Molecular Methods in the Characterization of Adenoviruses and *Chlamydia trachomatis* Detected in Clinical Specimens

THESIS

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CERTIFICATE

This is to certify that the thesis entitled **"Molecular Methods in the characterization of Adenovirus and *Chlamydia trachomatis* Detected in clinical specimens"** submitted by **J.Malathi ID No 1999PHXF402** for the award of Ph.D. Degree of the Institutes, embodies original work done by her under my supervision.

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LIST OF ABBREVIATIONS

%	percentage
°C	degrees centrigrade
µg	microgram(s)
µl	microlitre(s)
µM	micromoles
A	adenine
AFLP	Amplified fragment length polymorphism
ATCC	American type culture collection
AHC	Acute hemorrhagic cystitis
ARD	Acute respiratory disease
ARN	Acute retinal necrosis
ATP	Adenosine Tri Phosphate
ADP	Adenosine Di Phosphate
BHK	Baby Hamster Kidney
BP	Base pair
cDNA	Complementary DNA
CDC	Center for disease control
CHO	Chinese Hamster Ovary
CF	Complement fixation
CFU	Colony forming unit
CPE	Cytopathic effect
DEPC	Diethyl pyrocorbonate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
d NTPs	deoxyribonucleotide triphosphate
EB	Elementary body
EDTA	Ethylene diamine tetra acetic acid
EKC	Epidemic keratoconjunctivitis
EIA	Enzyme immune assay
EM	Electron Microscopy
EMEM	Eagle's minimum essential medium

EV	Enterovirus
FAT	Fluorescence Antibody test
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FSW	Female sex worker
FVU	First Void Urine
GH	Government general hospital
GH-CSF	Granulocyte Macrophage Colony stimulating factor
GM	Growth medium
GTC	Guanidine thiocyanate
GOH	Government ophthalmic hospital
GTP	Guanosine Tri Phosphate
HA	Hemagglutination
HBSS	Hank's Balances Salt Solution
HEK	Human embryonic kidney
HI	Hemagglutination inhibition
HIV	Human immuno deficiency virus
HSP	Heat Shock Protein
HSV	Herpes simplex virus
HVR	Hyper variable region
IF	Immunofluorescence
Ig	Immunoglobulin
IFU	Inclusion forming units
IL	Interleukin
IIF	Indirect immunofluoresence
IFN	Interferon
LGV	Lymphogranuloma
LPS	Lipopolysaccharide
Kb	Kilo bases
kDa	Kilo Dalton
LCR	Ligase Chain reaction
NAD	Nicotinamide Adenine dinucleotide
NGU	Non-gonococcal urethritis

Mab	Monoclonal antibodies
MBP	Maltose binding protein
MBC	Minimal Bacterial Concentration
MF	Microimmunofluorescence
MHC	Major Histocompatibility Complex
MIC	Minimal Inhibitory Concentration
MM	Maintenance Medium
MOLN	Murine
MOMP	Major Outer Membrane Protein
MRF	Medical Research Foundation
NASBA	Nucleic acid based amplification
NAAT	Nucleic acid amplification test
NIV	National institute of virology
No.	Number
NPA	Nasopharyngeal aspirate
OD	Optical density
ORF	Open reading frame
OMPA	Outer membrane protein A
OMPB	Outer membrane protein B
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween 80
PAGE	Polyacrylamide gel electrophoresis
PCF	Pharyngoconjunctival Fever
PCR	Polymerase Chain Reaction
PFGE	Pulse field gel electrophoresis
PID	Pelvic inflammatory disease
RB	Reticulate body
RBC	Red blood cells
RE	Restriction enzyme
RFLF	Restriction Fragment Length Polymorphism
RR	Ready reaction mix
rRNA	Ribosomal RNA
RSV	Respiratory syncytial virus

RT- PCR	Reverse Transcriptase based PCR
Pvt	Private
SDS	Sodium Dodecyl Sulphate
SP	Sucrose phosphate buffer
STD	Sexually Transmitted Diseases
T	thymidine
TCA	Tricarboxylic acid
TBE	Tris Boric acid EDTA
TEMED	N, N, N', N' – tetramethlylene diamine
TNF	Tumor necrosis factor
Tris-Cl	Tris-chloride
TR-FIA	Time resolved fluoro immunoassay
TRCF	Transcript repair coupling factor
TSR	Template suppressor
TWAR	Taiwanese Acute Respiratory
Vero	Venerial and enteric research organization
UV	Ultra violet
WI-38	Wister institute – 38

CONTENTS

Introduction

INTRODUCTION

Conjunctivitis is an acute or chronic mucosal inflammation of the conjunctiva. Inflammation of the conjunctiva can be due to Chlamydia, viruses, bacteria and rarely fungi & protozoa. Non-infective forms due to allergy or toxicity also exist. Toxicity may be due to preservatives, such as thiomersal, or associated with contact lens wear (Smith, 1986).

In 19th century in Europe, a mild type of "trachoma" that occurred in epidemics among the clients of swimming pools was described and was proved that infection of the eye occurred with *Chlamydia trachomatis* (*C. trachomatis*) through genital discharge coming in contact with eye (Dowson, 1996). During 1960s and 1970s the rapid evaluation of methods of serotyping chlamydial isolates led to the observation that isolates from patients with trachoma in endemic trachoma areas were largely due to serotypes A, B, Ba & C, whereas isolates from genital tract, were for the most part, serotypes D to K with a few types of B & Ba strains (Dean et al, 1992; Dowson, 1996).

Inclusion conjunctivitis caused by *C. trachomatis*

Conjunctivitis caused by *C. trachomatis* is called as paratrachoma, follicular conjunctivitis and inclusion conjunctivitis. Adult inclusion conjunctivitis is caused by sexually transmitted strains of *C. trachomatis* serovars D, E, F, G, H, I, J and K. Adult inclusion conjunctivitis is an eye disease resembling the early inflammatory phase of endemic trachoma but without the severe conjunctival scarring. Other uncommon chlamydial eye disease is caused by lymphogranuloma venereum (LGV) agent (Baron and Peterson, 1994). Conjunctivitis due to LGV strains are rarely seen and are severe as this strain is invasive with Perinaund oculograndular syndrome and massive periauricular and cervical lymphadenopathy. Chlamydial conjunctivitis has

a range of clinical features indistinguishable from viral, bacterial and allergic conjunctivitis or keratoconjunctivitis and the clinical presentations may be acute or chronic (Rao et al, 1996).

Ocular signs of untreated Chlamydial infection include corneal epithelial keratitis, sub epithelial and stromal inflammation, limbal keratitis, pannus formation, focal corneal neovascularization and scarring and chronic inclusion conjunctivitis (Stenson et al, 1981; Talley et al, 1992). The delay in diagnosing Chlamydial conjunctivitis in adults may result in increased ocular damage and further transmission to susceptible partners with systemic complications (Kowalskin et al, 1994). Severe form of adult inclusion conjunctivitis may be misdiagnosed as trachoma as in chronic severe infection the symptoms are overlapping. Data on the prevalence of inclusion conjunctivitis are based mostly on western literature (Woolland et al, 1992; Mellman-Rublin et al, 1995; Garland, 1995; Postema et al, 1996) except a few from India. The prevalence of conjunctivitis due to *C. trachomatis* in Chennai, India, was reported to be 17.0% (Madhavan, 1992) in1991 and 34.6% in 1996 (Rao et al, 1996). In a study conducted at New Delhi, India 53.37% positivity for *C. trachomatis* among individuals having follicular conjunctivitis was reported (Satpathy et al, 1999).

Neonatal conjunctivitis due to *C. trachomatis*

Newborns born to mothers with cervical infection due to *C. trachomatis* may develop conjunctivitis called as ophthalmia neonatorum (Hammerschlag and Rapoza, 1996). Conjunctivitis of the newborn or ophthalmia neonatorum is a conjunctivitis recognized in the 1st month of postnatal life. It occurs in 1.6 to12.5% of newborns. (Talley et al, 1994) In up to 73 percent of ophthalmia neonatorum *C. trachomatis* is the causative agent. (Schaller et al, 1997). Chlamydial infections have been reported in more than, 25 percent of newborn (Person et al, 1983). Approximately 30 percent

of infants exposed to *C. trachomatis* in the birth canal will develop conjunctivitis and 10 to 20% will develop pneumonia (Schatchter, 1999). In India the prevalence of neonatal conjunctivitis due to *C. trachomatis* is reported to be 24.0 percent (Mohile et al, 2002). Conjunctivitis of the newborn manifests as red eye accompanied by variable degree of chemosis, periorbital edema, conjunctival exudation and ocular discharge. It can be misdiagnosed as periorbital cellulitis. The condition is always treated as an ocular emergency and requires a specific etiologic diagnosis to initiate appropriate therapy. The other important sequelae of neonatal infection are sub acute pneumonia, which is rarely fatal but cause a prolonged and often debilitating illness and an increased risks of chronic respiratory disease.

Preventing maternal infections before the infants delivery is ideal (Salpietro et al, 1999). But frequently vaginal and cervical infections are asymptomatic and without obvious signs. Therefore it remains unrecognized. Ophthalmia neonatorum due to *C. trachomatis* develops 5-13 days after exposure. Ocular complications encountered if untreated include conjunctival scarring, micropannus and up to one third of affected infants may develop pneumonia (Talley et al, 1992).

Adenoviral conjunctivitis

Among conjunctivitis caused by viruses, adenoviruses are the most frequent causes occurring in sporadic and epidemic forms (Tai et al, 1974; Petit et al, 1979; Woshart et al, 1984; Saitoh-Inagawa et al, 1999). Conjunctivitis due to adenoviruses, like chlamydial conjunctivitis to a lesser degree may present with follicular conjunctivitis, a pharyngoconjunctival fever or as epidemic keratoconjunctivitis. Acute follicular conjunctivitis is a self-limiting condition without sequelae, caused by adenovirus serotypes1, 2, 4-6 and 10. In its acute form it lasts upto 21 days - full recovery may take 28 days or longer. Pharyngoconjunctival fever (PCF) is caused by serotypes 3 and 7 with features including fever, conjunctivitis, pharyngitis, headache, and lymphadenopathy occurring over a course of 10-14 days, usually with out sequelae (Seal et al., 1998).

A mild keratitis may occur with PCF. PCF responsible for community-based, conjunctivitis attributable to adenovirus was initially reported in Austria in 1889 (Dawson et al, 1996) and 1st report of an adenoviral epidemic took place in Bombay in 1901 (Herbert et al, 1901).

In Chennai, India, epidemics due to adenovirus type 4 in 1991 and type 3 in 1992-93 was observed (Madhavan, 1999). In Chennai, India, Epidemic conjunctivitis due to adenovirus 7a was reported during 1996 (Dalapathy et al, 1998).

Though adenovirus conjunctivitis is a self-limiting disease it causes significant amount of morbidity. There are no effective antiviral drugs available for adenovirus infections, and the prevention of spread to others by isolation of the affected individuals from susceptible individuals at work, school and family is the most important means for preventing adenoviral conjunctivitis.

Genital infections caused by *C. trachomatis*

Genital *C. trachomatis* infections and their sequelae have a major impact on individuals and the health care system (Wilson et al, 2003). In women genital infections caused by *C. trachomatis* include urethritis, endocervicitis, endometritis and salpingitis. Infection per se is confined to epithelial surfaces but an immune mediated host response can cause severe inflammation and damage affecting deeper tissues especially after repeated episodes. The most serious complications of chlamydial upper genital tract in women are infertility and ectopic pregnancy, which result from damage to fallopian tubes (Tyagi and Singh 1998; Gilbert, 1996). Cervical chlamydial infection during pregnancy is associated with an increased risk of preterm birth (Lan et al, 1995). The prevalence of Chlamydial infections of genital tract in western populations had been estimated 10–25 percent (Talley et al, 1994; Postema et al, 1996; Gomes et al, 2001; Pimenta et al, 2001; Sylvan et al, 2002).

In India, reports on the prevalence of *C. trachomatis* genital infection are only a few and many of these are based on antibody detection by enzyme immunoassay (EIA) using commercial kits. The prevalence of *C. trachomatis*, among women having vaginal discharge was reported to be 12.2 per cent (Viswanath et al, 2000). Prevalence of *C. trachomatis* in women with tubal infertility is 74.07 per cent (Tyagi et al, 1998). Prevalence of *C. trachomatis* among female sex workers (FSW) in Mumbai was 23.2 per cent (Divekar et al, 2000). The prevalence of *C. trachomatis* infection among women attending a gynecology outpatient department in Northern part of India was determined to be 24.5- 43.1% (Singh et al, 2002).

