCR oligodeoxynucleotide primers's sequences

S. No.	Gene		5'-3' sequence
1	MOMP (1130bp)	F	ATGAAAAAACTCTTGAAATCG
		R	CTCAACTGTAACTGCGTATTT
2	MOMP (584bp)	F	TGAACCAAGCCTTATGATCGACGGA
		R	TCTTCGACTTTAGGTTTAGATTGA
3	L4	F	GAAGTTTGAATTGCCTGATGC
		R	GGCTTAGGACCGAAAACAATC
4	L22	F	AGCTGCAGGATTGATGAGAAA
		R	GTTAGATGACTCGTGCGCTTC
5	23SrRNA	F	AAGTTCCGACCTGCACGAATGG
		R	TCCATTCCGGTCCTCTCGTAC
6	Efflux gene	F	ACGATCTTTCCGTGCATTGGTCGT
		R	GCCATGTAAGAGCCGACACCCA

Thermal conditions for PCR

The thermal conditions for amplification of *C. trachomatis* major outer membrane protein (MOMP) gene (1130bp) were initial denaturation at 95° C for 5 min followed by 35 cycles of denaturation at 94° C for 60 s, primer annealing at 49° C for 60 s and extension at 72° C for 80s, then a final extension at 72° C for 10 min. For re-amplification of the *C. trachomatis* major outer membrane protein (MOMP) gene (584bp) inner primer pair was selected by taking 3µl of the amplified product of 1130bp MOMP gene. The thermal

conditions were initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 50s, primer annealing at 58°C for 50s and extension at 72° C for 50s, then a final extension at 72° C for 10 min.

The thermal conditions for *C. trachomatis* L4 gene amplification were initial denaturation at 95°C for 10 min followed by 35 cycles of denaturation at 94°C for 30s, primer annealing at 57°C for 40s and extension at 72° C for 1 min, then a final extension at 72° C for 10 min. The thermal conditions for *C. trachomatis* L22 gene were initial denaturation at 95° C for 10 min followed by 35 cycles of denaturation at 94° C for 30 s, primer annealing at 57° C for 45 s and extension at 72° C for 45s, then a final extension at 72° C for 10 min. The thermal conditions for *C. trachomatis* efflux gene amplification were initial denaturation at 95° C for 10 min followed by 35 cycles of denaturation at 94° C for 30 s, primer annealing at 60° C for 1min and extension at 72° C for 2 min, then a final extension at 72° C for 10 min. The thermal conditions for *C. trachomatis* 23S rRNA gene were initial denaturation at 95° C for 10 min followed by 35 cycles of denaturation at 94° C for 30 s, primer annealing at 60° C for 1min and extension at 72° C for 1 min, then a final extension at 72°. The PCR products were visualized by electrophoresis on a 1, 1.5 - 2% agarose gel stained with ethidium bromide on Alpha Imager gel documentation system (AlphaInnotech, San Leandro, USA).

DNA Sequencing

The PCR products were purified using Qiagen gel extraction kit as per manufacturer's instructions. The sequencing of purified PCR products was carried out using BigDye terminator v3.1 (Applied Biosystems, Foster City, CA, USA) as per recommendations. Briefly, 75-150 ng/µl of purified PCR product and sequencing primers (diluted to 1 pmol/µl) were added to 4 µl Big Dye Terminator Reaction mix. Final volume was made up to 10 µl with autoclaved MilliQ water as per the Big Dye Terminator kit instructions. Sequencing PCR was set up with 30 cycles of 30 sec denaturation at 96° C, 30 sec annealing at 55°C and 4 min extension at 60° C. After sequencing PCR, the products were purified by adding 1µl of 125 mM EDTA, 1 µl of 3 M Sodium Actetate (pH 4.6) and 25µl absolute ethanol per tube. The tubes were centrifuged at 4000 rpm for 30 min at room temperature. Two washes were performed with 35 µl of 70% ethanol by centrifugation at 4000 rpm for 10 min and 5 min, respectively. The pellets were air dried and re-suspended in 10 µl of Hi-Di formamide (Applied Biosystems). The samples were denatured at 94° C for 5 min followed by a brief incubation on ice and loaded on the 3130XL Genetic Analyzer (Applied Biosystems). Sequence analysis was carried out using Sequence Analysis software (Applied Biosystems) and SeqMan module of DNASTAR v5.07 software.

Results

Propagation of Clinical C. trachomatis isolate(s)

Clinical isolate (s) of *C. trachomatis* (CT 227, CT 244) which were obtained from the treatment failure/ recurrently infected women and have shown *in vitro* decreased susceptibility towards the first line antichlamydial agents; azithromycin, doxycycline as described in chapter 4 were successfully propagated in cell culture for further experimental work. Isolate CT-235 was also passaged for further experimental work but presented with less number of inclusion bodies and finally it did not survived in cell culture condition.

Amplification and Sequencing of C. trachomatis gene(s)

Total genomic DNA was extracted from *C. trachomatis* infected cells. PCR amplification followed by DNA sequencing was carried out for the propagated clinical isolate(s) of *C. trachomatis.*

Major outer membrane protein (MOMP) gene

For genotype determination of the propagated *C. trachomatis* isolates major outer membrane protein gene was amplified. Primary amplification of 1130 bp fragment encompassing VS1–4 of ompl/A was performed using the outer primer pair, and re-

amplification of a 584 bp fragment spanning VS1–2 was conducted based on the inner primer pair (Table 3.1). From the first PCR step a single band was observed at 1130bp as compared to the 100bp ladder on a 1% agarose gel. For amplification of the 584bp of ompl/A inner fragment, second PCR step was carried out with 3 μl product taken from the first PCR step. The amplified products were observed as a single band on a 2% agarose gel. Purified strands of 584 bp and 1130 bp fragments of the ompl/A segment were sequenced bidirectionally (Figure: 6.1).

The sequences were compared to sequences of known *C. trachomatis* strains by BLAST searching GenBank (www.ncbi.nlm.nih.gov/GenBank). The sequences were assembled into alignments using reference sequences derived from GenBank and analyzed using Sequence Analysis software (Applied Biosystems) and SeqMan module of DNASTAR v5.07 software. It has been found that both of the *C. trachomatis* isolates (CT 227, CT 244) were of serovar D/UW-3/CX.

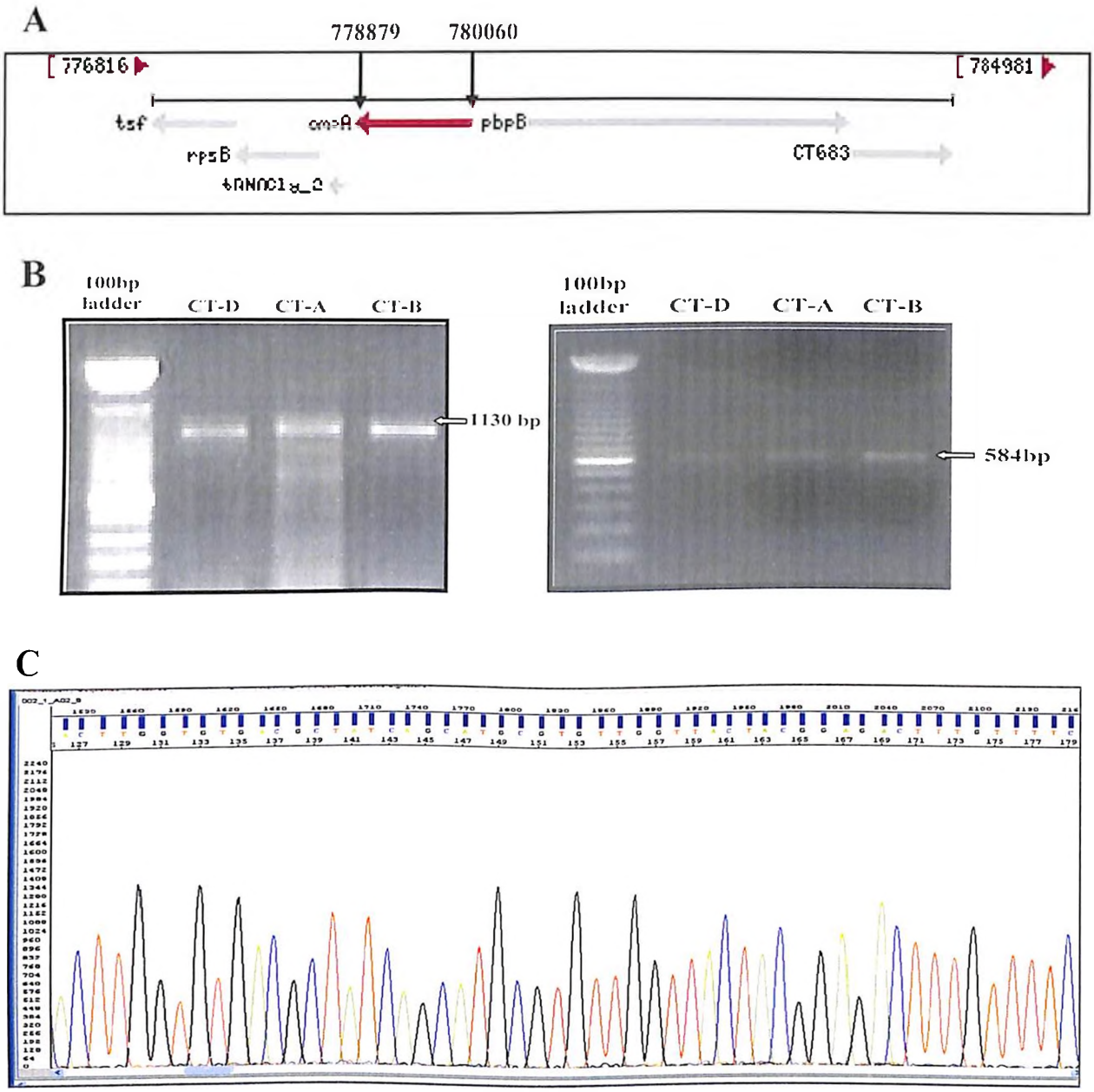
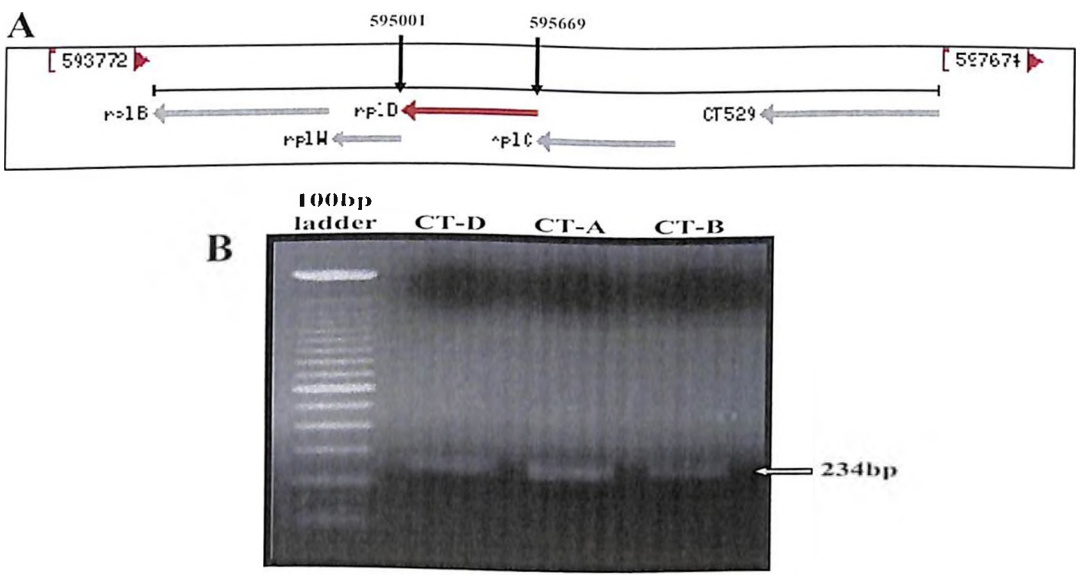


Figure 6.1: (A) Schematic representation of omp1/A gene orientation in *C. trachomatis* genome. (B) Agarose gel electrophoresis of 1130 bp and 584 bp MOMP amplicons. Lane 1 is 100 bp DNA Ladder, CT-D is laboratory standard serovar D, CT-A is CT-227 and CT-B is CT 244. (C) Representative picture showing sequencing of the 1130 bp MOMP.