Immune-mediated reactive cervicitis or Reiter's syndrome (arthritis, urethritis and conjunctivitis) can complicate genital chlamydial infection in either sex, but is more common in men. Intracellular presence of Chlamydial antigen, rRNA and DNA of *C. trachomatis* in the inflamed joints of patients with Reiter's syndrome or reactive arthritis after urogenital infections have been demonstrated (Kuipers et al,1995) .

Endemic respiratory tract infections in children are associated with upper respiratory tract symptoms and pneumonia. *C. trachomatis* and adenovirus are established as an important respiratory tract pathogens in children (Smith et al, 1989; Hierholzer et al,1989).

Respiratory infections caused by *C. trachomatis*

Respiratory infections due to adenoviruses occur endemically. Epidemics usually occur among infants, children and military recruits. The incubation period ranges from 5 to 8 days. Since adenoviruses are stable they are transmitted by fomites. The initial site of infection can be conjunctivae, oropharynx or the intestine and the infection subsequently spreads to the regional adenoid tissues. Adenovirus

serotypes 1-5 and 7 account for 87 percent of adenovirus infections. Pneumonia in children is caused by adenovirus serotypes 1-3 and 7. Pharyngo conjunctival fever and epidemic keratoconjunctivitis are caused by serotypes 3,4 and 7 and 14 (Hierholzer et al, 1989).

Laboratory methods of etiological diagnosis

Conventional methods

Given the devasting sequelae of untreated *C. trachomatis* infections, it is important to differentially diagnose the etiological agent and institute appropriate therapy. Though effective antiviral therapy is not available for adenoviral infections, the spread of infection among community can be prevented if differentially diagnosed by isolating the patient and unnecessarily the patient need not be given antibiotics or other antiviral therapy, which are toxic especially in respiratory tract infections in children. Rapid diagnostic methods used for the detection of Adenovirus and *C. trachomatis* from clinical specimens includes fluorescence antibody test (FAT) and EIA which are more sensitive than culture techniques which is considered to be the "gold standard" (Wilson et al, 1986; August et al, 1987; Sheppard et al, 1988;Hierholzer et al, 1989; Satpathy et al, 1994). But FAT technique needs trained personnel for interpreting the result and is often highly subjective.

Though culture technique is considered to be the "gold standard", its sensitivity is affected by type of the swab used for collecting the clinical specimen the transport medium, transport conditions, the site of collection, the culture media (Kuo et al, 1988; Jones et al, 1988).

Role of molecular biological techniques in detection and characterization of *C. trachomatis*

Polymerase chain reaction (PCR) a molecular biological technique is proven to be a rapid, sensitive, specific, reproducible method on comparison with FAT, culture and EIA for the detection of *C. trachomatis* (Mouton et al, 1997; Houng et al, 2002). Therefore PCR technique was applied to detect *C. trachomatis* in conjunctivitis, genital infections and lower respiratory specimens. Typing of *C. trachomatis* isolates is done with type specific antisera using FAT technique. These conventional methods are cumbersome and the antisera cross react among serovars and lead to mis-interpretations of serotypes (Frost et al, 1993). PCR- based sequencing of Major outer membrane protein (MOMP) gene of *C. trachomatis* and PCR based Restriction fragment length polymorphism (RFLP) are reported as a definite method to identify the serotypes (Dean et al, 1997; Bandea et al, 2001). So PCR-based DNA sequencing of major outer membrane protein gene for *C. trachomatis* and PCR-based RFLP for adenoviruses was proposed.

C. trachomatis infection are treated by quinalones (Ciprofloxacin, sparfloxacin etc.) macrolides (erythromycin, roxithromycin, azithromycin) and tetracyclines. Accurate determination of Minimum inhibitory concentrations (MIC) of antibiotics against isolate may help in institution of therapy particularly in recalcitrant infection. Determining Minimum inhibitory concentrations (MIC) and Minimum bactericidal concentrations (MBC) of antibiotics for *C. trachomatis* is a difficult, cumbersome work, as it requires tissue culture facility and due to the complicated life cycle of *C. trachomatis* (Cross et al, 1999). Therefore a molecular method, Reverse transcriptase based PCR (RT-PCR) was proposed to determine the MIC of antibiotics for *C. trachomatis.*

Role of molecular biological techniques in detection and characterization of Adenovirus

PCR for the detection of adenovirus from clinical specimens (Allard et al, 1990; Hierholzer et al, 1993; Kinchington et al, 1994) has been reported to be sensitive. Hence the technique was applied to detect adenovirus in conjunctivitis and interstitial pneumonia. Knowledge about the prevalent serotype associated with genital, ocular infection is essential to know the epidemiology of the disease. Isolates of adenovirus are typed by immunological methods, haemagglutination inhibition, neutralization, immunofluorescence or whole genomic DNA restriction analysis (Hierholzer, 1989). These methods are cumbersome and time consuming (Saitoh - Inagawa et al, 1996). Hence a definite method is needed to determine the serotypes. Therefore multiplex PCR using subgenus specific primers and PCR-RFLP techniques were proposed to determine the serotype of adenoviruses detected in clinical specimens.

Aims and Objectives

AIMS AND OBJECTIVES

Hypothesis

Molecular biological techniques will be rapid, more sensitive and may be highly specific in detecting *C. trachomatis* and Adenovirus from suspected clinical specimens.

OVERALL AIM

To standardize molecular biology technique for the detection and characterization of *C. trachomatis* and Adenovirus from suspected clinical specimens to compare the results with that of conventional methods. To standardize molecular methods for typing Adenovirus and *C. trachomatis* detected in clinical specimens.

SPECIFIC OBJECTIVES

I. Methodological

1. To evaluate an effective in house method of DNA extraction from clinical specimens.

2. To standardize PCR with MOMP and plasmid primers for the detection of *C. trachomatis* from conjunctivitis specimen and to evaluate their effectiveness.

3. To standardize PCR for the detection of *C. trachomatis* with plasmid primers from genital specimens.

4. To standardize RT-PCR for the detection of Minimum inhibitory concentrations of antibiotics against *C. trachomatis.*

5. To standardize nucleotide sequencing of PCR plasmid amplified products for typing *C. trachomatis* detected in clinical specimens.

6. To standardize nucleotide sequencing of PCR Major outer membrane (MOMP) protein gene DNA amplified products for typing *C. trachomatis* detected in clinical specimens.

7. To standardize multiplex PCR for the subgenus typing of adenovirus isolates recovered from conjunctivitis patients.

8. To standardize PCR based RFLP for typing Adenovirus detected in clinical specimens.

II. Conventional cell culture method in growing *C. trachomatis*

1. To determine the relative sensitivity of McCoy, HeLa, BHK-21, HEp-2, A 549, Vero cell lines for growing *C. trachomatis*

III. Conventional and molecular methods in the laboratory diagnosis of *C. trachomatis* in primary conjunctivitis

1. To evaluate PCR with plasmid primers against MOMP primers for the detection of *C. trachomatis* in primary conjunctivitis.

2. To evaluate PCR against conventional methods of antigen detection and isolation for the detection of *C. trachomatis* in primary conjunctivitis.

IV. **Conventional and molecular methods in the laboratory diagnosis of *C. trachomatis* in cervicitis and urethritis.**

1. To evaluate PCR with plasmid primers against direct antigen detection and isolation for the detection of *C. trachomatis* in cervicitis and urethritis.

V. **Conventional and molecular methods in the laboratory diagnosis of *C. trachomatis* in interstitial pneumonia.**

1. To evaluate PCR with plasmid primers against direct antigen detection and isolation for the detection of *C. trachomatis* in interstitial pneumonia.

VI. **Conventional and molecular methods in the laboratory diagnosis of Adenoviral epidemic conjunctivitis**

1. To evaluate PCR against conventional methods of antigen detection and virus isolation for the detection of adenovirus in epidemic conjunctivitis.

VII. **Conventional and molecular methods in the laboratory diagnosis of Adenoviral in interstitial pneumonia.**

1. To evaluate PCR against conventional methods of antigen detection and virus isolation for the detection of adenovirus in interstitial pneumonia.

VIII. Characterization of *C. trachomatis*

1. To determine the genotype of *C. trachomatis* isolated from conjunctivitis cases by MOMP sequencing.

2. To determine the genotype of *C. trachomatis* detected in clinical specimens.

3. To evaluate RT-PCR against conventional methods to determine the MIC, MBC concentrations of Ciprofloxacin, erythromycin, sparfloxacin and roxithromycin against *C. trachomatis* isolates from conjunctivitis cases.

4. To study the influence of conjunctival specimens on the MIC, MBC concentrations of Ciprofloxacin, erythromycin, and roxithromycin against *C. trachomatis*

IX. Characterization of adenovirus

1. To determine the serotype of adenoviruses isolated from epidemic conjunctivitis patients by neutralization.

2. To determine the serotype of adenoviruses isolated from epidemic conjunctivitis patients by multiplex PCR.

3. To serotype adenoviruses isolated from epidemic conjunctivitis patients by PCR-RFLP.

4. To serotype adenoviruses detected from interstitial pneumonia patients by PCR-RFLP.

Review of Literature

REVIEW OF LITERATURE

Chlamydia are obligate intracellular parasite that replicate in a vacuole in the cytoplasm of eukaryotic cells. Three species are recognized in this group Chlamydiales; family Chlamydiaceae (Stephens et al, 1999).

Chlamydia trachomatis

Chlamydia pneumoniae

Chlamydia psittaci

Chlamydia sps cause a variety of human and animal diseases and are one of the most prevalent causes of sexually transmitted disease (Kuipers et al, 1995;Sturm-Ramirez et al, 2000). Among the three species *Chlamydia trachomatis* (*C. trachomatis*) is an important human pathogens and human is the only known natural host for all strains of *C. trachomatis* except the mouse pneumonitis agent.

Chlamydia trachomatis species contain three biovariants: trachoma, lymphogranuloma venereum (LGV), and murine (Moln). Strains of *C. trachomatis* that are natural pathogens of humans are either the trachoma or LGV biovar. The trachoma biovars are closely related; however, numerous serovars have been identified among strains with in each biovar. Microimmunofluorescence is used to classify *C. trachomatis* into serotypes A to L. Types A to K infect epithelia of conjunctivae, pharynx, lower respiratory tract, urethra, endocervix, fallopian tubes and gastrointestinal tract. Type L1, L2 and L3 cause a systemic disease called LGV (Stephens et al, 1999).

C. trachomatis are small, non-motile weakly Gram-negative obligate intracellular parasites, which have two forms of life (Baron, 1994). There is a small, 300nm diameter form, which has a compact electron-dense nucleoid; this is the highly

infectious, stable, extracellular transport form. Then there is a larger form, 800-1200nm in diameter, without a dense nucleoid, 'the initial body' which is intracellular and constitutes the replicating form of the organism. Both forms stain well with the Macchiavello, Casteneda or Giemenez stains and by Giemsa (Smith 1989; Zampachova, 1991; Baveja et al, 1997). The organisms grow in the cytoplasm of their host forming characteristic micro-colonies or inclusion bodies made up of the larger and smaller cells; the larger inclusions sit on the cells nucleus as a 'helmet' or 'mantle'. *C. trachomatis* have compact inclusions with glycogen matrix (Baron, 1994).

Life cycle of *C. trachomatis*

Chlamydia trachomatis have a complex and unique developmental cycle (Baron, 1994; Wilson et al, 2003) with two distinct forms. The elementary body (EB), a rigidly enveloped structure approximately 3.0μm in diameter is the infectious form of the organism and adapted for extracellular survival and establish infection. Elementary bodies on contact with new susceptible host cell are actively ingested by endocytosis and are enclosed with in the cell interior in a cytoplasmic vesicle, a phagosome. With in 6-8 hours after entering the new host cell, the EB is reorganized into metabolically active Reticulate body (RB). Reticulate body divides by binary-fission with in a membrane bound vacuole. They get reorganized into EBs. These newly formed EBs are enclosed within the phagosomic intracellular vesicle, which is visible as an inclusion with fluorescence antibody test (FAT), Giemsa and iodine staining under microscope. These inclusion bodies are of great use in diagnosing *C. trachomatis* infections from clinical specimens. Inside the host cell they prevent lysosomal fusion, and survive intracellulary. At 48-72 hours after initial attachment, the cell ruptures to release many infectious EBs which infect new host cells.

History

The generic name Bedsonia was also used for *Chlamydia* in recognition of the pioneering work of Sir Samuel Bedson and his colleagues who isolated and characterized the agent of psittacosis in 1930.

In 1907, Halberstardter and Von Prowazek described cytoplasmic inclusion bodies in epithelial scrapings from orangutans inoculated with material from trachoma cases. He thought they were protozoan and named them "chlamydozoa" or "mantle bodies". Similar inclusion bodies were observed in infants with inclusion blennorrhea, in endocervical scrapings from their mothers, and in urethral scrapings from men with non-gonococcal urethritis (NGU).

In vitro cultivation of *C. trachomatis*

Linder, Halberstardter, Von Prowazek in 1910 had seen, the characteristic inclusion bodies in conjunctival epithelial cells of newborns born to mothers with infected cervix and in child suffering from blennorrhea and from urethra of men with 'non-specific' urethritis. T'ang in 1957 cultured *C. trachomatis* in the yolk sac at lower temperature of incubation and with antibiotics (Streptomycin) which suppressed the accompanying conjunctival bacteria and allowed survival of the chick embryos. Jones and his colleagues in 1959 propagated an inclusion conjunctivitis agent in the yolk sac.

In 1944, Machiavello attempted isolating the trachoma agent in the yolksac of an embryonated Hen's egg. T' ang and his colleagues (1957) in China isolated the trachoma agent in egg yolksac. After the isolation of trachoma agent in egg yolksac, a dramatic increase in Chlamydial research was done. *C. trachomatis* was isolated from inclusion conjunctivitis cases by Jones et al,1959. Serotyping of *C. trachomatis* showed that some serovars were specifically associated with trachoma while others

were associated with genital tract disease, although there have been exception. e.g Serovar B and Ba are rarely associated both with trachoma & genital tract disease (Wang and Grayston,1970, Morre et al, 1998). Association of *C. trachomatis* with urethritis was then discovered. Association of pneumonia was discovered in 1974 (Schachter et al, 1975; Beem and Saxon, 1977).

This was followed by studies from a Swedish group showing that *C. trachomatis* was major cause of pelvic inflammatory disease (PID) (Mardn et al, 1977).

Lymphograuloma venereum (LGV), caused by invasive serovars L1, L2, L3 of *C. trachomatis* was first discovered in 1786 by John Hounter. The causative agent was first isolated by intracerebral inoculation of monkeys in 1930 and its growth cycle was observed by growing it in mice.

The unique life cycle of these organisms were described first by Bedson in 1932 for psittacosis.

Structure

Electron micrographs of thin sections of the small cell show a thin cell wall and a limiting cytoplasmic membrane (Collier, 1983; Bowie, 1990). Inside the cytoplasmic membrane there are ribosome of the 70S type found in bacterial cells; protein synthesis on these is inhibited by broad-spectrum antibiotics such as tetracycline, erythromycin, chloramphenicol. The DNA is concentrated in the central nucleoid and is double stranded with a G plus C ratio in the range of 39-41 percent. The genome sequences encoded a number of new outer membrane proteins, including at least one predicted porin (OmpB) in addition to the major outer membrane protein (MOMP / OMPA). The cell wall structure is similar to that of Gram-negative bacteria and produce an endotoxin like lipopolysaccharide.

Although it was earlier proposed that *C. trachomatis* cell wall does not contain peptidoglycan (Baron, 1994), now it has been proved beyond doubt that they do contain peptidoglycan in their cell wall (Stephens et al, 1999).

GENETICS

The complete circular *Chlamydia trachomatis* genome consists of a 1,04,2519-base pair (bp) chromosome and a 7,493-bp plasmid (Stephens et al, 1998). The guanine plus-cytosine content is 42.5 percent. Two encoded gene products (CT 582 & CT 583) in the chlamydial chromosome were homologous to protein by ORF 7 and ORF 8 of chlamydial plasmid. *C. trachomatis* contain 23S, 16S and 5S ribosomal RNA as 50S and 30S subunits. Enzymes were identified in the genome, which account for the essential requirements for DNA replication, repair, transcription, and translation. Chlamydiae have substantial capacity for DNA repair and recombination, including mismatch repair (MutL, MutS, and three parologous MutY proteins), the exonuclease complex, transcription-repair coupling factor (TRCF), and a number of proteins implicated in recombination-coupled repair such as RecA, RecBCD, and RecJ. Like other bacteria, chlamydiae contain a core RNA Polymerase (Stephens, 1999).

One of the phenotypic hallmarks of chlamydial development is the remarkably condensed nucleoid structure observed in elementary bodies (EB) (Costerton et al, 1976). This condensation is mediated by two highly basic DNA-binding proteins (Wagar and Stephens, 1988). Each of these proteins has been called "histone-like" because of sequence similarity to eukaryotic histone H1. In addition to the denoted histone-like proteins, two proteins containing domains detected only in eukaryotic chromatin-associated proteins, two SWI/SNF helicases, and DNA topoisomerase I containing a SWIB domain support a complex eukaryotic-like mechanism for chlamydial nucleoid condensation and decondensation (Stephens et al, 1998).

Major outer membrane protein (MOMP)

The MOMP protein has a molecular weight of 40kDa. Antibodies to this MOMP neutralize the infectivity of the organism (Morre et al, 1998). The MOMP of *C. trachomatis* is one of the principle cell wall surface components that is responsible for the structural components, that is responsible for the structural integrity of the extracellular infectious EBs and the developmental into the plastic and fragile intracellular reticulate body (Hatch et al, 1984). This protein also has pore-forming capabilities that permit exchange of nutrients for the reticulate body form. The structural and porin functions of the MOMP are mediated by disulfide bond interactions within and between MOMP molecules (Bavoil, 1984). The chlamydial MOMP displays an array of molecular biological and immunologic characteristics that are important for the study of this protein. A major focus for chlamydial vaccine research is the MOMP, which is the most antigenically, and diverse and abundant surface protein of the organism (Dean et al, 1997). The MOMP has become an important target for epitope mapping to define surface exposed regions for antibody binding and neutralization studies, for determining host-cell mediated cell interactions and for immunotyping to identify the spectrum of strains responsible for human diseases (Dean et al, 1997).

The MOMP antigen of *C. trachomatis* contains four variable domains (VD I, VDII, VD III and VD IV) that are flanked and interspaced by five constant domains (Morre et al, 1998). Epitope mapping has revealed that VDI, II and IV are surface exposed. MOMP is the most prominent surface-exposed molecule on the EB, comprising up to 60 percent of the total cell wall protein (Caldwell et al, 1981; Caldwell et al, 1982). Several observations indirectly suggest a role for MOMP as an adhesin. Antisera to EBs neutralize infectivity in a serovar-sepcific manner and MOMP is the predominant serotyping antigen (Caldwell and Schachter, 1982). Serotype-specific monoclonal antibodies to surface-exposed variable sequence

"domains" I or II and IV of MOMP neutralize chlamydial infectivity by blocking attachment. These regions exhibit a net negative charge; thus, a blockage of attachment by antibody binding is proposed to reflect an inhibition of electrostatic interactions with the host cell. In addition, VD IV also contains a conserved hydrophobic domain that is inaccessible to antibody on native EBs. The conformation of this domain is altered by treatments such as thermal inactivation that inhibit EB attachment. These observations led to a proposal that MOMP might function as an adhesin by mediating both electrostatic and hydrophobic interactions with the host cell surface. The sequestered hydrophobic domain was further hypothesized to exist in a pocket, similar to the structure of haemagglutinin of influenza virus, such that close contact with a cell surface ligand would be required to establish this level of interaction (Su et al, 1990). Serovar L2 is remarkably resistant to trypsin treatment (Hackstadt, 1999), whereas serotype B attachment is dramatically reduced (Su et al, 1988). Comparison of the peptide sequence of VDs II and IV reveals a trypsin-sensitive lysine residue in each of these domains of serovar B MOMP that is absent from L2 MOMP (Su et al, 1988). Thus, although intact MOMP is not necessarily required for attachment, VDs II and IV appear to be critical.

The location of these variable domains in the MOMP aminoacids are VDI: 64-83, VD: 139-160, VDIII: 224-237, VDIV 288-317. Epitope mapping has shown that 3 domains VDI, VDII, VDIV contain contiguous antigenic determinants that elicit formation of serovar, subspecies specific antibodies.

VDI and VDII show greatest amount of inter sero group sequence variation and is located near the C terminus of the protein and is the location of sub-species-sero group and a highly conserved species specific antigenic determinant. Antigenic determinants have not been mapped to VDIII, the smallest and least variable domain of MOMP genes.

VDI, IV protrudes towards the external environment at the Chlamydial surface, and they are susceptible to cleavage by trypsinisation. Tryptic cleavage in both VDII and VDIV but not in VDIV alone decreases Chlamydial attachment to HeLa cells, which suggests that these domains are conformational MOMP structure that are dependent on the integrity of these domains (Moulder et al, 1991).

The nucleotide and deduced amino acid sequences of four VDs are as follows (Yuan et al, 1989; Dean et al, 1992; Poole and Lamont, 1992). VDI was found to contain 60 nucleotide encoding 20 amino acids for all serogroup of B complex, 66 nucleotide encoding 22 amino acids for K and L3 and all C serovars of C complex, 63 nucleotide encoding 21 amino acids for serovars F and G. The nucleotide sequences of VDI regions of MOMP from serogroup B are highly conserved, with three to five substitutions per serovar in comparison with the serovar B prototype sequences. The deducted amino acid sequence of VDI of sero groups of B serovars were also highly conserved, with only two to four substitutions per serovar: most of the substitutions occur within a 12-aminoacid region that resides in the central portion of the domain. In contrast, serovars in sero group C and K and L3 showed greater nucleotide and amino acid variations within VDI. In comparison with results for VDI of serovar C, the other sero group C serovars showed one to none nucleotide substitutions that results in zero to four amino acid changes. Again most of the substitutions occurred in the central regions of the domain. VDI regions of the serovar F and G were identical in their nucleotide and amino acid sequences.

VDII contained 63 nucleotide encoding 21 amino acids in MOMP of serovars D, E, L1, F and G. VDII of the remaining 10 serovars had 66 nucleotide encoding 22 amino acid. Comparison of the MOMPs of all serovars show that VDII had more sequence variation than did VDI. In comparison with serovar B, other sero groups of B serovars had between 2 to 19 nucleotide substitutions resulting in 2 to 8 amino acid changes. The least amount of variation occurred between serovars B and Ba, in which

there were two nucleotide substitutions that each resulted in an amino acid substitutions. Most of the amino acid substitutions occurred within an eight-amino acid region in the central part of the domain. VDII sero group C serovars also showed considerable sequence variation, with four to six amino acid changes. A similar amount of variation was found in VDII sequences between serovars F and G of the intermediate sero group. In comparison with serovar F VDII sequences, the sequence of serovar G VDII had 6 nucleotide substitutions resulting in 3 amino acid changes.

VDIII was the smallest and least variable domain among serovars. It contained 42 nucleotide encoding 14 amino acids. The sequences of serovars A, H, J and L3 were identical to each other. Serovars D, L1 and F had sequences identical to each other, differing from that of serovar B by three amino acid substitutions. The remaining serovars had variation in VDIII that does not group serovars into serogroups.

VDIV was the largest domain, containing 96 nucleotide encoding 32 amino acids in serovars A and I and all sero group B serovars. This domain could be separated into three distinct regions on the basis of sequence homology. Within serogroup B, serovars B and Ba had identical amino acid sequences in the N-terminal regions as did serovars D and E. There were three amino acid substitutions between B and Ba and serovars D and E. Serovars L1 and L2 contained one to two, respectively, amino acid respectively, additional substitutions in these regions.

The serovars of *C .trachomatis* could be separated into three groups on the basis of the amino acid homologies of their VDs. Group 1 consisted of serovars B, Ba, D, E, L_1, L_2 . Group 2 consisted of servers F, G and Group 3 consists of serovars A, C, H, I, J, K, L_3.

A mechanism for MOMP function in the attachment process and possible identification of the host ligand have been provided by studies of recombinant maltose binding protein (MBP)-MOMP fusion. An excess of heparin sulfate but not chondroitin sulfate inhibited MBP-MOMP bound specifically to human epithelial cells at 4^0C but was not internalized upon shifting the temperature to 37^0C. Binding to HeLa cells was inhibited by removal of cell surface proteoglycans by heparinase treatment but not chondroitinase treatment, and mutant Chinese Hamster Ovary (CHO) cell lines defective in glycosaminoglycan synthesis displayed reduced binding of both MBP-MOMP and native EBs. Primers targeted against the conserved region of MOMP gene are used in PCR techniques to amplify DNA of *C. trachomatis* from clinical specimens (Bobo et al, 1991; Palmer et al, 1991; Talley et al,1992).