Ribosomal L4, L22 and 23S rRNA gene

To detect changes in the L4 (rplD) gene sequence that were reported to be associated with macrolide resistance, the 234-bp fragments was amplified using the primers L4-F and L4-R of 669 bp L4 gene and a single band was observed for each isolate in 2% agarose gel. Products were sequenced in both directions and analyzed (Figure: 6.2). The studied region of gene L4 in all of the isolates showed no sequence variations from that of the published GenBank sequence (NC000117.1).



C

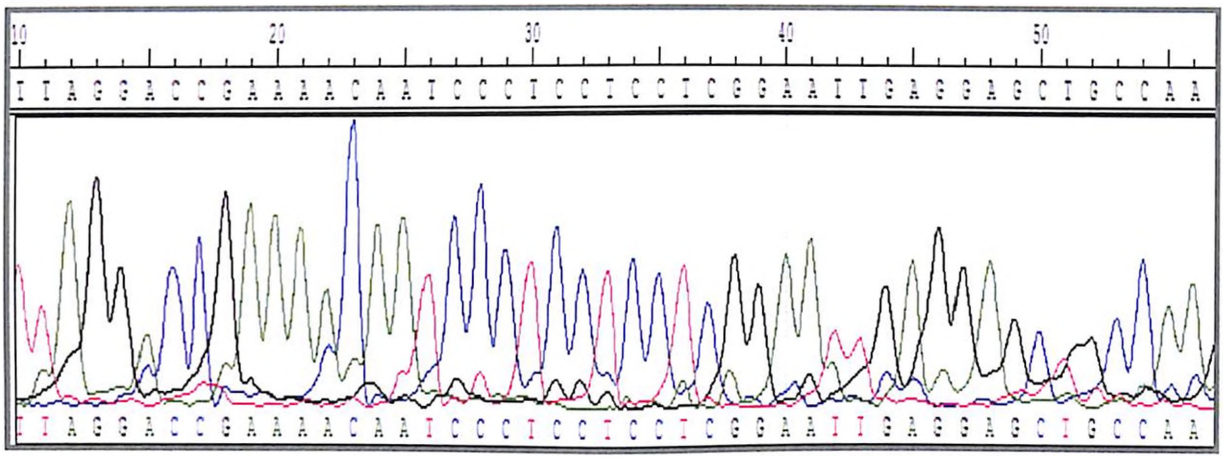


Figure 6.2: (A) Schematic representation of L4 (rplD) gene orientation in *C. trachomatis* genome. (B) 2 % agarose gel electrophoresis of 234 bp L4 amplicon. Lane 1 is 100 bp DNA Ladder, CT-D is laboratory standard serovar D, CT-A is CT-227 and CT-B is CT 244. (C) Representative picture showing sequencing of the 234 bp L4 gene by SeqMan module of DNASTAR v5.07 software.

Ribosomal 50S, L22 (rplV) gene was checked for presence of any mutation(s) in the *C. trachomatis* isolates. A fragment of 255 bp was amplified by L22-F and L22-R primers from total of 336 bp L22 gene and a single band was observed for each isolate in 2% agarose gel. Products were sequenced in both directions and then analyzed (Figure: 6.3). No sequence variations were observed in the studied region of gene L22 in all of the isolates from that of the published GenBank sequence (NC000117.1).

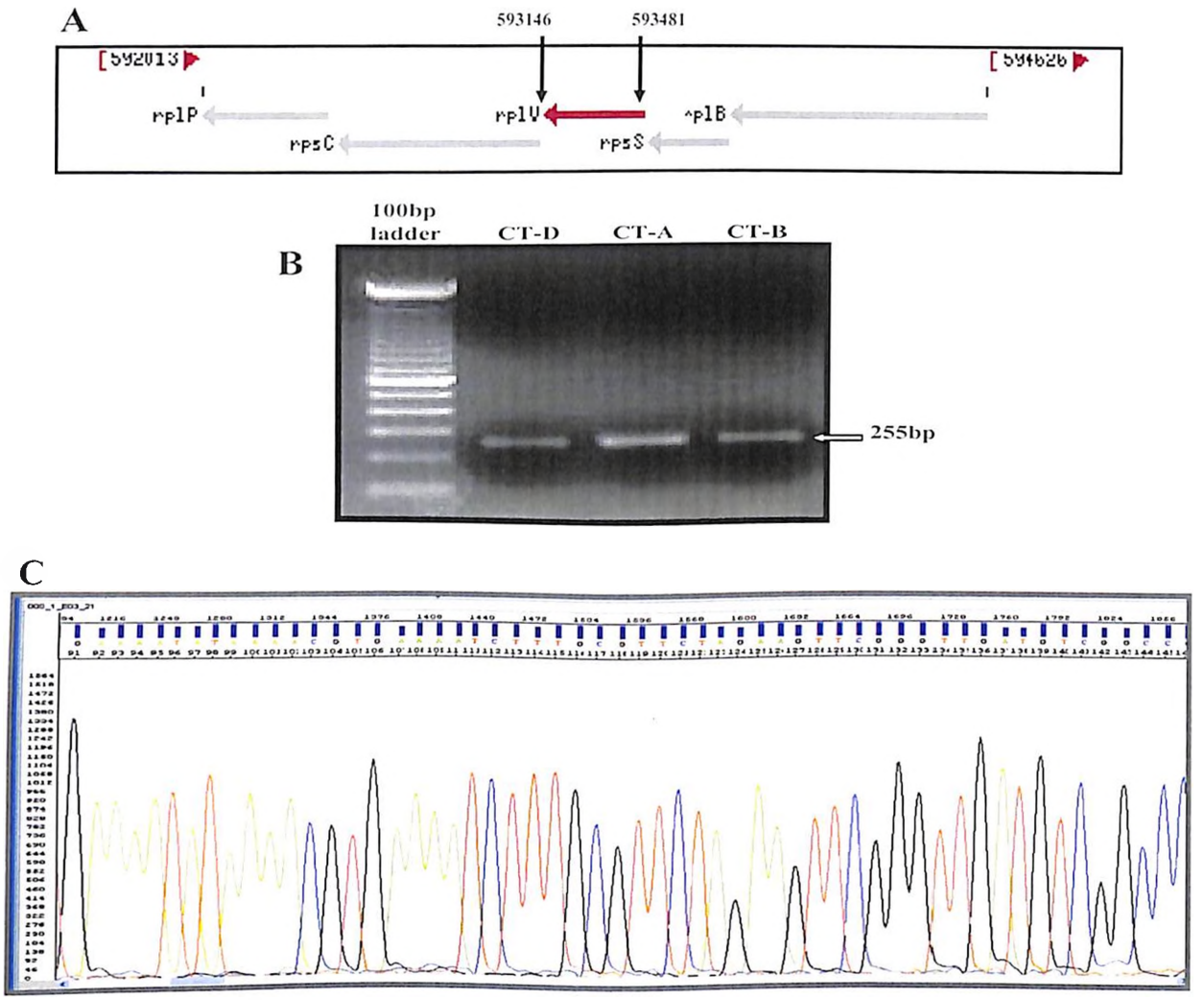


Figure 6.3: (A) Schematic representation of L22 (rplV) gene orientation in *C. trachomatis* genome. (B) 2 % agarose gel electrophoresis of 255 bp L22 amplicon. Lane 1 is 100 bp DNA Ladder, CT-D is laboratory standard serovar D, CT-A is CT-227 and CT-B is CT 244. (C) Representative picture showing sequencing of the 255 bp L22 gene.

To detect changes in the 23S rRNA gene sequences associated with macrolide resistance; total RNA for reverse transcription (RT) reactions was extracted from infected HeLa cells, and then amplified a 725-bp DNA fragment encoding the peptidyl transferase

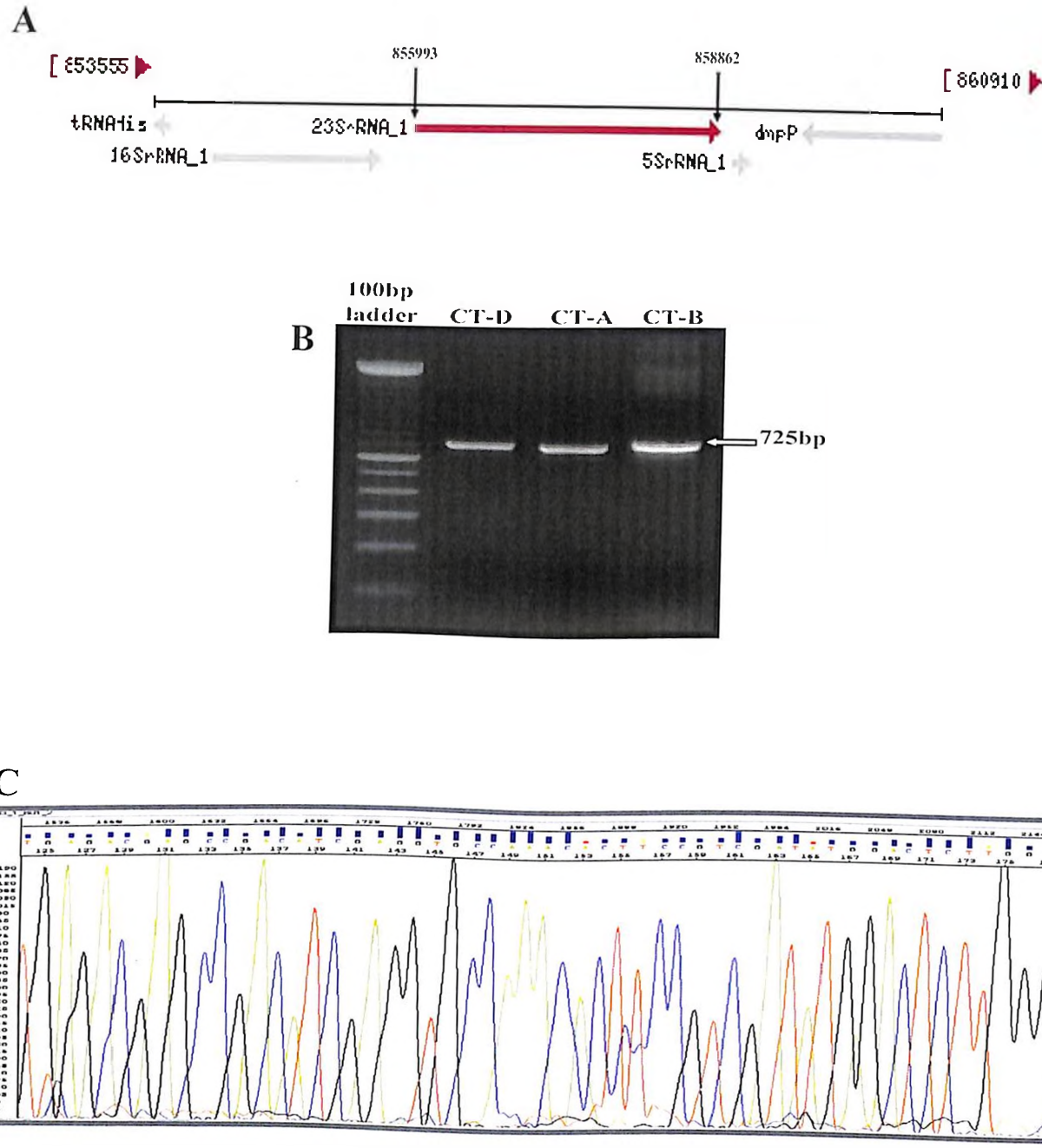


Figure 6.4: (A) Schematic representation of 23S rRNA gene orientation in *C. trachomatis* genome. (B) 1.5% agarose gel electrophoresis of 725 bp 23S rRNA gene amplicon. Lane 1 is 100 bp DNA Ladder, CT-D is laboratory standard serovar D, CT-A is CT-227 and CT-B is CT 244. (C) Representative picture showing sequencing of the 725 bp 23S rRNA gene.

loop of the 23S rRNA gene with the rr primers (Misyurina et al. 2004). The amplified products were observed as a single band on 1.5% agarose gel and sequenced in both directions. Sequence results were reviewed and compared with the blast program and also with the reference *C. trachomatis* serotype D (GenBank accession numbers NC000117) sequences deposited in the GenBank (Figure: 6.4). No alteration(s)/ mutation in nucleotide sequences were found in the studied clinical isolate of *C. trachomatis*.

C. trachomatis ygeD gene

C. trachomatis ygeD gene was checked for any changes in the genetic level. A fragment of 822 bp was amplified and a single band was observed in 1.5% agarose gel (Figure: 6.5 A, B). Products were sequenced in both the directions and reviewed by assembling into alignments using reference sequence *C. trachomatis* serotype D (GenBank accession numbers NC000117) and analyzed using Sequence Analysis software (Applied Biosystems) and SeqMan module of DNASTAR v5.07 software. In one of the studied *C. trachomatis* clinical isolate (CT-244) an alteration in a nucleotide at sequence position 734318 (T to G) was found (Figure: 6.6 and 6.7).