PLASMID OF *CHLAMYDIA TRACHOMATIS*

The obligate intracellular bacterium *Chlamydia trachomatis* has a genome comprising of 1.000kb of chromosomal DNA and 5 to 10 copies of well-conserved plasmid named as PCT and was described as PCHL1, PCTTL and PLGV44 (Comanducci, 1993). The plasmid of *C. trachomatis* is found in all isolates of human pathogens. The plasmid appears to be under positive selective pressure in the natural habitat of *C. trachomatis* essentially epithelial cells and mucosal tissues of humans. The plasmid was first identified in 1980. *C. trachomatis* (Stothard et al, 1998) plasmid occur in 5 to 10 numbers and are about 7.5 Kilo bases (kb) in size. The plasmid contains eight open reading frames (ORF's), the product of some of which bear similarity to known proteins for example ORF 1 contains a dnB-like gene: ORF 3 codes for a 28 KD protein antigen and ORF 8 encodes a hypothetical recombinase-like protein (Comanducci et al, 1988). However the functions of the eight PCT genes have not been characterized. The evolutionary preservation of the plasmid and its nucleotide sequence however suggests that it is vital to *C. trachomatis*. Functions of some of the ORF's and location of transcription for the 7.5kb PCT have been studied very well.

Nucleotide sequence analysis of cryptic plasmid of *C. trachomatis*

Complete plasmid genomes from eight serovars of *C. trachomatis* was isolated in *E.coli* as cloned sequence ligated to pBR322. Restriction enzymes cleavage site mapping indicated that these plasmids were closely related. Homologous plasmid sequences have been detected by DNA hybridization in all of the 200 clinically isolated strains of *C. trachomatis* which have been examined. DNA sequences homologous to the *C. trachomatis* plasmid were not found in eukaryotic DNA nor in a plasmid of similar size isolated from *C. psittaci* (Palmer,1986). *C. trachomatis* plasmid genes are expressed *in vivo* and the plasmid encoded gene products may play a role in the intracellular growth of this organism (Palmer,1986).

Transcription of the 7.5 kb plasmid of *C. trachomatis* serovar L2 was investigated (Fahr et al, 1992). Faint, diffuse transcripts of about 1.6 and 2.2 kb and intense short transcripts of about 250 and 430 bases were identified by Northern blot analysis (Fahr et al, 1992). The short transcript were found to have a common 5' end corresponding to bp 501 relative to the unique *Bam* HI site of the plasmid and to terminate at different downstream sites. Putative promoter sequence of TTGCCA and TATATT, which closely resemble the consensus recognition site of *E. coli* sigma 70, were identified at the 35 and 10 positions upstream from the 5' end of the short transcripts made in Chlamydia. Transcripts of similar size was also expressed from this promoter in *E.coli* harboring a recombinant plasmid encoding the short transcripts. The short transcript encode a common open reading frame of 34 codons; however, a strong ribosome binding site was not found in the vicinity of the initiator codon, and is not known whether the transcripts are translated *in vivo*. Large ORF of 330 codes, which has been shown to encode a hypothetical protein containing conserved domains of recombinase-like proteins, is anti sense to the short transcripts. Transcripts encoding the large ORF could not be detected directly by Northern blot or primer extension analysis.

Evidence of involvement in DNA replication

The plasmid was cloned at the *Bam* HI site into *E .coli* and the recombinant plasmid was designated PCTL1. A detailed restriction endonuclease map of PCTL1 was constructed (Hatt et al, 1988). A fragment of the chlamydial plasmid was shown to function as promoter in *E. coli* when placed upstream of the lac Z B gene. The entire plasmid was sequenced by chain termination method. Open reading frames were identified from the resulting consensus sequences together with a candidate for the plasmid origin of replication consisting of four perfect tandem repeats of a 22 bp sequence, an A: T rich sequence and an open reading frame which could generate a 34.8 kDa product. The predicted polypeptide products of the open reading frames were compared by computer with all reported protein sequences. Homology of the predicted polypeptide product of an open reading frame to the *E.Coli* dnaB protein and the analogous product of gene 12 bacteriophage P22 is described (Hatt, 1988).

Location of origin of replication for the 7.5kb *C. trachomatis*

The hypothetical origin of replication for the 7.5 kb plasmid common to *C. trachomatis* is believed to be in a region of the plasmid that contains four 22-bp tandem repeats preceded by an A-T region. To test this hypothesis, replication of the plasmid DNA in metabolically active reticulate bodies of the LGV biovar of *C. trachomatis* was examined by electron microscopy (Tam et al, 1992). The results showed that the origin of replication was near the tandem repeats of plasmid. The replication of the plasmid was unidirectional, and the copy number during replication was 5-10 (Tam et al, 1992).

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1988). But laboratory isolates of *C. trachomatis* lacking plasmid supports the view that plasmid is not necessary for the survival of this organism (Peterson et al,1990;Stothard et al ,1998).

METABOLISM

Given the obligate intracellular parasitism of Chlamydiae, it was unexpected to discover a diverse set of genes that appear to provide considerable metabolic capabilities. The central metabolism determined for *C. trachomatis* was found to be conserved (Stephens, 1998; Stephens et al, 1999). This delineates a common basis required for growth in this intracellular environment. Illustrated are capacities for transport of essential nutrients, including two porins, OmpA and OmpB (predicted), and inner membrane transport systems for ions and amino acids. It appears that chlaymadiae use fewer transport systems than free-living bacteria and rely on transport systems with broad specificity. Some transport systems that are highly conserved in other bacteria were conspicuously missing in the chlamydial genome. Both genomes lacked phospho phenyl pyruvate transport system permeases and the phosphate transport operon that is found even in archaea. The phosphate transport function appears to be provided by an alternative phosphate permease (CT694). Likewise, no homologs were found for bacterial siderophores or sideropohore receptors that bind and transport iron, suggesting that chlamydiae obtain iron from host cell pools (Stephens, 1999).

Chlamydiae have been called "ATP energy parasites" because they have been thought to obtain ATP entirely from their host cells (Iliffee-Lee and McClarty, 1999; Moulder, 1991). Both genomes contained two paralogs (evolutionarily related genes within a genome) for ADP/ ATP translocases, which support the acquisition of ATP, and perhaps GTP, from the host cell (Hatch, 1988). One of the most surprising findings was the constellation of identified gene assignments that suggests

Chlamydiae are capable of substrate level and oxidative ATP production and reducing power and may not be strictly auxotrophic for ATP. The presence of an apparently complete glycolytic pathway was complemented by a tricarboxylic acid (TCA) cycle. Although the TCA cycle pathway was incomplete, it is sufficient to function potentially as a branched pathway or is fed by uptake of glutamate and decarboxylaser for which transporters present (Iliffee-Lee and McClarty, 1999). Reducing power generated by these central pathways is used, as evidenced by the presence of complete complexes for NADH oxidoreductase, cytochrome, and a V-type ATPase. In addition to superoxide dismutase (Koshiyama and Stephens, 1988), protection from oxygen intermediates is mediated by a thiol-specific antioxidant that is common to several microbial pathogens and has both thiol antioxidant and peroxidase activities (Yim et al, 1994). It is likely of significance in terms of the inclusion environment that most of the chlamydial transport systems, the NADH oxido reductase, cytochrome, and a V-type ATPase, each have high homologies to systems that use sodium ions rather than hydrogen. Thus, the Na+/K+ differential within eukaryotic cells has been exploited by chlamydiae. Moreover, the use of Na+/K+ rather than H+ may be related to the lack of acidification of the inclusion during chlamydial growth. *C. trachomatis* synthesize folio and are sensitive to sulphonamides (Fan et al,1992).

Infection and disease epidemiology

Human infections caused by *C. trachomatis* includes trachoma, a blinding disease, paratrachoma or adult inclusion conjunctivitis, ophthalmia neonatorum and sexually transmitted diseases.

Trachoma, a chronic follicular conjunctivitis is the leading cause of preventable blindness in the world (Adu-el-Asrar et al, 1998). Trachoma, which once had a much wider distribution but still has a potential for affecting 400 million people who live in areas where the disease is endemic. It is still considered to be the world's

leading preventable cause of blindness, which about 6 million people blinded as a result of this disease (Thylefors et al, 1995). About 5.5 million people are blind or at risk of blindness as a consequence of trachoma (Thylefors et al, 1995).

The name "trachoma" was first introduced in AD 60 and referred to the "roughness of conjunctiva". The disease was endemic in many parts of the world (Tabbara, 1996a). But during the last century, the disease has disappeared from many parts of the world due to improvements in the standard of living and of hygienic practices. The disease remains endemic in hot, dry climates and a country where there is a dramatic population increases.

Areas where trachoma is endemic, conjunctival epithelium of children is the main reservoir of infection. Disease spread from eye to eye by direct contact. Based on human volunteer studies, the incubation period is reported to be 5 to 12 days (Bowie, 1990).

Transmission may be potentiated by flies. These flies are moisture seeking and feed on ocular discharge. In countries like Egypt seasonal epidemics of bacterial conjunctivitis precede the peak of trachoma infectivity by a few weeks. The prevalence of trachoma among children is essentially 100 percent in areas of hyper endemicity. Active infection becomes infrequent by adulthood, but sequelae continues to progress and causes corneal scarring (Schachter, 1999).

Trachoma is endemic primarily in tropical and subtropical countries like Middle East and northern part of India. The disease is associated with poverty and poor hygienic conditions (Schachter, 1999).

The world prevalence of trachoma is 150 million and most of them are children. About 5.5 million people are blind or at risk of blindness as a consequence of trachoma (Mabey et al, 2001).

Clinical features

Initially there is congestion, edema in both palpebral and bulbar conjunctivae with associated papillary hypertrophy prominent in palpebral conjunctivae. Then there is follicle formation. On rupturing of the follicle, a shallow pit termed, Herbert's pit is formed (Gordan, 1996; Schachter, 1999).

Immunity

Reinfection is necessary for severe blinding trachoma and if so what is the role of immunity. Infection in humans and in experimental animals produces abundant local and circulatory immune responses, but the source of the protection is not known. Cytoplasmic IL-1 alpha and IL-1 beta expression was noted in the conjunctival epithelium in trachoma specimens and Tumor Necrosis Factor (TNF)-alpha was detected in macrophages (Brunham, 1999). So the up regulated local production of IL-1 alpha, IL-1 beta and TNF-alpha are attributed to conjunctival damage and scarring (Bobo et al, 1996; Abu-el-Asrar et al, 1998). In experimental animals, after infection there is a short period of immunity to reinfection with homologous strains (but little immunity to heterologous rechalleng). In studies that were made, it was recognized that with relatively crude and antigenically weak vaccines, there was transient immunity to challenge (Brunham, 1999). When this immunity waned, not only were the experimental animals susceptible to reinfection, but also there was more severe disease after challenge. It was also suggested that there occurs a suppression of Chlamydia-specific IgA antibody-secreting cells, during the proinflammatory response to ocular chlamydial infection in trachoma (Ghaem-Maghami et al, 1997).

The epidemiology of trachoma continues to provide hope for those trying to produce an effective vaccine for trachoma. In all cases studied, active trachoma decreases and with age. Although the age, at which this decrease occurs, varies with

the intensity of the disease in the population, the older segment of the population eventually has little active disease, suggesting that immunity eventually prevails (Brunham, 1999). Proponents of vaccine development thus aim to create a vaccine that would mimic that natural occurrence by immunizing with protective antigens while excluding the harmful sensitizing antigens. Using genetic engineering, it has been possible to insert chlamydial genes into poliovirus vaccine (Murdin et al, 1993). By use of serovar-specific portions of the major outer membrane protein (MOMP), it has been possible to induce neutralizing antibodies in mice and monkeys. Thus, models have been created which allow for development of vaccines that present potentially protective chlamydial antigens while deleting the sensitizing antigens that may contribute to disease. Such vaccines have been targeted for prevention of pelvic inflammatory disease as well as trachoma. None of these vaccines have been evaluated in humans, but they have not produced the type of protection observed in the monkey studies that used cruder vaccines for a trachoma vaccine. So the ultimate goal of current chlamydial vaccine efforts is to utilize either conventional or modern vaccinology approaches to produce a suitable immunization regimen capable of inducing a sterilizing, long-lived heterotypic protective immunity at mucosal sites of infection to curb the severe morbidity and world wide prevalence of chlamydial infections (Igietseme et al, 2002).

For elimination of trachoma, a single dose of oral azithromycin, an azalide antibiotic is given than long courses of topical tetracycline treatment (Tabbara et al, 1996b; Schatcher et al, 1999; Fraser – Hurt el al, 2001). But azithromycin is relatively expensive; the other commonly used antibiotic doxycycline requires longer dosing and is contraindicated in pregnant women in children below 6 weeks (Dawson and Schachter, 1985). Macrolides are also recommended for the treatment of trachoma, but they also require long-term dose of treatment and the intestinal side effects are more (Schatcher, 1999).

Genital infections

Trachoma biovars D, E, F, G, H, I, J, K of *C. trachomatis* infects superficial columnar epithelial cells in endocervix, urethra, epididymitis, endometrium, oviduct, conjunctiva, nasopharynx, lower respiratory tract. *C. trachomatis* does not invade deeper tissues (except the LGV biovars). Infection due to *C. trachomatis* in columnar epithelial cells induces a brisk inflammatory response but consequences of chlamydial infection seem to be produced by host inflammatory cells (Schachter, 1999). Infections due to *C. trachomatis* might be asymptomatic. In women, urogenital infections results in a broad spectrum of clinical manifestations including urethritis, cervicitis and pelvic inflammatory disease leading to serious complications such as ectopic pregnancy and tubal infertility (Smith et al, 1989; Gilbert et al, 1996). In men urogenital infection causes urethritis and rarely ascending infections like epididymitis (Mounton et al, 1997; Morre et al, 2000). Salpingitis is the most important genital infection caused by *C. trachomatis* in women as it causes infertility (Lan et al, 1995).

Serum antibody levels have been seen in 90 percent of men and 99 percent of women (Schachter, 1999). Presence of antibody alone cannot a protective factor. *C. trachomatis* is the most common STD agent in the women (Corey, 1984). The rate of infection among women is more than among males, because of the fact that females are mostly affected of infection. *C. trachomatis* infection is seen among people from all strata of society, but the higher infection rates reported among young people and the poor (Schachter, 1999).

Incubation period for *C trachomatis* genital infections varies with the specific site. For urethritis it is 5 to 14 days (Bowie, 1990). But for cervicitis no definite incubation period is observed. In case of salpingitis, first the cervix gets infected and is followed by the tubal infection with in 2 to 3 weeks. Incubation period for conjunctivitis in infants varies from 5 to 19 days (Hammershlag, 1996). But in ophthalmia neonatorum, the infection starts in the 2nd week of infection (Hammershlag, 1996; Flach, 1996).

Clinical feature

When an individual is infected with *C. trachomatis*, polymorphonuclear inflammatory response is observed first. This is followed by sub epithelial infiltration of lymphocytes, plasma cells, monocytes (Schachter, 1999). During this stage inclusion are observed in the cervix, urethra. Redness, mucopurulent discharge and photophobia are the clinical features observed in individuals having inclusion conjunctivitis with mild swelling of the eyelid. A preauricular lymph node may be palpable. There is hyperemia, chemosis in bulbar conjunctiva and follicle formation is seen in palpebral conjunctiva, superficial punctate keratitis is observed in the cornea. A small micropannus measuring 1 to 2 mm may be observed. Tiny subepithelial infiltrates may be seen near the limbus (Tabbara, 1996a; Dawson, 1996).

In case of urogenital infections the patient complaints of genital discharge with associated pain (Bowie, 1999).

The common symptoms associated with endocervicitis due to *C. trachomatis* are discharge with edema, erythema or induced mucosal bleeding of the cervix (Sellors et al, 1998). Considerable percentage of the patients with a chlamydial conjunctivitis have been reported to have concomitant asymptomatic genital Chlamydial infection (Postema et al,1996).

Infection of urethra due to *C. trachomatis* may be symptomatic or asymptomatic. *C. trachomatis* has been isolated from 0.11 percent of asymptomatic sexually active men (Podgore et al, 1982; Bowie 1990). It is reported that 30.50 percent of men diagnosed to have non-gonococcal urethritis (NGU) are culture positive for *C. trachomatis* (Bowie, 1990).

Non-gonococcal urethritis due to *C. trachomatis* causes less dysuria, less profuse and less purulent urethral exudate than gonococcal infections (Gilbert, 1996).

Non-gonococcal urethritis cannot be differentiated clinically from gonorrhea. Clinical symptoms include itching, dysuria and a white mucoid discharge that typically occurs 1-3 weeks after sexual exposure. Signs include tenderness and urethral discharge. Diagnosis of *C. trachomatis* NGU requires documentation of leucocyte exudate as well as exclusion of urethral gonorrhea by Gram stain and culture. Presence of 15 or more leucocytes per high – power field in the urine sediment is suggestive of urethritis (Bowie, 1990; Schachter, 1999).

Epididymitis is responsible for the passage of semen into urethra through vas deferens. *C. trachomatis* is the usual cause of epididymitis in sexually active young men. Presence of urethral discharge in association with epididymitis suggests the diagnosis of Chlamydial infection. Serious complications include abscess formation, testicular infarction, chronic pain and infertility (Schachter, 1999).

Among homosexual men, non-LGV serovars of *C. trachomatis* have been associated with proctitis (inflammation restricted to rectum), while LGV serovars are less associated with proctitis and Proctocolitis (Smith, 1989). Symptoms of proctitis due to non-LGV strain include mild-to-moderate rectal discharge, mild anorectal pain, tenesmus and constipation. Rectal mucosa is erythematous and bleeds easily when swabbed. With LGV serovars the clinical symptoms are severe. Rectal mucosa is ulcerated and shows hemorrhages with accompanied fever and inguinal lymphadenopathy (Bowie, 1990).

C. trachomatis is an important cause of cervicitis. Patients with cervical infections due to *C. trachomatis* experience urethral symptoms. Frequency of urination, dysuria are associated with urethral chlamydial infections. Women with cervicitis have erythematous vaginal mucosa, hypertrophic cervical erosion and purulent or mucopurulent cervical discharge.

Pelvic inflammatory disease (PID)

Infection due to *C. trachomatis* may spread through endometrium and cause infection of the fallopian tube (Gilbert 1996). The chances of sterility due to *C. trachomatis* infection of the fallopian tube are 25 percent after the first attack and 75 percent after the third episode. PID develops in 1 percent of young women in addition. Risk factor that pre-dispose a women to develop PID are use of certain contraceptive devices, multiple sex partners, and chlamydial, and gonococcal infections (Schachter, 1999). A broad spectrum of clinical signs and symptoms can be associated with PID, but mostly the history of lower abdominal pain, cervical motion tenderness, and adnexal tenders are the most important clinical symptoms association with PID.

In addition to the above symptoms, one of the following findings may be associated with PID; fever, leucocytosis elevated erythrocyte sedimentation rate; inflammatory adnexal mass on sonography or culdocentesis revealing bacteria and while blood cells in the peritoneal fluid. From inflamed fallopian tubes, *C. trachomatis* may spread to the liver surface and cause peri hepatitis, women with peri hepatitis presents with fever, severe right upper guardrant pain, tenderness and spasm of the abdominal wall.

Children born to mothers with cervical *C. trachomatis* infection can develop pneumonia, conjunctivitis due to transmission of the organism from infected birth canal, some infants have conjunctivitis in addition to lower respiratory tract infection (Bowie, 1990; Flach, 1996). One or both eyes may be infected with lacrimation, profuse mucopurulent discharge and swelling of the lids (Seal, 1998).

In 1980, *C. trachomatis* was recovered from sputum, lung biopsy, bronchial washings and nasopharynx specimens collected from 6 patients with clinical diagnosis of acute bronchitis and severe interstitial pneumonia. (Tack et al, 1980).

Inclusion conjunctivitis

In patients with inclusion conjunctivitis, respiratory tract infection, otitis media are also seen. LGV conjunctivitis is a rare conjunctival infection caused by serotypes L1, L2, L3 and infected individuals have severe swelling erythema of the eyelids. Large granuloma is seen in the conjunctiva with follicular hypertrophy, hyperemia, and chemosis. Large periauricular lymph node is observed (Tabbara, 1996a).

Treatment

Oral tetracyclines 500mg three times daily for 4 weeks or curative doxycycline 100mg orally twice daily may be given for a period of 4 weeks. Oral azithromycin may also be given. Doxycycline 100mg orally twice for 3 weeks or 1000mg single dose may also be given. Topical tetracycline ointment may be given for eye infections (Darougar et al, 1981; Schachter, 1999). Azithromycin and doxycycline are recommended for treatment of genital infections (Lau et el, 2002; Adimra, 2002). A single dose of azithromycin have been proven to a more effective and convenient treatment on comparison with Ciprofloxacin and doxycycline for *C. trachomatis* genital infections (Rustomjee et al, 2002). Because of azithromycin's less frequent side effects and improved adherence, it is used to treat pregnant women with uncomplicated *C. trachomatis* infections (Adimra, 2002).

Pregnant women should not be given systemic tetracycline. But oral erythromycin may be given. Neonatal inclusion conjunctivitis can be treated with erythromycin 50mg/kg/day in 4 doses and topical therapy consist of tetracycline 1.0 percent ointment or erythromycin 5mg per gram ointment twice daily for 2 weeks.

Prevention: Vaccines are not available for chlamydial infection.

Immunity to *C. trachomatis* infections

Based on human challenge experiments and vaccine trials it was inferred that *C. trachomatis* infection induces immunity in humans.

Vaccine trials demonstrated that immunization intramuscularly with whole inactivated chlamydial bacterial cells was also successful. This was proved by a trail conducted by Grayston and Wang (1978) on 332 children. These children were initially free of trachoma but at high risk of acquiring active trachoma. They were followed up to 3 years to observe signs of development of disease. Vaccinated individuals had a 50 to 73 percent reduction in trachoma incidence. But by the third year, this protection waned and became undetectable. In a study conducted in primate models of ocular infection, it was demonstrated that vaccination induced only strain specific immunity. This finding was further supported by studies conducted by Taylor et al, (1990), Brunham et al, (1996) on virulence and immunity.

Among the serovars, LGV serovars are the more virulent strains than the others. Virulence is marked by faster in vitro growth and greater capability for systemic dissemination of L serovars than other serovars. Horizontal transmission of pathogens with a high probability of multiple infection of a common host evolves towards virulence. Repeated exposure of chlamydial infection among sex workers selects virulent L serovars infection and that CD 4 T cell responses are particularly important for immunity against these strains (Brunham, 1999).

C.trachomatis activates complement and gets inactivated by a normal serum lysozyme found in mucosal secretions can act on *C. trachomatis.* Chlamydia infected cells produce gamma interferon and Interferon (IFN)-β and growth of chlamydia are inhibited by interferon (Rottenberg et al, 2002). Chlamydial infected cells also produce number of cytokines and chemokines other than interferon. Following are some of cytokines produced Chlamydial infected cells interleukin 8 (IL-8), GRO-2

sxpan, granulocyte-macrophage colony stimulating factor (GM-CSF), 1L-6, 1L-1B and procoagulant and antifibrolytic factors (Lu et al, 2002). These cytokines are produced only from cells, where replication of *C. trachomatis* is going on. Cellular contact with inactivated chlamydial does not evoke this response. Many of these cytokines are transcriptionally activated by NF-KB 1L-8 particularly relevant among the several cytokines produced by chlamydial infected cells since 1L-8 is a strong chemo attractant for neutrophils, which would accumulate at sites of chlamydial infection. *C. trachomatis* gets readily inactivated by both oxidative and non-oxidative mechanisms and thus 1L-8 production is a potent host defense against chlamydial infection (Rottenberg et al,2002)..

Chlamydial lipopolysacchirde (LPS) is less toxigenic than enterobacterial LPS, but this adaptation does not help the organism form innate defenses. Quick, efficient entry of the organism into epithelial cells provides sanctuary for this organism from innate defenses. Moreover chlamydial infection may not induce expanded natural killer cell activity and thus infected cells escape detection by innate cellular defenses. Histopathologic studies with biopsy samples from conjunctivae of active and inactive cases of trachoma, cervical, endometrial, fallopian tube tissues showed infiltration of lamina propria by activated T and B lymphocytes, histocytes, macrophages and lymphoblasts. Neutrophilic exudation into the mucous layer was also seen. Epithelial cells infected with Chlamydia express Major histocompatibility complex (MHC) class I molecules. Since the organism grows within a membrane bound vacuole, chlamydial peptides are not freely available to class I molecules. Chlamydial infected cells are unlikely to be antigen- presenting cells to native T cells since they lack co- stimulatory molecules. However it is known that activated CD8 and effector CD4 T cells can recognize MHC presented peptides on cells other than professional antigen-presenting cells. If chlamydial peptides are able to access class I and class II molecules, effector T cells may be able to target infected cells directly (Bowie, 1990).