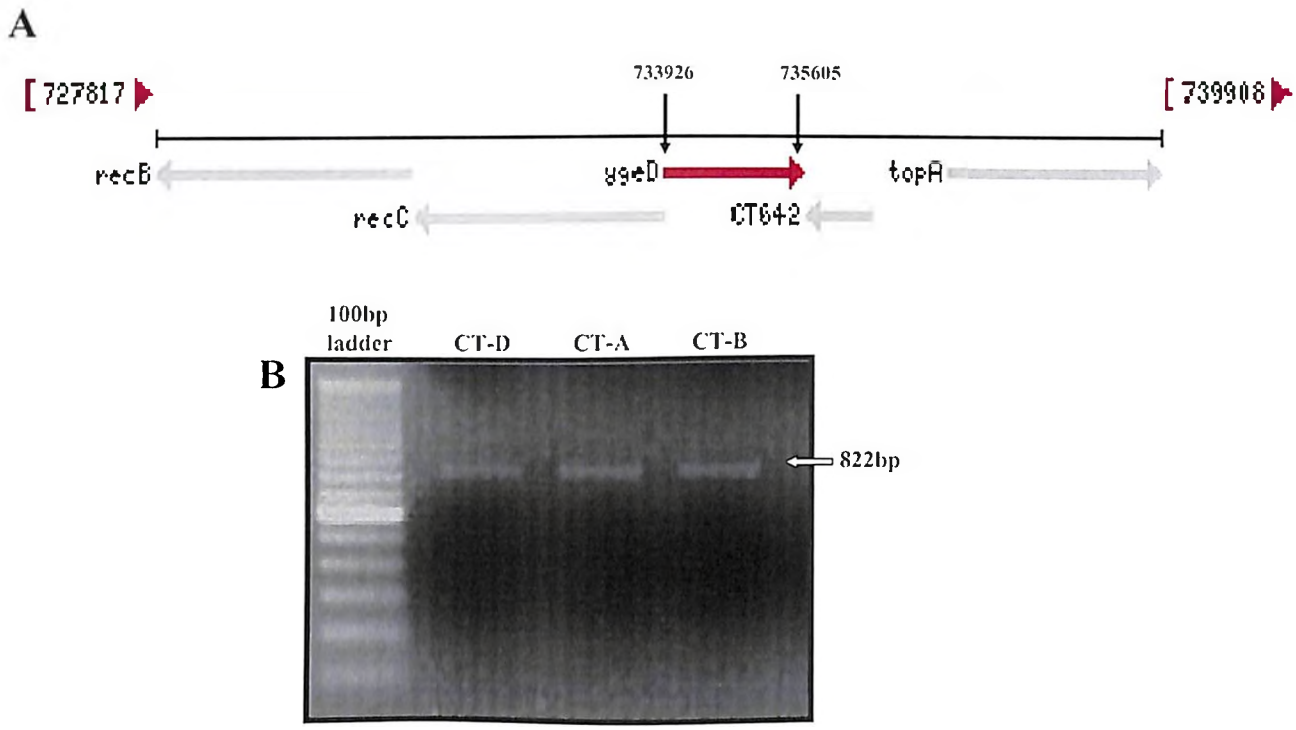


Figure 6.5: (A) Schematic representation of ygeD gene orientation in *C. trachomatis* genome. (B) 1.5 % agarose gel electrophoresis of 822 bp ygeD gene amplicon. Lane 1 is 100 bp DNA Ladder, CT-D is laboratory standard serovar D, CT-A is CT-227 and CT-B is CT 244.

In addition, *C. trachomatis* isolates were also checked for presence of insertion elements (Tet C) by using primer sequences as described previously [Lenart, 2001 #86]. No Tet C gene product was identified from these *C. trachomatis* isolates. Thus, no mechanism related to Tet gene(s) was found to account for the shift in the MIC for these isolates.

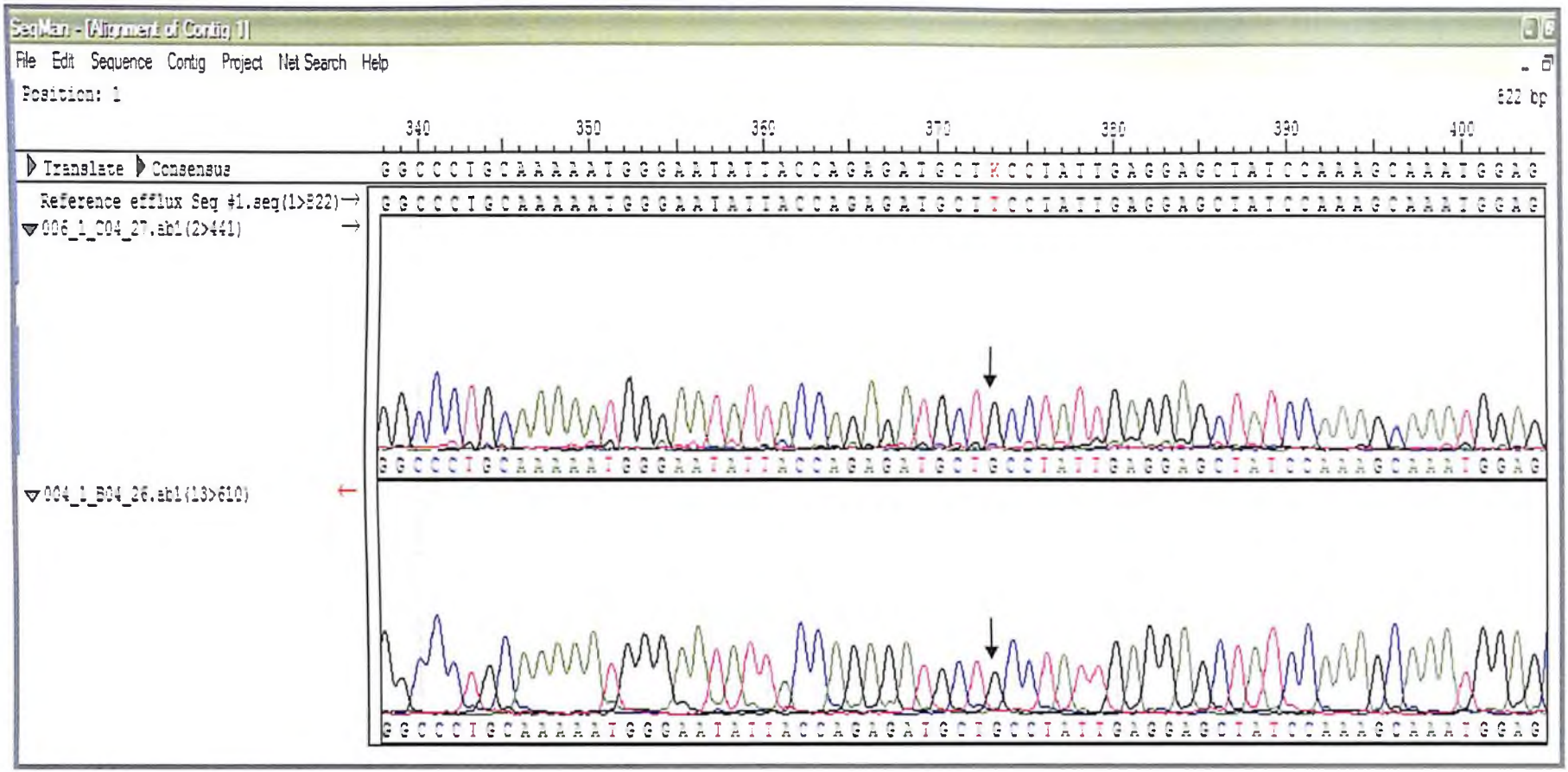


Figure 6.6: Representative picture showing sequencing of the 822 bp of *ygeD* efflux gene of *C. trachomatis* clinical isolate (CT-244) with T to G alteration in a chromosomal position 734318.

```
>gb|AE001273.1|  Chlamydia trachomatis D/UW-3/CX, complete genome Length=1042519
 Features in this part of subject sequence:
   hypothetical protein
   predicted Efflux Protein
 Score = 1513 bits (819),  Expect = 0.0
 Identities = 821/822 (99%), Gaps = 0/822 (0%)
 Strand=Plus/Plus

Query  1       ACGATCTTTCCGTGCATTGGTCGTTACACATTTTCTTACAATTATAAATGACAATCTCTA  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  733946  ACGATCTTTCCGTGCATTGGTCGTTACACATTTTCTTACAATTATAAATGACAATCTCTA  734005

Query  61      TAAGTTTCTTTTAGTTTTTTCCTTGTTAGAGGGAAAAACCTTAGAAGAAAATGCTAAAAT  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  734006  TAAGTTTCTTTTAGTTTTTTCCTTGTTAGAGGGAAAAACCTTAGAAGAAAATGCTAAAAT  734065

Query  121     TCTGTCTTTAGTTAGTTTCTTTTTTGCTCTCCCGTACATTTTGTTGGCTCCCTTTTCAGG  180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  734066  TCTGTCTTTAGTTAGTTTCTTTTTTGCTCTCCCGTACATTTTGTTGGCTCCCTTTTCAGG  734125

Query  181     TAGTTTGGCAGATAGGTTTCAAAAGAGAAATATTATTTTATTCACTCGGGTCATCGAAAT  240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  734126  TAGTTTGGCAGATAGGTTTCAAAAGAGAAATATTATTTTATTCACTCGGGTCATCGAAAT  734185

Query  241     CTTTTGTGCTATTCTAGGAGTATATTTCTTCCACATCCATTCCGTTGTAGGAGGGTATTT  300
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  734186  CTTTTGTGCTATTCTAGGAGTATATTTCTTCCACATCCATTCCGTTGTAGGAGGGTATTT  734245

Query  301     TGTCTTAGTATTGATGGCTTGCCATTCTGCTATTTTCGGCCCTGCAAAAATGGGAATATT  360
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  734246  TGTCTTAGTATTGATGGCTTGCCATTCTGCTATTTTCGGCCCTGCAAAAATGGGAATATT  734305

Query  361     ACCAGAGATGCTGCCTATTGAGGAGCTATCCAAAGCAAATGGAGCAATGACAGCAGCTAC  420
               |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  734306  ACCAGAGATGCTTCCTATTGAGGAGCTATCCAAAGCAAATGGAGCAATGACAGCAGCTAC  734365

Query  421     CTATTCTGGCAGTATTTTAGGGTCTTGTCTAGCTCCTCTTATGGTGGATTTAACGAAAGA  480
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  734366  CTATTCTGGCAGTATTTTAGGGTCTTGTCTAGCTCCTCTTATGGTGGATTTAACGAAAGA  734425

Query  481     TTTTGTCACAAATAGTTACGAGTTATCGGCCTGTTTCTGTGTAGTCTCTTCTGTTTTAAG  540
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  734426  TTTTGTCACAAATAGTTACGAGTTATCGGCCTGTTTCTGTGTAGTCTCTTCTGTTTTAAG  734485

Query  541     TTTATTTATTGCGTTAGGCATTCGTGCAAGTAATGTAAAAAATAAAGGACAGAAAATTGC  600
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  734486  TTTATTTATTGCGTTAGGCATTCGTGCAAGTAATGTAAAAAATAAAGGACAGAAAATTGC  734545

Query  601     TTATGTAAGCTTTAGAAATCTTTGGAAGGTCTTTCAAGAGACGCGTAATATTGCTTATCT  660
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  734546  TTATGTAAGCTTTAGAAATCTTTGGAAGGTCTTTCAAGAGACGCGTAATATTGCTTATCT  734605

Query  661     GATGATTTCCGTATTCCTTGTTGCCTTCTTTCTTTTTGTTGGGGCCTACGTCCAATTACA  720
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Sbjct  734606  GATGATTTCCGTATTCCTTGTTGCCTTCTTTCTTTTTGTTGGGGCCTACGTCCAATTACA  734665

Query  721     AATCATTCCTTTTGTAGAATTTACTTTAGGATACTCCAAACATTATGGAGCGTATCTATT  780
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Sbjct  734666  AATCATTCCTTTTGTAGAATTTACTTTAGGATACTCCAAACATTATGGAGCGTATCTATT  734725

Query  781     TCCTATTGTAGCAGTAGGTATGGGTGTCGGCTCTTACATGGC  822
               |||||||||||||||||||||||||||||||||||||||||
Sbjct  734726  TCCTATTGTAGCAGTAGGTATGGGTGTCGGCTCTTACATGGC  734767
```

Figure 6.7: Picture showing alignment of *C. trachomatis* isolate (CT-244) ygeD predicted efflux gene with reference sequence in NCBI genbank.

Discussion

As *C. trachomatis* plays an important role in human disease, the development of effective antimicrobial agents for use in the treatment of *C. trachomatis* infection is very important (Binet, 2005; Byrne, 2001). Further the determination of their efficacy in treatment of chlamydial infection is an important area of concern. Clinical isolates showing resistance to antichlamydial drugs and associated with a recurrent infection have been described previously (Stamm, 2001; Wang, 2005). However, understanding of both, the clinical significance and the causes of *C. trachomatis* treatment failures are still not known hence it is crucial as research priorities for treatment and prevention strategies for chlamydial infections. In particular, the potential for *C. trachomatis* to develop antimicrobial resistance has not been well studied, despite some published case reports (Somani, 2000; Samra, 2001; Rice, 1995; Jones, 1990) that suggest resistance as a cause for clinical treatment failures. There have been no descriptions either of isolation of *C. trachomatis* strains that display stable resistance to antimicrobial agents recommended for therapy or of mechanisms of putative antimicrobial resistance for isolates obtained from patients with treatment failures. However, studies suggests that chlamydiae can evolve *in vitro* resistance to antibiotic stressors through the accumulation of point mutations, and these resistance properties can be circulated among strains via horizontal gene transfer and homologous recombination (DeMars, 2008; Demars,

2007; Gomes, 2004; Suchland, 2009). Despite this ability to evolve in the laboratory, stable genetic antibiotic resistance in clinical settings has yet to be documented. Results of one study of isolates from women with recurrent *C. trachomatis* infection suggested that treatment failures were more common in women infected with heterotypic resistant strains, as determined in McCoy cells infected with 5000–20,000 IFUs/culture well, than in women infected with susceptible isolates (Wang, 2005; Whittington, 2001). The term "heterotypic resistance" refers to the replication of a heterogeneous population of resistant and susceptible bacteria from a subculture of a single resistant organism propagated on antimicrobial-containing medium. In contrast, "homotypic resistance" refers to replication of a homogeneous, clonal population of resistant organisms from a subculture of a single resistant organism.

In the previous study (Chapter 4) of clinical isolates of *C. trachomatis* in which minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) were determined against first line antichlamydial drugs (azithromycin and doxycycline) showed decreased susceptibility pattern. Majority of the isolates having decreased susceptibility profile were obtained from recurrently infected women. Two (CT 227 and CT 244) of the isolates of serovar D as determined by amplification of their MOMP genes, presented resistance in *invitro* condition in the given concentrations of antichlamydial drugs.

The observed resistance was heterotypic; as the inclusions observed were small and their number in the presence of antibiotics was much lower (Chapter 4) as also described by Suchland et al., 2003. Resistant isolates could be visually distinguished from sensitive ones, which showed no inclusions at the drug concentrations defined as the MIC.

Further, in the study these clinical *C. trachomatis* isolates were investigated for presence of any genetic evidence for antichlamydial drug resistance. Hence, ribosomal rplD (encoding L4 protein), rplV (encoding L22 protein), 23S rRNA protein genes and ygeD an efflux gene were selected for the study. 50S ribosomal protein L4 is important during the early stages of 50S assembly; it initially binds near the 5' end of the 23S rRNA and L22 is a core protein of the large ribosomal subunit. It is the only ribosomal protein that interacts with all six domains of 23S rRNA, and is one of the proteins important for directing the proper folding and stabilizing the conformation of 23S rRNA. L22 is the largest protein contributor to the surface of the polypeptide exit channel, the tunnel through which the polypeptide product passes (Davydova, 2002; Unge, 1998). Mutations in these genes have been suggested to confer macrolide resistance in *C. trachomatis (*Canu, 2002). PCR amplification and DNA sequencing were used to determine whether heterotypic resistance to antichlamydial drugs in theses isolates was due to mutation(s) in the rplD (encoding L4 protein), rplV (encoding L22 protein), 23S rRNA gene or in efflux gene. However, no genetic alterations (mutation) were

found in the studied gene(s) (L4, L22 and 23S rRNA) of the two heterotypic resistant *C. trachomatis* isolates. The sequenced gene(s) were showing no sequence variation with respect to the reference *C. trachomatis* gene sequences as given in the NCBI genbank (Accession number NC000117).

Solomon et al has also reported antimicrobial drug susceptibility in 9 chlamydial isolates 2 months after mass antimicrobial drug treatment in Tanzania (Solomon, 2005; Hong, 2009). They have also observed a slight increase in the MIC after treatment but found no resistant strains. However, in Nepal, azithromycin-resistant pneumococcal strains were observed only after consecutive annual treatments (Fry, 2002). Similar findings were also observed in 2006, in ocular *C. trachomatis* 18 months after 4 biannual treatments (2003–2004) in a trachoma-endemic region of (Lakew, 2009; Hong, 2009). This suggests that antimicrobial drug resistant *C. trachomatis* might emerge if multiple treatments have occurred. Hence, patients with recurrent *C. trachomatis* infections or multiple treatment failures could be suspected as infected with resistant chlamydia isolate(s). In relation to the genotypic characteristics of the resistant *C. trachomatis* isolates previous studies showed quadruple mutation: G274A, C276T, C339T, C466G in the L4 gene of 13 macrolide-resistant *C. trachomatis* mutants. But the mutations reside in a non-conserved region of the L4 protein. Such mutations have not been found in other macrolide-resistant bacteria (Tait-Kamradt,

2000; Vester, 2001; Zhu, 2010). Mutations in 23S rRNA conferring erythromycin resistance have been described for clinical strains of Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium chelonae, Mycobacterium abscessus, Brachyspira (Serpulina) hyodysenteriae, Helicobacter pylori and Proprionibacterium spp. (Vester & Douthwaite, 2001). The mutations A2058G, 2611C in the peptidyl transferase region of the 23S rRNA gene in macrolide resistant *C. trachomatis* were introduced for the first time by (Misyurina et al., 2004). The positions of 2057, 2058, 2059 and 2611 (E. coli numbering, which correspond respectively to 2037, 2038, 2039 and 2592 in the homologous *C. trachomatis* gene sequences, are significant for developing drug resistance to macrolides. Mutations at A2058 or at A2059 for certain macrolides confer the highest levels of resistance (Vester & Douthwaite, 2001). In addition, other studies also suggest that a clinical *C. trachomatis* isolate can contain organisms differing in their levels of susceptibility to antibiotics, some of them resistant to macrolides and carrying mutations in the 23S rRNA gene and others sensitive to macrolides and lacking these mutations. It seems that the viability of bacteria carrying the mutations in both alleles of the 23S rRNA gene is low and the phenomenon of reduced viability of bacteria resulting from acquisition of mutations that lead to drug resistance is well known.

Further to explore if the phenotypically resistant characteristics of the studied *C. trachomatis* clinical isolates is due to efflux protein, *C. trachomatis* ygeD protein gene was

selected. The predicted efflux ygeD is a member of the major facilitator superfamily of membrane transporters that use the transmembrane proton gradient (Putman, 2000; Riska, 2004) and most homologous to acyltransferases. The functions, natural substrates, and potential for macrolide efflux of the proteins YgeD are unknown, but few studies predict that it has some role in the resistance. Hence, the ygeD gene of *C. trachomatis* isolates were amplified and sequenced in both the directions and compared with the reference sequence in the NCBI genbank (Accession number NC000117). The sequence showed 99% identities with a point mutation T (purine) to G (pyrimidine) in 734318 position of *C. trachomatis* genome. There is no difference in the products of the mutated nucleotide with respect to the reference sequence of *C. trachomatis*. As reference sequence has CTT – Leucine amino acid and mutated nucleotide has CTG which also code for the same amino acid, leucine. Hence, this can be concluded that the mutation may not be directly related to the resistant characteristic of the bacteria but it might be possible that it has some indirect role in modifying the life cycle, which may make bacteria more refractory to the drugs.

In summary, no genetic mechanism was identified in the ribosomal L4, L22 and 23S rRNA genes for the phenotypic change in the *C. trachomatis* isolates. However a point mutation has been detected in the *C. trachomatis* predicted efflux ygeD gene, suggesting that it may play a role in resistant characteristics of a bacteria. Chlamydiae are obligate

intracellular bacteria that can multiply only in the cytoplasm of a susceptible host cell, and acquisition of antimicrobial drug resistance genes from other organisms through horizontal transfer is probably rare. Arguments used to explain heterotypic resistance might also be applicable to the studied isolates. Specifically, within the complex life cycle of the chlamydiae, there are metabolically inert forms such as the elementary body that are likely to be unaffected by antibiotics, as well as intermediate and aberrant forms that may be preferentially induced under antibiotic pressure which may results in treatment failures. Hence, emergence of antimicrobial drug resistance to chlamydia treatments would be detrimental to the goal of eliminating chlamydial infection, so surveillance for resistance should be a part of the treatment programs. The application of these findings in characterizing resistance in clinical isolates against antichlamydial drugs will enhance our understanding of the nature and transmission of resistant *C. trachomatis*.

Chapter 7

Characterization of the heterotypic resistant Chlamydia trachomatis clinical isolate towards antichlamydial drugs at phenotypic level

Characterization of the heterotypic resistant Chlamydia trachomatis clinical isolate towards antichlamydial drugs at phenotypic level

Introduction

Chlamydia trachomatis is an obligatory intracellular bacterial pathogen that causes a spectrum of clinically important chronic inflammatory diseases of human. (Belland et al. 2003b). It infects the genital and ocular mucosa causing sexually transmitted disease and trachoma respectively. In females, *C. trachomatis* is a common cause of cervicitis, urethritis, with sequelae including ectopic pregnancy, pelvic inflammatory disease, tubal factor infertility and chronic pelvic pain. In males, *C. trachomatis* infections can lead to urethritis, epididymitis and Orchitis and it may also contribute to male infertility by directly damaging the sperm (Amirshahi et al.; Cunningham and Beagley 2008). Recurrent genital *C. trachomatis* infections reported often results from failure of antibiotic therapy or from reinfection due to unprotected sexual contact with either an untreated existing partner or a new infected Partner (Hillis et al. 1994; Hillis et al. 1997). However, *C. trachomatis* atypical intracellular characteristics as persistent bodies are suggested to have a role resulting in refractory to antichlamydial drugs and recurrent infections (Dreses-Werringloer et al. 2000;

Beatty et al. 1994; Wyrick). Further, the increasing antibiotic resistance among pathogens creates severe problems in the clinical treatments of bacterial diseases. Drug resistance may arise due to the effects of three different mechanisms: modifications of drug target sites, chemical modifications of drugs and increased drug efflux from the bacterial cells (Poole 2005; Dugan et al. 2004). Few studies with clinical isolates of *C. trachomatis* from treatment failure patients demonstrated *in vitro* heterotypic resistance (Wang et al. 2005; Sandoz and Rockey). These isolates were characterized at genotypic level for presence of any mutational changes at the target site. In the previous study (chapter 4) of recurrent infections and treatment failures few clinical isolates of *C. trachomatis* have also demonstrated *in vitro* heterotypic resistance against first line antichlamydial drugs (azithromycin and doxycycline). Genotypic characterization did not revealed any mutational changes at the drug target site(s); however a point mutation was observed in a predicted efflux gene of resistant *C. trachomatis* isolate (chapter 6).

Further, it has also been suggested that genotypic changes may not be only responsible for the resistant characteristics of clinical *C. trachomatis* isolate(s) obtained from multiple treatment failure patients. Different drugs have different targets for their action in bacteria hence; mutation in a single gene may not be suggested to result in multiple treatment failures. It has been reported that in Gram-negative pathogens, efflux is the predominant

mechanism of tetracycline resistance in several organisms including *Chlamydia suis* [Poole 2005, Dugan 2004]. Hence, studies are needed to explore the role of efflux gene(s) in emerging resistance in *C. trachomatis*.

In addition, in the presence of stress conditions host cell might play a role in altered drug sensitivity profile of bacteria. Resistant bacteria may act on various system of a cell directly or indirectly for its survival in the presence of drugs (Hasdemir 2007). According to many studies *C. trachomatis* changes host cell plasma membrane and actin organization, by modifying its arrangements to complete its life cycle (Kumar and Valdivia 2008a, 2008b; Scidmore 2008). Hence we also checked for plasma membrane and actin organization of host cells in the presence of heterotypic resistant bacteria with antichlamydial drugs to know if it has a role in altered drug susceptibility characteristics.