At sites of chlamydial infection, professional antigen-presenting cells are present in abundance. These include macrophages, dendritic cells, B-lymphocytes. Dendritic cells do not appear to support chlamydial replication but quickly route the chlamydial endosome to lysosomes for antigen processing and class II presentation. Chlamydial-pulsed dendritic cells and they give protective immune response in murine model (SU et al, 1998).

T lymphocyte responses: T lymphocyte responses play a central role in host resistance to chlamydial infection and class II restricted CD 4 T cells are essential for immunity to chlamydial infection (Morrison et al, 2000). Persistent chlamydial infection is best seen among individuals having tubal infertility. In individuals with tubal infertility chronic infection is best seen and chlamydial DNA are found in macrophages and smooth muscle cells deep within lamina propria. As macrophages, smooth muscle cells are long-lived cells and not shed by epithelial cell turnover. The organisms are not in a replication –competent state and can be detected only by nucleic acid replication methods. These organisms express reduced amounts of MOMP protein and sustained amounts of Heat shock protein 60 (Hsp60) (Ward, 1999). Chlamydia possesses a powerful anti apoptosis mechanism and may be able to create a long-lived sanctuary within cells through inhibition of mitochondrial cytochrome C release and prevention of programmed cell death.

The basis for chlamydial immunity appears to depend on soluble Immunoglobulin A (IgA) and IFN-gamma responses that facilitate rapid clearance of infected epithelial cells. IgA binds to surface determinants of the chlamydial MOMP and interfere with nutrient acquisition at intracellular sites or cell attachment in the extracellular environment. T cells then collect beneath the epithelium and secrete IFN-gamma. IFN-gamma alters epithelial cell biochemistry and limits chlamydial metabolism in part through altered nutrient acquisition. Thus intracellular and extracellular IgA and IFN-gamma appear to alter cellular and chlamydial metabolism and thereby interact to maximally inhibit chlamydial growth and accelerate clearance of infection (Brunham, 1999).

LABORATORY DIAGNOSIS OF INFECTION

Direct Examination

Giemsa stain

Epithelial cell scrapings/swabs obtained from genital or ocular sites are transferred to a glass slide and the presence of intra epithelial cytoplasmic inclusions is considered to be a positive diagnostic Giemsa test (Sheppard et al, 1988). Traditionally, the Giemsa stain has been the standard by which the efficiency of all other methods was measured. The Giemsa stain requires substantial experience and time (20-30 min per slide) to interpret results and is therefore not applicable to most diagnostic facilities. Further more, if Giemsa-stained cell are not examined with an oil immersion objective (1000X), many inclusions may be missed. The sensitivity of the Giemsa stain for the diagnosis of chlamydial infections stain inclusions produced by both *C. trachomatis* and *C. psittaci* depends upon the site from which the specimen was obtained and it is reported to be 43 percent (Sheppard et al, 1988). Only 15 percent (male urethra) to 41 percent (female cervix) of genital infections can be diagnosed with the Giemsa stain, but 95 percent of conjunctival specimens from infants with chlamydial infections are positive (Smith 1989; Baveja, 1997).

Iodine stain

This stain is not recommended for the detection of *C. trachomatis* infections in cell scrapings because of poor sensitivity (15%) and specifically, since other cells-particularly those in cervical preparations-may have endogenous glycogen yielding false-positive results (Gilbert, 1996)

Papanicolaou stain

This stain has been used for over 40 years to identify changes in cervical epithelial cells that indicate malignant cells. More recently, the cytomorphology of *C.*

trachomatis infection in cervicovaginal smears by three stages (Smith, 1989). In the first stage, fine uniformly-sized acidophilic and/or basophilic coccoid bodies may be distributed randomly in the cytoplasm but they tend to localize in the perinuclear region. The cytoplasm associated with the bodies is porous. In the second stage, cytoplasmic vacuoles with clearly defined membranes occur; they may be small with central homogenous condensations surrounded by a halo, or they may be larger coccoid structures randomly dispersed in the vacuole. In the final stage, intracytoplasmic vacuoles develop that generally contain a target-like condensation surrounded by a halo.

Immunofluorescence (IF) Using Monoclonal Antibodies (mabs)

Mabs prepared against the major outer membrane protein of *C. trachomatis* have been conjugated with fluorescein and distributed in a protein-stabilized buffer solution containing the counter-stain Evan's blue (MicroTrak, Co., Palo Alto, CA). These monoclonal antibodies are available as kit with *C. trachomatis*-positive and negative control slides. In addition, specimen collection kits are available that contain acetone in a glass ampoule with disposable dispenser, a single-well slide that allows specimen and reagent concentration for optimal sensitivity, large and small swabs, and a transport container with instructions for specimen collection.

After the slides have arrived in the laboratory, the smears are fixed with acetone and then stained with the fluorescein-conjugated Mabs to *C. trachomatis* at room temperature in a moist chamber. After a short incubation period (15 min, room temperature), the slides are examined with a fluorescence microscope for the presence of elementary bodies of *C. trachomatis.* They appear as individual pinpoints of medium to bright, uniform apple-green fluorescence that have a smooth edge and are disc shaped. Other forms of the organism may also be present. Some Chlamydia (approximately 2-3 times the size of an immature body) may stain with a peripheral halo. These represent immature organisms; reticulate bodies released from ruptured

inclusions. With the MicroTrak kit, finding at least 10 chlamydial elementary bodies make a positive laboratory diagnosis. This criterion has been established in order to ensure accurate reading of specimens (Smith, 1989).

Criteria for identification of *C. trachomatis* by the direct IF technique. Interpretation of IF results is subjective; therefore, the laboratory must strictly adhere to definitive criteria in order to eliminate false-positive reports.

1. Fluorescing material is only accepted as positive if it is found in mucus or within cells.

2. More than one fluorescing body is needed per high-power field (40X objective) to qualify as positive.

3. The fluorescing body must be distinctly green (fluorescein color), not yellow (auto fluorescence)

4. The fluorescing bodies must be punctate or small and round. They must not be refractive.

5. A distinct body must not be visible at the point of fluorescence when viewed by transmitted or phase illumination.

6. Chlamydia fade with prolonged exposure to the exciting light whereas debris often shows little fading.

In addition to the above, one must realize that certain strains of bacteria (particularly staphylococci) that contain protein A can bind to the Fc portion of the Mabs and appear fluorescent. This problem has been recognized mainly with examination of rectal specimens. Chlamydial elementary bodies (300nm) are smaller and tend to stain more uniformly than bacteria, which tend to stain at the rim of the cell, producing a doughnut appearance.

Laboratory evaluations

Sensitivity of the IF test has ranged from 61-100 percent; however, 11 of 18 (61%) studies achieved sensitivities >90 percent. Similarly, specificity has varied from 63-100 percent with (72%) studies achieving specific results of >90 percent (Sheppard et al, 1988; Rapoza et al, 1990).

Direct IF tests, compared with Giemsa staining, cell culture methodology are rapid, inexpensive, do not require viable organisms, and the procedure can be incorporated into any laboratory that has a fluorescence microscope (Taylor et al, 1988; Rapoza et al, 1990; Chan and Cunnungham, 1994; Baveja et al, 1997). Disadvantages are that the direct test is less sensitive than culture and the amount of specimen material on the slide is often not adequate for examination. At this time, use of the IF technique has not been approved by the Food and Drug Administration for respiratory tract specimens.

Enzyme Immunoassay:

Chlamydiazyme (Abbott Laboratories, North Chicago, IL) was the first enzyme immunoassay (EIA) to be available commercially. For this procedure, endocervical or urethral swabs are placed in a transport tube containing 0.1ml of storage reagent. When received in the laboratory, 1ml of specimen dilution buffer is added to the tube containing the swab. The tubes are incubated for 10 min at room temperature and then agitated on a multi-tube Vortex apparatus for 3 cycles of 15 seconds each. The swab is then pressed against the side of the tube to remove excess fluid and discarded. Samples (200µl) of the swab extract are placed in a reaction tray wells of plastic plates and then reacted sequentially with a treated polystyrene bead, rabbit antibody to *C. trachomatis* and horseradish peroxidase-conjugated antibody to rabbit IgG. After addition of o-phenlyenediamine and H_2O_2 substrate and H_2SO_4 to stop the reaction, the optical density is measured with a spectrophotometer.

Laboratory evaluation

In general, EIAs have resulted in sensitivities that range from 64-100 percent compared with cell culture recovery of the agent (Mabey et al, 1987; Gann et al, 1990; Mohanty et al, 1996) however, most studies have reported performance characteristics that are comparable to those carried out by the Micro Track fluorescence assay. It should be emphasized that not all studies were performed using the chlamydiazyme assay, and the method employed for the cell culture recovery of *C. trachomatis* will greatly influence the performance characteristics of the assay (Wilson et al, 1986; Rahi et al, 1988; Wiesmeier et al., 1988, Van Dyck, Ieven et al, 2001)

Decision for use of antigen detection methods (direct IF or EIA) will depend on the size and capabilities of each individual laboratory and the prevalence of chlamydial infections in the population that they support. Generally, EIA is more suitable for those laboratories that process many specimens, since the procedure is somewhat automated and objective. On the other hand, IF technology is more adaptable to facilities testing only a few samples each day; however, more technologist time per sample is required for subjective interpretation of each cell preparation on individual slides.

Isolation and Identification of the Agent

C. trachomatis from clinical specimens was earlier isolated by inoculation into yolk sac of chick embryo. Growth of Chlamydia in yolk sac cell cultures can be detected by staining with Giemsa, Machiavello, Giemenez stain inclusion may also be stained by immunofluorescence with an antiserum directed against the group antigen. Sensitivity of yolk sac inoculation for the isolation of strains is low and serial 'blind' passage is sometimes required which is time-consuming, expensive and open to the

danger of cross-contamination. Chlamydiae will also grow in cell culture, particularly if their penetration into cells is aided by centrifugation of the inoculum onto the cells. The introduction of irradiated McCoy, HeLa or (Baby Hamster Kidney) BHK cells for the primary isolation of *C. trachomatis* has provided a much more sensitive alternative to yolk sac culture (Smith, 1989).

Preparation of cell culture

McCoy, BHK-21 or HeLa cell lines are trypsinized and cells are planted into glass shell vials containing a piece of cover slip in Eagle's minimal essential medium (MEM) that contains 10% fetal calf serum. Monolayers should develop in 24 hours. At the end of 24 hours the cells are treated with cycloheximide at a concentrations of 1-3µg/ml for 24 hours. The specimen is then inoculated onto shell vials after aspirating out the growth medium. The shell vials with the specimen are centrifuged at 3,000 rpm at room temperature. After centrifugation, 9ml of MEM containing 1µg/ml of cycloheximide is added to each vial. The vials are incubated at 35°C for 24-72 hours.

Staining of monolayers on cover slips

Growth of *C. trachomatis* is confirmed by Immunofluorescence. The methodology followed is as follows.

1. The medium is aspirated from each vial and the monolayer on the circular cover slip is rinsed with (Phosphate Buffered Saline) PBS.

2. The cover slips are rinsed with cold absolute methanol and then cover slips are fixed in methanol at room temperature for 10 min.

3. Methanol is aspirated and the cover slips are allowed to air-dry for 15 min.

4. Fluorescein-conjugated monoclonal antibody are added to each vial and incubated at room temperature for 30 min.

5. At the end of 15 minutes the cover slips are washed with 4 ml of PBS thrice.

6. Cover slips are mounted with a mounting fluid.

7. The cover slips are examined at 100X to 400X magnifications. Positive results are indicated by the presence of distinct fluorescent inclusion bodies in the cytoplasm of infected cells (Madhavan et al, 1992; Madhavan et al, 1994).