Thus the aim of this study is to detect the expression of efflux gene (ygeD) in *C. trachomatis* heterotypic resistant isolate in cell culture condition in the presence of antichlamydial drugs (azithromycin and doxycycline). In addition to that host HeLa cell plasma membrane and actin was also studied to know if *C. trachomatis* indirectly affects on it in the presence of antichlamydial drugs to complete its life cycle, which may result *in vitro* altered drug susceptibility characteristics.

Materials

Unless otherwise noted, all fine chemicals used in the study were purchased from Sigma Aldrich (St. Louis, USA). Eagle's Minimum Essential Medium (EMEM) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from HiMedia; Mumbai, India. Fetal bovine serum (FBS) was from PAA Laboratories GmbH; Colbe, Germany. Plasticware and glassware used in cell culture was purchased from Greiner, Germany. Trizol, DNase I, DNA ladder was from Invitrogen, USA. Primers were synthesized by MWG-Biotech, Germany. The antimicrobial agents tested in this study were azithromycin and doxycycline purchased from Sigma - Aldrich, (St. Louis, Missouri, USA). Azithromycin and doxycycline were supplied as powder form and solubilized according to the manufacturer's instructions.

Experimental methods

HeLa cell culture and Propagation of C. trachomatis clinical isolate

The human cervical epithelial adenocarcinoma cell line HeLa 229 was procured from National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained and cultured in Eagle's Minimum Essential Medium (EMEM; HiMedia, India) as described in chapter 4. *C. trachomatis* isolate (CT-244) was propagated as described earlier (chapter 4)

in DEAE- dextran (30 μg/ml) treated HeLa 229 cells, purified and stored at -80°C in sucrose phosphate glutamate medium (SPG, pH 7.0). Infectious titers were determined by titration on HeLa 229 cell monolayers by staining with fluorescein isothiocyanate (FITC) labeled monoclonal antibody against chlamydial major outer membrane protein (MOMP; MicroTrack, San Jose, CA) and are expressed as inclusion forming units (IFU). Immediately prior to use, the purified organisms were thawed and diluted in SPG.

Antimicrobial Agents

Azithromycin and doxycycline (Sigma-Aldrich) were dissolved according to the instructions of the manufacturer. Two-fold serial dilutions were prepared before each testing in DMEM cell culture medium without antibiotics.

RNA isolation and real-time RT-PCR analysis

Fresh monolayers of HeLa 229 cells were prepared by seeding (3×10^5 cells/well) in six-well tissue culture plates and infected with chlamydial inoculum at a MoI of 2 as described in chapter 4. Dilutions of drugs (0.5, 5 and 10μg/ml) were added at 2 hour post infection (hpi) and incubated at 35°C with 5% CO_2. Total RNA was isolated at various time points (8, 24 and 48 hpi) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according

to the manufacturer's instructions and quantified using a UV-VIS spectrophotometer. RNA was treated with DNase I to prevent DNA carryover. The isolated RNA was tested by PCR to check any carryover DNA contamination. No amplification of product was detected and the RNA was considered as DNA free. Complementary DNA (cDNA) was prepared using SuperScript™ First-Strand Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Real-time PCR was performed with the DyNAmo™ SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland). The primer sequences used for efflux (ygeD) gene were F 5'ACGATCTTTCCGTGCATTGGTCGT3 and R 5' GCCATGTAAGAGCCGACACCCA3'. Sequence used for endogenous control (16S rRNA) gene are 5'CTGCAGCCTCCGTAGAGTCTGGGCAGTGTC3' and 5'TTCAGATTGAA CGCTGGCGGCGTGGATG 3' and as described earlier (Mpiga and Ravaoarinoro 2006). All primers were of HPLC-purified grade and were commercially synthesized (MWG-Biotech AG, Ebersberg, Germany). The negative control consisted of nuclease free water substituted for cDNA. PCR amplification was performed in an Applied Biosystems 7000 Real-Time PCR System (Applied Biosystems, CA, USA). For data analysis, the $2^{-\Delta\Delta Ct}$ method was used to calculate fold change (Livak and Schmittgen 2001).

Transduction of HeLa cell

For targeting host cell actin and plasma membrane proteins HeLa cells were transduced with Cellular-Lights and Organelle Lights transduction reagents (Molecular probes, Invitrogen, Carlsbad, CA, USA) respectively. The transduction was based on the BacMam technology of viral delivery for specific expression of a targeted (fluorescent) protein in mammalian cell.

Transduction was carried out according to the manufacturer's instructions. Hela cells ~1 × 10^6 to 4 × 10^6 were seeded in a 50cm^2 tissue culture Flask (Greiner, Germany). Cells were allowed to adhere and grow for approximately 24 hours at 37°C, 5% CO_2. BacMam enhancer solution were prepared by reconstituting the enhancer in 25 µL DMSO for a 1,000X solution. Cellular Lights transduction solution were prepared in Dulbecco's Phosphate Buffered Saline (D-PBS) without Ca_2+ or Mg_2+ by combining 2.0 mL of Cellular Lights reagent with 3.5 mL of D-PBS. Upon reaching of 70–80% confluency of the adhered cells, culture medium was aspirated and 5.5 mL of the diluted transduction solution were added. The cells were incubated at room temperature (20–25°C) in the dark for 4 hours with gentle rocking. Cellular Lights transduction solution from the cell culture flask were again aspirated and cell culture medium without serum plus 1X enhancer (10 µL of enhancer per 10 mL media) were added. Cells were incubated for 2 hours at 37°C and 5% CO_2. After

incubation enhancer solution from the cell culture flask were replaced with the appropriate cell culture medium and incubated the cells at 37°C, 5% CO_2 for >16 hours. For transduction of HeLa cells with organelle lights same methods were followed as of cellular lights. Myristoylation/palmitoylation sequence from Lck tyrosine kinase was targeted in host cell plasma membrane.

Transduced HeLa 229 cells were plated in 6 well tissue culture plates with cell density of 3×10^5 cells/well in EMEM containing 10% fetal calf serum (FCS). On reaching the sub-confluence, cells monolayer were washed twice with PBS and infected with chlamydial EBs at multiplicity of infection (MoI) of 2. For homogenous infection tissue culture plates were placed on a rocker for 2 hour at 35°C after addition of serum free media containing EBs. Media containing unbound EBs were aspirated and supplemented with complete Dulbecco's modified eagle's medium (DMEM) containing 10% FCS. Infected HeLa 229 cells were incubated at 35°C with 5% CO_2 in a humid environment. Thereafter at 2 hours post infection (hpi) media was aspirated and replaced with fresh media containing dilutions of azithromycin or doxycycline. After 48 hpi cells were analysed for fluorescence using flow cytometer (BD FACS Caliber) in FL-1 and FL-2 channel. For negating auto-fluorescence same pool of untransduced cells were used and appropriate setting was used

for further acquisition and analysis. Every experiment was done in triplicate. Flow histogram was analyzed for geometric mean using FCS V3 express (DeNovo Inc).

Statistical analysis

Differences between two groups were evaluated using Student t test and $p < 0.05$ was considered as significant.

Results

Real-time RT-PCR analysis

C. trachomatis isolate (CT-244) efflux ygeD gene was studied for any changes in gene expression in the presence of antichlamydial drugs (doxycycline and azithromycin) in host HeLa 229 cells.

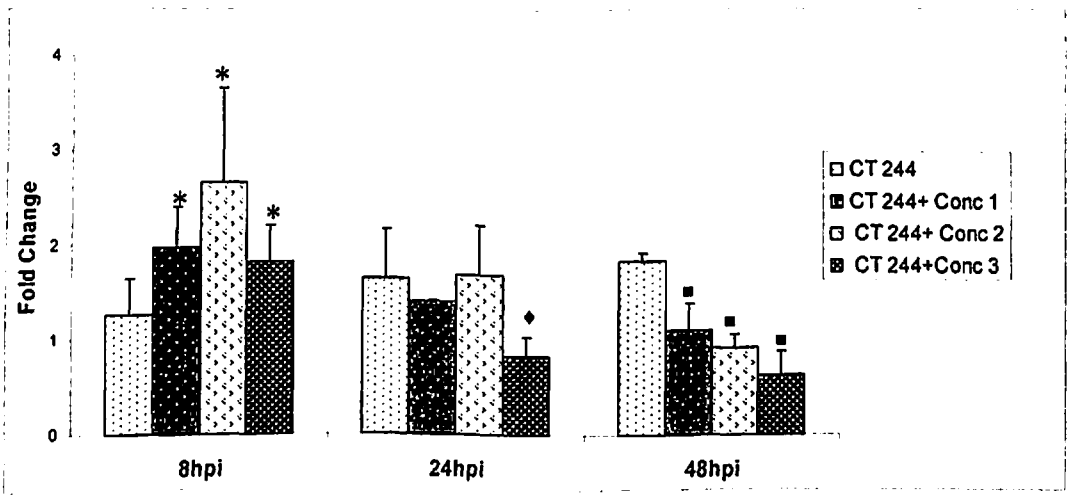


Figure 7.1: Expression of *C. trachomatis* isolate (CT-244) efflux ygeD gene in HeLa 229 cells in the presence of doxycycline at 8hpi, 24hpi and 48hpi. The graph show results as fold change represented by bars. Bars represent mean ±SEM for all experiments. * represents $p < 0.05$ as compared to "CT 244" at 8 hpi, ♦ represents $p < 0.05$ as compared to "CT 244" at 24 hpi and ▪ represents $p < 0.05$ as compared to "CT 244" at 48 hpi. Conc 1, 2 and 3 represents 0.5, 5 and 10μg/ml of doxycycline respectively. hpi = hour post infection.

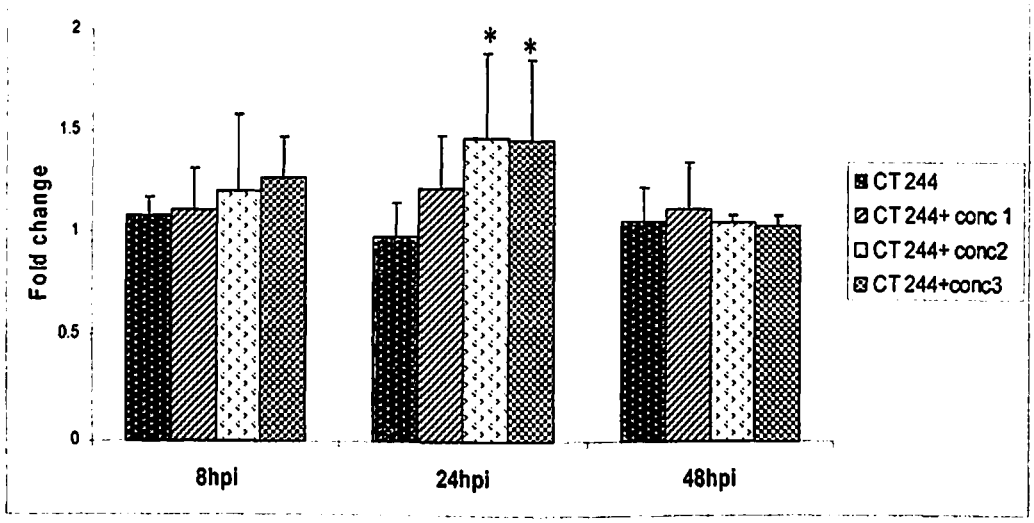
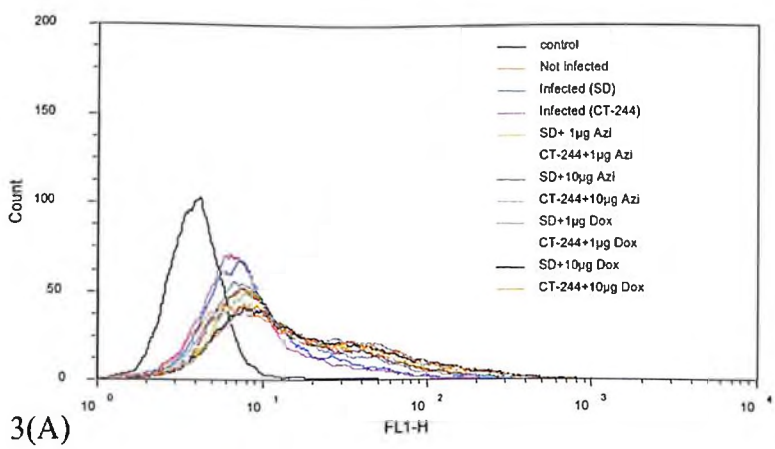


Figure 7.2: Expression of *C. trachomatis* isolate (CT-244) efflux ygeD gene in HeLa 229 cells in the presence of azithromycin at 8hpi, 24hpi and 48hpi. The graph show results as fold change represented by bars. Bars represent mean ±SEM for all experiments. * represents $p < 0.05$ as compared to "CT-244" at 24 hpi. Conc 1, 2 and 3 represents 0.5, 5 and 10μg/ml of azithromycin respectively. hpi = hour post infection.

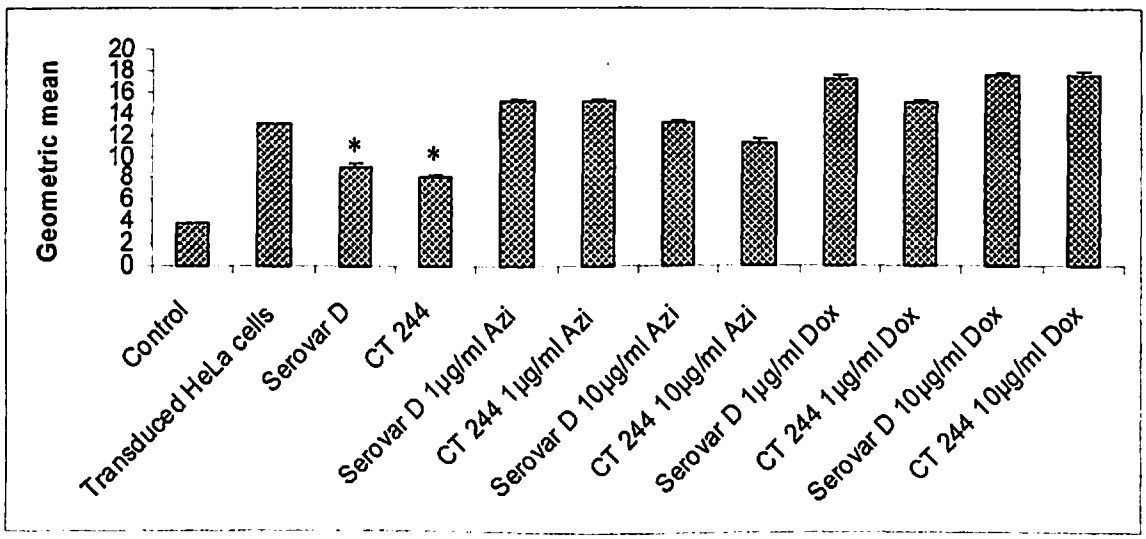
Increased expression of ygeD gene was detected with respect to the 8, 24 and 48 hpi in the absence of doxycycline. However, in the presence of doxycycline significantly increased expression was observed only at 8 hpi while at 24 and 48 hpi it was found to be decreased in presence of all three concentrations of doxycycline used (Figure 7.1). On addition of azithromycin there is no significant changes detected at 8 and 48 hpi with all the three concentrations of drug. However, at 24 hpi expression of ygeD gene was observed to be significantly increased (Figure 7.2).

Host cell analysis

Host HeLa 229 cells harbouring heterotypic resistant *C. trachomatis* isolate (CT-244) were studied for any changes in plasma membrane proteins in the presence of antichlamydial drugs by transducing with Organelle Lights transduction reagents. Upon infection with serovar D and CT-244 isolate expression of green fluorescent protein (GFP) - tagged plasma membrane protein in host HeLa cells were found to be significantly reduced as compare to the uninfected transduced HeLa cells. On addition of drugs expression was found to be up-regulated in comparison to the absence of drugs and comparable to the uninfected cells. Further there is no difference in expression was observed in between the serovar D and CT-244 isolate in the absence of antichlamydial drugs. However non significant difference was observed in the protein expression at higher concentration of azithromycin and lower concentration of doxycycline used (Figure 7.3).



3(A)

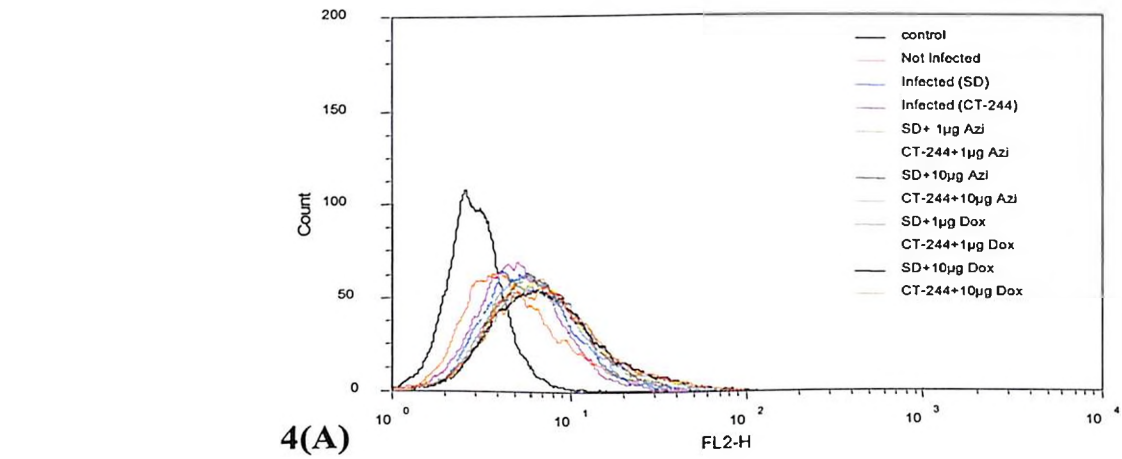


3(B)

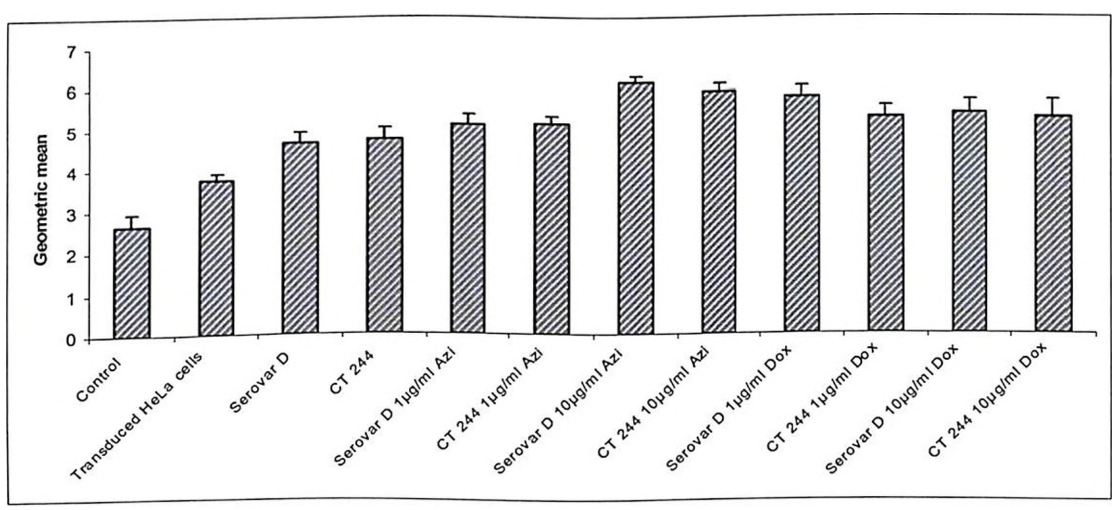
Figure 7.3: (A) Flow histogram and (B) Flow cytometric quantification showing geometric mean of expression of GFP-tagged plasma membrane protein in host HeLa cells harbouring *C. trachomatis* isolate (CT-244) in the presence of antichlamydial drugs (Azi: azithromycin and Dox: doxycycline), $p<0.05$ was considered as significant.

Host HeLa 229 cells harbouring heterotypic resistant *C. trachomatis* isolate (CT-244) were studied for any changes in actin protein expression in the presence of antichlamydial drugs by transducing with Cellular-Lights transduction reagents. The red fluorescent protein (RFP) -tagged actin protein expression was found to be up-regulated on infection with serovar D and CT-244 isolate in comparison to the uninfected HeLa cells. There is no difference in expression was observed in between the serovar D and CT-244

isolate. However, addition of drugs increased the expression of RFP-tagged proteins in infected transduced HeLa cells (Figure 7.4).



4(A)



4(B)

Figure 7.4: (A) Flow histogram and (B) Flow cytometric quantification showing geometric mean of expression of RFP-tagged actin protein in host HeLa cells harbouring *C. trachomatis* isolate (CT-244) in the presence of antichlamydial drugs (Azi: azithromycin and Dox: doxycycline).

Discussion

Repeated or chronic genital infections of *C. trachomatis* may result in increased tissue damage and scarring via acute inflammation (Belland et al. 2003a). To avoid severe sequelae of genital *C. trachomatis* infection, such as pelvic inflammatory diseases (PID), ectopic pregnancies, and tubal infertility, antibiotic strategies are important to eradicate the pathogen in asymptomatic and also uncomplicated infections. First-line antichlamydial drugs have proven successful for the treatment of *C. trachomatis* infection however, treatment failures have been observed in a notable number of cases (Dreses-Werringloer et al. 2000; Horner 2006; Jones et al. 1990; Lefevre and Lepargneur 1998; Lefevre et al. 1997; Samra et al. 2001; Somani et al. 2000; Wang et al. 2005). The role of genetic resistance in the recurrence of chlamydial infections is still not clear and needs further attention. Extensive use of drugs has been known to favor the selection of resistance in bacterial pathogen, including *Chlamydia suis* in the pig (Dugan et al. 2004; Lefevre and Lepargneur 1998; Di Francesco et al. 2008) resulting in chlamydial persistence. The obligate intracellular nature of *Chlamydia* may limit the emergence of resistance *in vivo* due to the strength of the immune response induced by the wild-type antibiotic-sensitive bacteria at the time of antibiotic treatment (McOrist 2000; Abdelrahman and Belland 2005; Sandoz and

Rockey). However, the relation between the emerging *in vitro* resistance in chlamydia and treatment failures in vivo are needed to be explored.

In the previous study *C. trachomatis* isolates showed *in vitro* resistance to antichlamydial drugs and they were from the recurrently infected patients with treatment failures (Chapter 4). Few other studies also suggest resistance as a cause for clinical treatment failures (Somani et al. 2000); further the potential for *C. trachomatis* to develop antimicrobial resistance has not been well studied, despite few published case reports (Lefevre and Lepargneur 1998); (Mourad et al. 1980; Bragina et al. 2001).

The well known mechanisms of antimicrobial drug resistance are alterations of drug targets or enzymatic inactivation of antimicrobial agents (Paulsen et al. 2001; Hasdemir 2007). Besides these well known mechanisms, recent studies have shown a further resistance mechanism, active drug efflux, which has become increasingly important in the current threat of multidrug resistance. It involves certain bacterial transport proteins which pump out antimicrobial compounds from the cell as a result of overexpression of these pumps due to mutations hence decreasing intracellular antibiotic concentration (Poole 2005). Efflux pumps possessed by other pathogens are also likely contribute to their pathogenic mechanisms by escaping a number of antimicrobial compounds that bathe mucosal surfaces [Pages, #35; Putman, 2000 #195].

Hence, we studied the efflux ygeD gene of heterotypic resistant *C. trachomatis* isolate in order to explore the resistant characteristics. The studied sequence showed variation with a point mutation T to G with reference sequence of serotype D (Chapter 6). This may be a non-significant mutation in developing *in vitro* resistance. In another study of efflux (ygeD) gene in clinical isolates of *C. trachomatis*- resistant to high and intermediate level of FQ concentrations several silent mutations and mutations resulting in amino acid substitutions were observed. Hence, this can be concluded that the mutation may not be directly related to the resistant characteristic of the bacteria but it might be possible that it has some indirect role, which may make bacteria more refractory to the drugs. Further expression of the efflux gene was also analyzed in the isolate and it was observed that efflux gene was actively expressed at 8hpi in presence of doxycycline, suggesting its expression may have helped in reducing doxycycline pressure at the initial time point. On addition of azithromycin, expression of ygeD gene was observed to be significantly increased at 24 hpi suggesting that in the presence of azithromycin efflux gene was capable in reducing the drug pressure at 24 hpi but not at the initial time point. Hence, we may conclude that *C. trachomatis* isolate with altered drug susceptibility profile may have an active efflux strategy for its survival in the presence of antichlamydial drugs.