Serological Diagnosis

Complement Fixation (CF):

This procedure detects antibody induced by the group antigen common to both *C. trachomatis* and *C. psittaci* (Schachter, 1976; Paavonen et al,1979). In systemic chlamydial infection, such as pneumonitis (psittacosis, infant pneumonia), LGV, and PID, the CF tests detect high levels of antibodies to these organisms (>1:16) (Schatcher, 1985). In uncomplicated ocular and genital infections, titers of about 1:16 are very unusual. Complement fixation lacks sensitivity and is recommended for evaluating other types of *C. trachomatis* infections.

Microimmunofluorescence (MIF)

The most sensitive serological procedure for detecting all chlamydial infections is the MIF assay (Rettig, 1986; Clad et al, 2000). The test requires growth of the chlamydial L2 serotype in embryonated hen's eggs or cell cultures followed by placing the concentrated antigen suspensions on a slide using separate pinpoint dots for each antigen, according to a specific pattern. Serial dilutions of sera from patients

are then placed over the antigen clusters. The reaction of antibodies, specific to each chlamydial serovar, are then measured using the indirect IF test (Kumar et al, 1991; Schmidt et al, 1992; Deak, 1994). Preparation of the test slides with the multiple antigens required for the assay is tedious, thus the procedure is limited to major chlamydial research laboratories (Schatcher, 1985).

Adenoviruses

Classification

Adenoviruses are non enveloped deoxyribonucleic acid (DNA) viruses 70-90 nm in diameter with a buoyant density in cesium chloride of 1.33- 1.34 g/cm 3 and a molecular weight by sedimentation of 170-175 X 10 6. DNA is linear, double-stranded with a molecular weight of 10^6 X 0-30 X 10^6 (Hierholzer, 1989).

Structure of the virus

The capsid proteins of Adenoviruses are arranged in an icosahedron having 20 triangular faces, 12 faces and 10 structural proteins. In each virion 240 hexons are dispersed on the triangular faces, edges and 12 pentons are located in the vertices of the icosahedron. Each penton contains a based called vortex capsomere and a fiber which is a rod like outward projections of variable length with a terminal knob. Adenoviruses are chloroform resistant and are stable for prolonged periods at 20 to 100°C in a pH environment of 6-9 (Hierholzer, 1989; Gordon, 1996).

There are at present 51 Adenovirus serotypes of human. Adenoviruses belong to the family Adenoviridae that includes two genera, Mastadenovirus and Aviadenovirus. These two genera do not immunologically cross react between them. Adenoviruses are classified into four major subgroups based on haemagglutinating properties (HA). The HA classification is based on complete agglutination of monkey or rat red blood cells (RBC), partial agglutination of rat RBC and absence of agglutination and secondarily on complete agglutination of human, chicken and other RBC.

Based on antigenic relationships, oncogenicity, fiber length, present DNA homology within and between subgroups, GC content of DNA, number of cleavage fragments after digestion with *Sma* I endonucleases and molecular weight of certain internal proteins human adenoviruses are classified into six subgroups (Table 1).

Diseases caused by Adenoviruses

Ocular infections caused by Adenoviruses are Pharyngo conjunctival fever (PCF), epidemic keratoconjunctivitis (EKC), Acute hemorrhagic conjunctivitis (AHC) (Asbell, 1996). Mild, non specific follicular conjunctivitis is the most common form of clinical presentation and are caused by serotypes 1,2,4 and 6. Epidemic keratoconjunctivitis (EKC) has an incubation period of 8-10 days and occur as sporadic or in epidemics and is most often associated with serotypes 8,19 and 37 and less often with serotypes 3,4,7,10,11 and 21 (Darougar et al, 1983; Aoki et al, 1982;O, Day et al, 1976).

PCF is caused by Adenovirus serotypes 1-7 and is usually seen in discrete out breaks among school-age children, young adults. Clinical symptoms include fever, pharyngitis, conjunctivitis, malaise and cervical lymph adenopathy with headache, diarrhoea and rash. Follicular conjunctivitis becomes evident 5-6 days after exposure with infected individuals shedding virus for about 10 days (Woshart et al, 1984).

Adenoviruses are non-enveloped viruses, which have distinct icosahedral morphology, 70-90 nm in diameter. The genome is 35.9 kilobase pair linear dsDNA that codes for 11 to 15 polypepetides.

Replication of the virus

Virus replication takes place entirely within the nucleus of the infected cell. Adenovirus replication occurs by genome transcription in the nucleus, followed by migration of (ribonucleic acid) RNA into cytoplasm. Here structural peptides are synthesized, after which the proteins migrate back to the nucleus where the viral DNA

TABLE - 1

Subdivision of Human Adenoviruses by Oncogenic, Haemagglutinating, and Morphologic Characteristics: Properties of Adenovirus Subgenera A to F'

Sub-group	Serotype	Oncogenicity	HA	Length of fibers	DNA		
					Homology (%)	G+C (%)	No. of small fragments after cleavage at CCC/GGG
A	12,18,31	High	Rat (incomplete)	28-31	48-69 (8-20)	47-49	4-5
B	3,7,11,16,34,35	Weak	monkey	9-11	89-94 (9-20)	50-52	8-10
	14,21	Weak	monkey	9-11	89-94 (9-20)	50-52	8-10
C	1,2,5,6	negative	Rat (incomplete)	23-31	99-100 (10-16	57-59	10-12
D	10,19,26,27,36,38,39	negative	Rat+H,M,G,D	12-13	95-99 (4-17)	57-60	14-18
	13	negative	Rat+H	12-13	95-99 (4-17)	57-60	14-18
	15,22,23,30	negative	Rat+H	12-13	95-99 (4-17)	57-60	14-18
	17,24,32,33	negative	Rat+H	12-13	95-99 (4-17)	57-60	14-18
	20,25,28,29	negative	Rat+H	12-13	95-99 (4-17)	57-60	14-18
E	4	negative	. Rat (incomplete)	17	(4-23)	57	19
F	40,41	negative	rat	Not detected	Not detected	Not detected	9

H-Human RBC, M-Guinea pig RBC, D-Dog RBC,

replication has occurred and the virus particles are assembled. The complete virion appears as intracellular inclusions. Although the usually recognized infection caused by adenovirus involves the surface mucous membranes reactive periocular inflammation, acute posterior multifocal placoid pigment epitheliopathy has been described. Adenovirus retinal pigment epithelial infection may be similar to the acute retinal necrosis (ARN) due to herpes virus group (Gordon et al, 1996). Jawetz and associates first isolated etiological agent of keratoconjunctivitis in 1955.

Treatment

The treatment of epidemic keratoconjunctivitis is mainly to palliate symptom. An ocular lubricant, topical vasoconstructors, cold or warm compresses and sometimes cytoplegic agents may be of benefit. Topical prophylactic antibiotic treatment is not necessary since the rate of bacteria super infection is exceedingly low. Topical steroid therapy in patients with severe conjunctival reactions with pseudomembrane formation does provide dramatic symptomatic relief with decreased inflammation and appear to result in a deceased number of corneal infiltrates during the convalescent period. Treatment of epidemic conjunctivitis with idoxuridine (Dudgeon et al, 1969), adenine arabinoside (Pavan-Langston et al, 1972) and trifluridine (Ward et al, 1993) has been unsuccessful, the disease progresses in spite of treatment with these agents. The ineffectiveness of these antiviral agents may be caused in part by the delay in the initiation of treatment. Laboratory studies in cell culture have demonstrated the effectiveness of trifluridine in decreasing replication of adenovirus types 8,13,19. Subsequent clinical confirmation is not recorded. Because the epithelial opacities are probably immunological in origin and the conjunctival disease is self-limited, no antiviral therapy is recommended at present for treatment of adenovirus epidemic keratoconjunctivitis. Chronic adenovirus keratoconjunticitis with several adenovirus serotypes have been reported. However the association of chronic adenovirus infection is infrequently recognized. The syndrome is associated with a

prolonged intermittent course, with the exacerbations associated with photophobia, tearing occurring after an episode of acute conjunctivitis. The cornea may or may not have subepithelial corneal opacities. The presence of active focal superficial keratitis is variable. The diagnosis is made by isolation of the virus from cornea or conjunctiva and the presence of serotype –specific neutralizing and haemagglutination inhibition (HI) antibody in the absence of other etiological agents (Asbell, 1996). However intravenous injection of ribavirin, a nucleoside analogue is given for treating children suffering with severe adenoviral respiratory tract infections (Chacon et al, 1998; Shetty et al, 2000;Gavin et al, 2002). The most common adverse effect of this drug is reversible mild anemia (Gavin et al, 2002).

History

Epidemic keratoconjunctivitis was first described in 1889 as 'subepithelial keratitis'. In late 1930s epidemics of EKC passed by maritime travel from east to Hawaii, where 10,000 cases were documented in naval shipyards of the west cost of the United States (Kemp et al, 1986; Asbell, 1996).

Immune response

During the early stage of infections process, temporary immunosuppression is induced by Adenoviruses including reduced levels of lysozyme, complement and other factors. During the later stage serum and mucosal IgM, IgG antibodies are produced and neutralize the virus infectivity. These antibodies also produce long-term protective immunity. No recommended drugs or therapy are available to treat Adenoviruses infection. Interferon has been demonstrated to have inhibitory effects on human Adenoviruses. However a therapeutic trail with exogenous interferon with stimulates of oncogenous interferon shown to have minimal effects (Knopf and Hierholzer, 1975; Gupta et al, 1986).

Collection and processing of specimen

In cases of conjunctivitis, swab moistened with saline is used to collect epithelial cells from the palpebral conjunctiva. The swab is placed in 1.5 ml of Hank's Balanced Salt Solution (HBSS) and vortexed vigorously to remove the cells sticking to the swab. The swab is then squeezed against the wall of the container and transported to the laboratory.

For isolation from nasopharyngeal aspirates, specimen should be taken within the 1st three days of illness. Nasopharyngeal secretions are collected by gentle suction with a polyethylene catheter and mucus trap. After collection viral transport medium HBSS is passed through the tube and transported to the laboratory. In the laboratory the specimen is transferred to a centrifuge tube.

The specimen is decontaminated with 100 µg/ml concentration of Gentamicin and 10µg/ml concentration of amphotericin. The specimen is then centrifuged at 400Xg for 10 minutes supernatant fluid is removed to make smears for staining and inoculation onto cell cultures.

Growth in cell culture: All human Adenoviruses except type 40, 41 replicate and produce Cytopathic effect (CPE) in human cell lines of epithelial origin such as HeLa, (Human Epidermoid) HEP-2, primary human embryonic kidney (HEK).

Inflected cells become enlarged, rounded and highly refractive and aggregate into irregular clusters with associated increased acidity of the medium. Viral detection from clinical specimen is greatly enhanced by proper collection of specimen early in the disease and by prompt transport to the laboratory. For serological diagnosis paired blood samples collected after on set of symptoms and 2-4 weeks later are needed. Serum sample should be stored at -20^0C.

Direct detection of virus in clinical specimen

Tissue sections, specimens concentrated by ultra centrifugation or membrane ultra filtration are fixed, stained for election microscopy (EM). Sensitivity of EM is enhanced by increasing the virus with animal (Hierholzer et al, 1989).

Time resolved fluoro immunoassay (TR-FIA), where a purified Adenovirus Mab is used as capture antibody in plastic wells to which specimen is added and a europium-labeled Ad Mab is used as detector antibody (Hierholzer et al, 1983). Once all the auto fluorescence disappear, specific fluorescence of the sample is measured by a single-photon counting fluorometer. Enzyme immunoassay is also used for the direct detection of adenoviruses antigen. Sensitivity of EIA is increased by using Mabs in EIA as the detector antibody. This way specificity of the test is improved and the background staining is reduced (Hierholzer, 1989; Anderson, 1983).

Direct specimens are stained with immuofluorescence staining with group specific antiserum for rapid identification (Schwartz et al, 1976; Vastine et al, 1977).

In situ DNA probe technology is also used for direct detection of Adenoviruses from clinical specimens (Hyypia, 1985; Virtamen, 1983; Lehtomaki, 1986). Smears made with clinical specimens are fixed with acetone and covered with a hybridization mixture containing the denatured biotinylated DNA probe sonicated hering sperm DNA to prevent non specific sticking of the DNA, formamide & dextran sulfate. The smears are sealed and the mixture is steam heated at 80^0C to denature the DNA, followed by hybridization at 37^0C. The smears are then washed with buffers at different temperatures and the slides are covered with, streptavidin-biotinylated peroxidase. After a washing step the slides are reacted with diaminobenzidine. Hydrogen peroxide smears are counter stained with fast green, mounted with balsam, and examined by light microscopy.