Antimicrobial susceptibility profile of *C. trachomatis* may be dependent on the host cell environmental conditions and host cell-specific factors. It is reported that oxygen concentrations in female urogenital tract affects the removal of chlamydia upon antibiotic treatment (Shima et al.). In addition it has been observed that pathogenic microbes exploit the host cytoskeleton for entry, colonization, and intracellular survival in eukaryotic cells (van Ooij et al. 1997). *C. trachomatis* also co-opts host actin and intermediate filaments to form a dynamic scaffold for providing structural integrity to the chlamydial vacuole and minimizing immune detection for its survival.(Kumar and Valdivia 2008a) Hence, host cell factors should be studied to know if this affects the antibiotic susceptibility profile. Therefore, host HeLa cells harbouring the heterotypic resistant *C. trachomatis* isolate were studied for any phenotypic changes at the cellular level. The significantly reduced expression of GFP-tagged plasma membrane protein in HeLa cells detected may be due to the use of proteins for the invagination of infectious elementary bodies of *C. trachomatis*. However, on addition of drugs expression was found to be comparable to the uninfected cells. Further for detecting any changes in actin protein expression in the presence of antichlamydial drugs host cells were studied for RFP-tagged actin protein expression and it was found to be up-regulated upon infection. However, addition of drugs increased the expression in infected cells. There is no difference observed in expression of plasma membrane and actin protein in between the

serovar D and CT-244 isolate. Hence, this may be suggested that *C. trachomatis* isolate with altered drug susceptibility profile do not affect its host cell plasma membrane or actin organization for its survival in order to resist the antichlamydial drugs.

In conclusion, this study support the emergence of clinical antibiotic resistance, not an impossible scenario for *C. trachomatis* despite their isolated niche which limits the opportunity for acquisition of antibiotic resistance genes from other organisms.(McOrist 2000) Successful treatment is necessary for preventing sequelae of chlamydial infections hence, treatment failures and *in vitro* antibiotic resistance characteristics of *C. trachomatis* is of great concern. The results of present study in characterizing resistance in clinical isolate may enhance the understanding of chlamydial therapy and the nature or transmission of resistant *C. trachomatis*.

Chapter 8

Conclusions and Future Scope of Work

Conclusions and Future Scope of Work

1. Treatment of genital *Chlamydia trachomatis* infections is presently based on antichlamydial antibiotics. Hence this study presents the *in vitro* evaluation for efficacy of first line antichlamydial drugs (azithromycin and doxycycline). Recurrent infections were determined in *C. trachomatis* infected symptomatic female patients after treatment and it was found to be 23.5% within the study period of 1 year. Further, *C. trachomatis* isolates were obtained from the positive patients to know the *in vitro* drug susceptibility profile towards the current regimen (doxycycline and azithromycin). Drug susceptibility profiling of these isolate(s) were performed by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against azithromycin and doxycycline. Modified antibiotic susceptibility profiles were observed in isolates (n=8), were majorly obtained from recurrently infected patients. Azithromycin was more active as compared to the doxycycline. The MIC values of all these isolates were significantly higher than the standard isolate and complete eradication could not occur at higher concentration. However, the number and size of inclusion bodies were found to be decreased with increasing concentration of antibiotics and this small percentage of organisms may reflect the presence of heterotypic resistance (n=2).
2. In addition, the study was undertaken to evaluate the potential clinical utility of a novel polyherbal formulation "Basant" on *C. trachomatis* to develop an effective microbicide. Basant has no cytotoxic effect on the epithelial adenocarcinoma cells (HeLa 229), indicating the safety of Basant on epithelial cells, which are the primary target of various pathogens. Basant was found to have the inhibitory activity against *C. trachomatis*, in

both forms of its life cycle i.e. elementary bodies (EBs) and also on reticulate bodies (RBs). Subsequently its effective inhibitory activity was also determined on clinical isolates of *C. trachomatis*. Hence, it can be used topically for direct killing of the pathogens at the site thus, preventing the pathogen's entry into the host

3. Further, to explore if the *in vitro* heterotypically resistant characteristic of *C. trachomatis* isolates was associated with mutational changes, genotypic characterization of the resistant isolates (n=2) was performed towards antichlamydial drugs. Resistance markers were selected and target gene(s) were amplified from the *C. trachomatis* isolate(s) which have shown decreased susceptibility and sequencing was done to test them for possible mutation(s). No sequence variation was found in the ribosomal L4, L22, 23SrRNA with reference sequences of *C. trachomatis* serovar D/UW-3/CX given in NCBI genbank. In addition, efflux (ygeD) gene of *C. trachomatis* isolates was also checked for the presence of any mutational changes. The studied sequence showed 99% identities with the reference sequence of *C. trachomatis* serovar D/UW-3/CX given in NCBI genbank with a point mutation T (purine) to G (pyrimidine) in 734318 position of *C. trachomatis* genome. There is no difference in the products of the mutated nucleotide with respect to the reference sequence of *C. trachomatis*. As reference sequence has CTT – Leucine amino acid and mutated nucleotide has CTG which also code for the same amino acid, leucine. Hence, this can be concluded that the mutation may not be directly related to the resistant characteristic of the bacteria but it might be possible that it has some indirect role in modifying the life cycle, which may make bacteria more refractory to the drugs. In summary, no genetic mechanism was identified in the ribosomal L4, L22 and 23S rRNA genes for the phenotypic change in the *C. trachomatis* isolates. However, a point mutation

detected in the *C. trachomatis* efflux ygeD gene, suggesting that it may play a role in resistant characteristics of bacteria.

4. Heterotypic resistant *C. trachomatis* clinical isolate was further studied for efflux (ygeD) gene expression at various time points of its life cycle in the presence of antichlamydial drugs. It was observed that efflux gene was actively expressed ($p < 0.05$) at 8 hpi in presence of doxycycline, suggesting its expression may have helped in reducing doxycycline pressure at the initial time point. On addition of azithromycin, expression of ygeD gene was observed to be significantly increased ($p < 0.05$) at 24 hpi suggesting that in the presence of azithromycin efflux gene was capable in reducing the drug pressure at 24 hpi but not at the initial time point. Hence, this study may suggest that *C. trachomatis* isolate with altered drug susceptibility profile may have an active efflux strategy for its survival in the presence of antichlamydial drugs. In addition, host cell factors were also studied to know if this affects the antibiotic susceptibility profile of resistant *C. trachomatis* isolate. Therefore, host HeLa cells were transduced with GFP-tagged plasma membrane and RFP-tagged actin protein and assessed for any phenotypic changes at the cellular level. There is no difference observed in expression of plasma membrane and actin protein in host HeLa cells harbouring susceptible (serovar D) and resistant (CT-244) *C. trachomatis* isolate. Hence, this study may suggest that *C. trachomatis* isolate with altered drug susceptibility profile do not affect its host cell plasma membrane or actin organization for its survival in order to resist the antichlamydial drugs.

Overall, this study suggests decrease in efficacy of first line drugs for genital chlamydial infection which can hamper the treatment effectiveness. Presence of heterotypic resistant isolates, their genotypic and phenotypic characterization points towards the emerging problem of antichlamydial drug resistance in *C. trachomatis*.

Overall the findings may have important implications in the management of human chlamydial infections and thus may help in reducing the reproductive morbidity and associated complications.

Future Scope of Work

Despite the *in vitro* heterotypic resistance presentation by *C. trachomatis* clinical isolates against antichlamydial drugs, its full characterization has not been possible. Resistant *C. trachomatis* isolates are known to recover in culture with varying success and could not be expanded or survive long-term passages in cell culture. Studies suggest that homotypic resistance can emerge in clinical settings and disseminate amongst chlamydia. Hence, further research is warranted to define the potential ability in developing resistance and its characterization. In addition study needed larger number of clinical isolates to know its biological relevance to *in vivo* conditions which can be correlated with cases of treatment failure. Further, in this study a new polyherbal formulation Basant has shown its effective inhibitory activity against *C. trachomatis*, suggesting as a potential microbicide candidate for prevention of genital chlamydial infections. The mechanism by which Basant inhibits *C. trachomatis* is not known and requires further investigation.

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Appendix

Appendix

Preparation of reagents (Stock solution of commonly used reagents)

Cell Viability assay: MTT stock solution: 5mg/ml MTT (Sigma Aldrich, USA) in EMEM (Sigma Aldrich, USA) without phenol red. This solution is filtered through a 0.22 μm filter and stored at 2-8^{0}C. MTT working solution:1:10 dilution of the 5mg/ml stock (MTT in EMEM without phenol red).

0.5M EDTA

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

Ethidium Bromide (10 mg/ml)

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

DEPC water

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

Phosphate Buffer Saline (PBS)

8 gm of NaCl, 2 gm of KCl, 1.44 gm of Na_2HPO_4 and 0.2 gm of KH_2PO_4 were dissolved in 800 ml of distilled water. pH was set to 7.4 with HCl. Final volume was made up to 1 liter and sterilized by autoclaving at 15 lb/sq.in for 20 min. and stored at 4^{0}C.

Sucrose Phosphate Glutamate (SPG) media

1.38 gm of Na_2HPO_4, 0.39 gm of NaH_2PO_4, 85.6 gm of sucrose and 0.94 gm of L-Glutamate were dissolved in DMEM (Sigma Aldrich, USA) media (pH 7.4) with final volume made up to 1 liter and sterilized by filtering through 0.22 µm filter. Aliquots prepared and stored at -20^{0}C.

10 X TAE buffer (Tris acetate, EDTA)

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

10X TBE buffers (Tris borate, EDTA)

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

DNA loading dye (6X)

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

Gentamicin (stock solution):

1mg/ml Gentamicin stock was prepared in autoclaved distilled water and sterilized by filtration through 0.22µm filter. 100µl aliquot was stored by freezing at –20^{0}C.

Amphotericin (stock solution):

1mg/ml Amphotericin stock was prepared in autoclaved distilled water and sterilized by filtration through 0.22µm filter. 100µl aliquot was stored by freezing at -20^0C.

Cycloheximide (stock solution):

1mg/ml Cycloheximide stock was prepared in autoclaved distilled water and sterilized by filtration through 0.22µm filter. 100µl aliquot was stored by freezing at -20^0C.

List of Publications

RESEARCH PUBLICATION

1. **Bhengraj AR**, Srivastava P, Mittal A. Lack of mutation in macrolide resistance genes in *Chlamydia trachomatis* clinical isolates with decreased susceptibility to azithromycin. *International Journal of Antimicrobial Agents* 38 (2011) 177– 189
2. **Bhengraj AR**, Vardhan H, Srivastava P, Salhan S, Mittal A. Decreased susceptibility towards azithromycin and doxycycline in clinical isolates of *Chlamydia trachomatis* obtained from recurrently infected female patients in India. *Chemotherapy* 2010;56(5):371-7
3. **Bhengraj AR**, Goyal A, Talwar GP, Mittal A. Assessment of antichlamydial effects of a novel polyherbal tablet Basant. *Sexually transmitted infections.* 2009 Dec;85(7): 561
4. **Bhengraj AR**, Dar SA, Talwar GP, Mittal A. Potential of a novel polyherbal formulation BASANT for prevention of *Chlamydia trachomatis* infection. *International Journal of Antimicrobial Agents.* 2008 Jul;32(1): 84-8
5. Agrawal T, **Bhengraj** AR, Vats V, Salhan S, Mittal A. Expression of TLR 2, TLR 4 and iNOS in Cervical Monocytes of *Chlamydia trachomatis*-infected Women and Their Role in Host Immune Response. *Am J Reprod Immunol.* 2011 Dec;66(6):534-43.
6. Jha HC, Srivastava P, Vardhan H, Singh LC, **Bhengraj** AR, Prasad J, Mittal A. *Chlamydia pneumoniae* heat shock protein 60 is associated with apoptotic signaling pathway in human atheromatous plaques of coronary artery disease patients. *J Cardiol.* 2011 Nov;58(3):216-25.
7. Vardhan H, Gupta R, Jha R, **Bhengraj** AR, Mittal A. Ferritin heavy chain-mediated iron homoeostasis regulates expression of IL-10 in *Chlamydia trachomatis*- infected HeLa cells. *Cell Biol Int.* 2011 Aug 1;35(8):793-8.
8. Vardhan H, **Bhengraj AR**, Jha R, Srivastava P, Jha HC, Mittal A. Higher expression of ferritin protects *Chlamydia trachomatis* infected HeLa 229 cells from reactive oxygen species mediated cell death. Biochemistry and Cell Biology 2010 Oct;88(5):835-42

9. Vardhan H, **Bhengraj AR**, Jha R, Mittal A. *Chlamydia trachomatis* alters iron-regulatory protein-1 binding capacity and modulates cellular iron homeostasis in HeLa-229 cells. *Journal of biomedicine & biotechnology.* 2009;Aug 16: 342032

10. Agrawal T, Gupta R, Dutta R, Srivastava P, **Bhengraj AR**, Salhan S, Mittal A. Protective or pathogenic immune response to genital chlamydial infection in women--a possible role of cytokine secretion profile of cervical mucosal cells. Clinical Immunology. 2009 Mar;130(3):347-54.

11. Vardhan H, Dutta R, Vats V, Gupta R, Jha R, Jha HC, Srivastava P, **Bhengraj AR** Mittal A. Persistently elevated level of IL-8 in *Chlamydia trachomatis* infected HeLa 229 cells is dependent on intracellular available iron. *Mediators of inflammation.* 2009;May 26: 417658

12. Srivastava P, Gupta R, Jha HC, Jha R, **Bhengraj AR,** Salhan S and Mittal A. Serovar specific immune responses to peptides of variable regions of *Chlamydia trachomatis* Major Outer Membrane Protein in serovar D infected women. *Clinical and Experimental Medicine* 2008 Dec;8(4):207-15.

13. Srivastava P, Vardhan H, **Bhengraj AR**, Singh LC, Salhan S, Mittal A. Azithromycin treatment modulate ERK mediated pathway and inhibits inflammatory cytokines and chemokines in the process of resolution of recurrent *Chlamydia trachomatis* infection in infertile women. *DNA and Cell Biology* 2010.

14. Srivastava P, **Bhengraj AR,** Jha HC, Vardhan H, Jha R, Singh LC, Salhan S, Mittal A. Differing effects of azithromycin and doxycycline on production of cytokines in cells from *Chlamydia trachomatis* infected women. *DNA Cell Biol.* 2011 Aug 17

Article submitted:

1. **Bhengraj AR**, Srivastava P, Vardhan H, Yadav SY, Singh LC, Mittal A. Study of *ygeD* gene in survival of *Chlamydia trachomatis* in the presence of antichlamydial drugs and its phenotypic characterization. 2011 (Submitted)

Poster presentations:

- **Bhengraj A R**, Dar S A, Talwar G P, Mittal A. The inhibitory effect of a new polyherbal formulation Basant on *C. trachomatis* and its potential clinical use as an antichlamydial agent. National Conference on Emerging Trends in Life Sciences Research, 6-7 March 2009 at BITS, Pilani, India.
- **Bhengraj A R**, Dar S A, Talwar G P, Mittal A "Challenges in the treatment of *Chlamydia trachomatis* infection: potential of natural herbal formulation Basant as alternative treatment." In Microbicides 2008 International Conference (February 24–27, 2008) at New Delhi, India
- Gupta R., Srivastava P., **Bhengraj A.R.**, Jha R.,. Jha H. C, Vardhan H, Salhan S, Mittal A. *Chlamydia trachomatis* heat shock protein 60-specific antibody and cell-mediated responses can predict tubal factor infertility in infected women. Sixth Meeting of the European Society for Chlamydia Research, 1-4 July, 2008 at University of Aarhus, Aarhus, Denmark.
- Jha R, Jha HC, Vardhan H, Gupta R, Srivastava P, **Bhengraj AR**, Mittal A. Three-dimensional Structure of *Chlamydia trachomatis* Heat Shock Protein 60 Reveals Cross Presentation of Epitopes in Infected Women Causing Infertility. International Conference in Structural Biology, 19-22 November, 2007 at The Chinese University of Hong Kong, Hong Kong.
- Gupta R, Dutta R, Srivastava P, **Bhengraj AR**, Salhan S, Mittal A. Differential cytokine responses to *Chlamydia trachomatis* infection in cervical lavages of women may indicate extent of disease progression. 17th Meeting of the ISSTDR and the 10th IUSTI World Congress, 29 July – 1 August, 2007, Seattle (WA), USA.
- Jha R, Jha H C, Gupta R, Vardhan H, Srivastava P, **Bhengraj AR**, Mittal A. Similarity in domains of HSPs in humans and *Chlamydia trachomatis* are suggestive of their role in causation

of damaging sequalae in infertile women.11th ADNAT Convention, 26 February-8 March 2007 at Centre for Cellular and Molecular Biology, Hyderabad, India.

- Second Best Poster prize: Jha HC, Srivastava P, Jha R, Gupta R, Vardhan H, **Bhengraj AR**, Prasad J, Mittal A. Role of *Chlamydia pneumoniae* and its association with other risk factors in coronary artery disease. Biosparks, 5th Annual Research Festival, 23 -24 February 2007 at School of Life Sciences, Jawaharlal Nehru University, , New Delhi, India.
- Best Poster prize: Jha HC, Gupta R, Srivastava P, Vardhan H, Jha R, **Bhengraj AR**, Prasad J, Mittal A. *Chlamydia pneumoniae* and associated risk factors in coronary artery disease patients. International symposium on recent advances in cardiovascular sciences (RACS). "Global conference on heart disease, 14 -15 February, 2007 at Delhi Institute of Pharmaceutical Sciences and Research, New Delhi, India.

Biography of Candidate

Biography of Candidate

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Date of Birth: 12th DEC, 1978; Gender: Female; Marital status: Married; Nationality: Indian

Current Position (Present):

Graduate (Ph.D.) student at the Microbiology and Tissue Culture Division, National Institute of Pathology (ICMR), New Delhi, India.

Ph.D. thesis title: "Antichlamydial drugs sensitivity and emergence of resistance in *Chlamydia trachomatis* isolated from clinical treatment failures"

Education:

Ph D

National Institute of Pathology, New Delhi, India — Pursuing

(Off campus affiliated to

Birla Institute of Technology & Science,

Pilani, India)

M.Sc. Biotechnology (2004) — 6.28/10 CGPA

Indian Institute of Technology, Roorkee, Uttaranchal, India

B.Sc. Biotechnology (2001) — 68.5 % Marks

Vinoba Bhave University, Hazaribagh, Jharkhand, India.

Professional experience:

1. UGC Senior Research Fellow at Institute of Pathology (ICMR) under Dr. Aruna Mittal. My PhD involves the "Antichlamydial drugs sensitivity and emergence of resistance in clinical treatment failures". January 9th 2008 to January 8th 2011.
2. Worked as UGC Junior Research Fellow at Institute of Pathology (ICMR) under Dr. Aruna Mittal. My PhD involves the "Antichlamydial drugs sensitivity and emergence of resistance in clinical treatment failures". January 9th 2006 to January 8th 2008.
3. Worked as a JRF at School of life science (JNU) in a project entitled "Replication of ribosomal DNA in *Entamoeba invadens* trophozoites and cysts" Under the supervision of Dr.Jayshree Paul, School of life science (JNU). April 2005 to July 2005

4. Project work entitled "The Preliminary Screening of Thalassemia Carriers" under the Supervision of Prof.G.S.Randhawa in Deptt. Of Biotechnology, I.I.T. Roorkee from Jan 2003-April 2003.

Research Experience:

- DNA, RNA and Plasmid Isolations; PCR, RT-PCR.
- Transformation of competent cell with different construct.
- Cell Biology: Culture of different Cell lines, transfection with constructs and siRNAs.
    - Tissue culture, Cell culture and maintenance of cell lines (Adherent, non-adherent).
    - Intracellular obligate bacteria *Chlamydia trachomatis* culture. Bacterial culture.
    - *In vitro* drug sensitivity assays.
    - ELISA, Western blotting, IFA
    - Isolation of Nucleic Acids; DNA, RNA. PCR, RT-PCR
    - DNA sequencing.
    - Various staining techniques (Giemsa, Direct fluorescent assay (DFA).

Research Interest:

- Pathogens and pathogenesis
- Host – pathogen interactions
- Drug resistance in microorganisms

Academic honors:

1. Awarded Senior Research Fellowship by University Grants Commission (UGC-SRF), India (January 2008).

2. Awarded Junior Research Fellowship by University Grants Commission (UGC-JRF), India (June 2005).
3. Scholarship awarded for participation in the Microbicides 2008 International Conference (February 24–27, 2008) in New Delhi, India.
4. Qualified National examination for Junior Research Fellowship by Department of Biotechnology (DBT-JRF), India (June 2005).
5. Qualified Joint CSIR-UGC National examination leading to award of Lectureship (June 2003).
6. Scholarship for M.Sc. from Department of Biotechnology, Government of India (2001-2003).
7. Qualified All India Entrance exam conducted by Indian Institute of Technology Roorkee for admission to post graduation in Biotechnology (2001).

Conferences attended:

1. National Conference on "Emerging Trends in Life Sciences Research" held at BITS PILANI, India, March 6-7, 2009.
2. Conference attended "Antimicrobial Resistance" (18th January 2008) at Safdarjung hospital, New Delhi.
3. International Conference attended "Microbicides 2008" (February 24–27, 2008) in New Delhi, India.
4. 21st annual conference of Indian Association of Pathologists and Microbiologists, Delhi Chapter organized by Institute of Pathology (ICMR) and Safdarjung Hospital and V.M.M. College held on 16 April 2006.
5. International Workshop on Education and Capacity Building in Biophysics: Needs of the Asian African Region. Organized by the Department of Biotechnology, Indian Institute of Technology Roorkee held on 24-25 February 2003.

Biography of Supervisor

Biography of Supervisor

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

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

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

Email: amittal_iop@yahoo.com

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

Awards/Special recognitions:

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

Patent filed: 3

Technology Transfer: 1

Membership of National/International bodies:

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

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

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

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

Extramural Projects awarded:

National: 7

International: 2

Supervisor:

Ph. D students =10,

MSc students dissertation done/completed=11,

MD Thesis=1

Joint supervisor for Ph. D student= 1

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

1. Nath I and Mittal A. Subpopulation of lymphocytes. In manual of symposium-workshop 'New Directions in foetal liver transplantation', 20: 1986.
2. Mittal A. Rapid radiometric in vitro assay for evolution of M. leprae viability, drug screening and drug resistance: In Handbook of Practical Immunology. ed. G.P. Talwar, 288, 1992.
3. Talwar GP, Upadhyay SK, Garg S, Kaushik C, Kaur R, Dhawan S, Mittal A. Introduction of cell-mediated immunity in genital tract. In book: 'Neem Research and Development', eds. N.S. Randhawa and B.S. Parmar, 227-234, 1993.
4. Mittal A. Diagnostic methods for Chlamydia trachomatis-a chapter in manual. National workshop on Cytology and Immunohistochemistry of the Female Genital Tract, 19, 1994.
5. Kapur S, Singh R, Mittal A. Correlation of HIV infection with Hepatitis B and syphilis. In HIV/AIDS research in India, eds. Aggarwal, O.P., Sharma, A.K. Indrayan, 1997, pp. 465-468.
6. Mittal A. Laboratory Diagnosis of C. trachomatis Infection. Chapter in book: 'Chlamydial Infections', eds. V. Talib, 43-67, 1999.

No. of publications in peer reviewed journals: 88

Recent publications:

1. Huston WM, Harvie M, **Mittal A,** Timms P, Beagley KW. Vaccination to protect against infection of the female reproductive tract. **Expert Rev Clin Immunol.** 2012 Jan; 8(1):81-94
2. Bhengraj AR, Dar SA, Talwar GP, **Mittal A.** Potential of a novel polyherbal formulation BASANT for prevention of *Chlamydia trachomatis* infection. **International Journal Antimicrobial Agents.** 2008; 32:84-88.

3. Jha HC, **Mittal A.** Coronary artery disease patient's first degree relatives may be at a higher risk for atherosclerosis. **International Journal of Cardiology**.2008, doi: 10, 1016/j.ij card 2008. 03 .031.
4. Agarwal T, Vats V, Wallace P, Singh A, SalhanS, **Mittal A.** Recruitment of myeloid and plasmacytoid Dendritic cells in cervical mucosa during C.trachomatis infection. **Clinical Micobiology Infection 2008**,15:50-59.
5. Dutta R, Jha R, Salhan S, **Mittal A.** Chlamydia specific heat shock protein 60 antibodies can serve as prognostic marker for chronic Chlamydia infection. **Infection.** 2008; 36; 374-378.
6. Jha HC, Prasad J, **Mittal A.** High IgA seropositivity for combined *Chlamydia pneumoniae*, *Helicobacter pylori* infection and high sensitive C-reactive protein in Coronary Artery Disease patients in India can serve as atherosclerotic marker. **Heart and Vessels.** 2008; 23:390-396.
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