Dot-blot or spot hybridization techniques are also used for the direct detection of Adenoviruses from clinical specimens. Here purified Adenoviruses DNA containing the segment coding for the hexon antigen common to all Adenovirus are labeled with 32 p-Dgtp / by nick translation. Denatured and added to a hybridization solution with Sodium Dodecyl Sulphate (SDS) and citrate buffer. Clinical specimens are protein digested using proteinase, extracted with phenol and their DNAs precipitated with ethanol. Exacted DNA is dissolved in alkali, boiled, neutralized and then spotted onto nitrocellulose paper. The filters are baked at 80^0C for 2 hours, and prehybridized at 65^0C for 2 hours with denatured sperm DNA. Hybridization solution is added and reacted at 65^0C for 16 hours. The filters are then washed with SDS-citrate buffer and radioactivity is measured by autoradiography.

Virus isolation

Except Adenovirus 40 and 41 others grow in cell cultures of human origin. Human embryonic kidney cells are the best cells for the isolation of Adenovirus from clinical specimens. As HEK cells are not readily available, continuous epithelial cells of human origin HEP –2 or HeLa are commonly used. Human embryonic lung or foreskin fibroblast cells can also be used for the isolation of Adenoviruses from clinical specimen. But these cells are less sensitive for isolation than other cell lines mentioned. Respiratory and urine specimens are decontaminated with antibiotics and centrifuged at low-speed for 3 minutes to remove fibers, cells and other debris. Clinical specimens are brought to 10-20% suspensions with PBS or maintenance medium and centrifuged at low speed. For 30 minutes undiluted supernatant specimen is inoculated into cell culture tubes, absorbed for 1 hour at ambient temperature, overlaid with 1 ml of maintenance medium (MM) and incubated at 35^0C-37^0C. Cultures are observed daily for the presence of cytopathic effect (CPE). Uninoculated tubes should be incubated and observed for evidence of non-specific changes. Sometimes particularly stool, urine specimens themselves may have toxic effects on

cell cultures. These toxic changes can be observed within 24 hours after inoculation. The appearance of CPE in inoculated cell cultures depends upon the type of Adenovirus, viral load in the clinical specimen and the sensitivity of host cell (Hierholzer, 1989). Rapid shell vial technique was reported to be more sensitive for the isolation of adenoviruses (Darougar et al, 1984; Jain et al, 1991; Madhavan et al, 1992; Maitreyi et al, 2000). Adenoviruses isolated in cell cultures are identified by indirect immunofluorescence staining (IIF), heamagglutination TRFIA, RIA. Ezyme immunoassay and DNA hybridization assays are genus-specific but can be made type specific, by using specific monoclonal antibodies or DNA fragments. Haemagglutination with monkey, human and rat red blood cells (RBC) is done to place the virus into a subgroup and finally haemagglutination inhibition test, neutralization tests with selected antisera are utilized to classify the virus as a specific type. For performing haemagglutination tests virus showing 75-100 percent CPE should be harvested in order to obtain sufficient yields of soluble antigens.

For confirming, Adenovirus infection by serological techniques 4 fold rise in antibody titer should be demonstrated. Serological tests performed for the detection of Adenovirus antibody includes EIA and CF in which the hexon-anti hexon reaction is utilized. Purified Antigen extracted from Adenovirus grown in cell cultures is absorbed to the plates and human serum samples are added then anti-IgG serum conjugate is added followed by substrate and developing system. False-negative results may occur in serological tests due to persistent infections or to poor serologic responses in infants and children and due to heterotypic anamnestic responses.

Shortcomings of conventional methods

Rapid diagnosis of etiological agent in infectious diseases will help in timely institution of therapy and the spread of disease will be prevented. Timely institution of appropriate therapy therefore depends on the sensitivity, rapidity, specificity and

accuracy of the laboratory tests employed to detect the infectious agents from the clinical specimen. Culture technique is considered to be the "gold standard" but its application is limited by the time taken by the technique to identify the infectious agents. Many direct detection methods have been evaluated for the rapid detection of infectious agents from clinical specimens and each of these tests have their own merits and demerits. In search of a better method DNA-DNA hybridization technique was developed in 1970s and practical applications were rapidly developed.

The molecular method commonly employed in earlier days was *in situ* DNA hybridization technique. Here DNA present in the clinical specimen was denatured and probes having complementary sequences against the target DNA, labeled with enzymes, antigenic substrates chemiluminescent moieties or radioisotopes are added. Suitable detection methods are employed depending on the nature of the probe used in the test. Oligonucleotide probes have the advantages of hybridizing more rapidly to target molecules and can even detect change of point mutations within a given nucleic acid sequence under stringent conditions. (Wallace et al, 1979; Tenover, 1993).

In 1980 enterotoxigenic *E.coli* was detected by DNA- DNA hybridization in stool samples (Moseley et al, 1980). The direct labeling of oligonucleotide probes with enzymes, like alkaline phosphatase or horse radish peroxidase was introduced in 1980s. Affinity labels such as biotin and digoxigenin may be incorporated into probes by enzymatic methods. After the hybridization reaction is over, enzyme linked to streptavidin or a similarly labeled antibody is used to detect the moiety attached to the probe. The detection is completed through the use of calorimetric or chemiluminescent substrates.

Commercially made probe kits for the detection of *C. trachomatis* and *N. gonorrhea* had the greatest market and *C.trachomatis* direct probe test was evaluated by Woods et al, (1990).

Gen-probe assay for detection of *C. trachomatis* was compared with culture and chlamydiazyme EIA and PCR (Ossewaarde et al, 1992). It was demonstrated that probe assay had 99 percent and 64 percent sensitivity.

Molecular methods used for the detection of *C. trachomatis* includes Polymerase chain reaction, Ligase reaction (LCR), and Transcription based amplification (Persing, 1993)

Ligase chain reaction

Ligase chain reaction is a technique used for the detection of target DNA in clinical specimens. All amplification techniques depend on Polymerases to copy information *in vitro*. Wu and Wallace described an alternative probe amplification technique, based on target-dependent ligation of nucleotide probes in 1989. This method uses DNA ligase to join two pairs of complementary nucleotide probes after they have bound to a target sequence in vitro. Successful ligation depends on the contiguous positioning and perfect base pairing of the 3' and 5' ends of oligonucleotides on a target DNA molecule. Once the probes have been ligated, the ligated product, which now mimics one strand of the original target sequence, can serve as a template for ligation of complementary oligonucleotides. The components are then heated to denature the templates and allowed to anneal to new probes at lower temperature. In the original procedure fresh DNA ligase was added at the end of each round and the material is incubated for 30 minutes to 5 hours. Repetition of this procedure resulted in a doubling of the ligation products, leading to a geometric accumulation.

In the last 2 years, LCR was used for the detection of specific pathogen *C. trachomatis*. LCR do not copy new sequence information into the amplification products so they are called as probe amplification methods. However many

applications for detection of infectious agents do not require this capability; simple detection of nucleotide signature sequence suffices in many cases. Although LCR based methods cannot detect RNA targets, prior reverse transcription into cDNA results in detectable target, thus further expanding the range of potential application.

Conditions can be chosen, where only exactly complementary primers anneal allowing sensitive discrimination between alleles differing by a point mutation. This called as allele-specific ligation.

Potential problems that limit the usefulness of ligase-dependent amplification as originally described were high levels of background amplification due to blunt end ligation of probe duplexes the need to add ligase after each cycle, the relatively long reaction times. But recently the use of themophilic DNA ligase isolated form *T. aquaticus* that remain unaltered during the thermal cycling steps required for ligase-dependent amplification was described. This greatly simplified the LCR process because, like PCR the enzyme can be added at the beginning of the reaction. Furthermore the problem of blunt end ligation precipitated largely by low annealing temperature used is substantially decreased because probe annealing can be performed at higher temperatures. These improvements in the basic methodology will greatly accelerate development of amplification-based tests on ligation. Target dependent ligation amplification is now commonly referred to as the ligase amplification or ligase chain reaction.

LCR has been used by many researchers for the detection of *C. trachomatis* from urine specimens, as this technique is less sensitive to inhibitors.

Sensitivity of ligase chain reaction assay of urine from pregnant women for *C. trachomatis* was evaluated by Inge Panum et al, (1997). In their study, first-void urine samples from 1136 pregnant women at mean gestation age of 17 weeks and

mean age of 28.7 years were collected. From them endocervical swabs were also collected. Urine samples were centrifuged and 1 ml of urine was tested for *C. trachomatis* by LCR detecting plasmid DNA. Cervical swabs were tested by Syva Micro track Chlamydia immunoassay. Of them 27 women were positive for *C. trachomatis*, 25 cervical swabs positive by IF and 12 urine samples were positive by LCR.

Chernesky et al, (1994) examined the ability of ligase reaction (Abbott laboratories) to diagnose *C. trachomatis* in men and women by examining their first-void urine sample (FVU). LCR was compared with culture and enzyme immunoassay performed on swabs and FVU. Thirty milliliters of urine was collected from men and women. A urethral swab was collected from men and two-endocervical swabs were collected from women.

Urethral swab and endocervical swabs were inoculated onto monolayer cultures of McCoy grown in micro titer plates. FVU samples were centrifuged and the pellet was processed and added to 100 microlitre of LCR mixture. The LCR mixture consisted of 4 *C. trachomatis* plasmid oligonucleotide probes, thermus ligase NAD, magnesium ions, dNTPs. The sensitivity of LCR was determined by them to be 96%. Sensitivity of Chlamydiazyme was 37% in FVU (women), 67% (men).

Culture was found to be least sensitive (female cervix 55.6%, male urethra 37.5%). They have concluded that testing of FVU from men or women diagnosed the greatest number of genitourinary tract infections with no false positive.

Gaydos et al, (1997), evaluated the PCR and LCR for *C. trachomatis* in urine specimens form high school female students and monitored the persistence of Chlamydial DNA.

In their study they showed that PCR was positive in 40 percent of cases. A total of 33 infected patients were followed up after the institution of therapy. After 1-3 days of therapy, PCR and LCR were positive for 40 percent, 73.3 percent respectively. The biological explanation they have described is that the target sequence of DNA plasmid gene that is detected by PCR is longer than that for LCR. As Chlamydial DNA is degraded perhaps the smaller DNA segment of the PCR are fragmented and thus available as interrupted sequence for annealing of primers and subsequent amplification.

LCR procedure is marginally more sensitive than other Nucleic Acid Amplification tests (NAATs). The technique has got the advantages that it does not involve many steps to perform or many laboratory personnel required to perform this test.

LCR has good diagnostic potential but only a few applications have been published. The major limitations of LCR are the expensive requirement of four synthetic deoxyoligomers two of which must be 5'-phosphorylated. By comparison, PCR requires just two primers.

Mohany et al 1998 have analyzed and identified inhibitors present in urine samples by using PCR, LCR, TMA. In their study, consecutive urine specimens from 101 pregnant women and 287 non-pregnant women were processed by three commercial assays amplicor CT/NG, Chlamydia TMA). They have found the prevalence of inhibitors resulting in inhibition of amplification was 4.9% for PCR, 2.6% for LCR, and 7.5% for TMA. Nitrites were found to be inhibitory to LCR, to remove inhibitors form urine samples phenol chloroform extraction, storage of specimens at 4.0^0C and dilutions of DNA.

Transcription based amplification or Nucleic acid sequence based amplification (NASBA)

NASBA is an indigenous method based on retroviral replication of RNA. This amplification process, while more complex than PCR, has the advantage that it does not require thermal cycling. It proceeds more rapidly and isothermally at 37°C. The process involves the copying of a target RNA sequence at one end. This is synthesized by reverse transcriptase extension of primer, which is complementary to the 3' end of RNA target to give RNA: cDNA hybrid. From this a double-stranded cDNA intermediate product is generated by the DNA Polymerase activity of reverse transcriptase, which extends a second primer complementary to the 5' end of the target (now DNA) following RNAse degradation of the original RNA target. This intermediate acts a template for T7 RNA Polymerase, which continuously generates numerous progeny RNA copies. The progeny RNA copies then participate in generation of cDNA products in a process essentially the same as described above. This product, in turn, automatically enters the continuous cyclic amplification process and leads to the cDNA and RNA products accumulating rapidly and exponentially. This technique appears to be less sensitive to inhibitors than PCR, sensitivity is equivalent with of LCR. It does involve many steps and is thus more labor intensive.

One of the problems with NAATs is presence of inhibitors in the specimens. Especially urine samples are known to contain inhibitors that would prevent amplifications of the target DNA.

Polymerase chain reaction (PCR)

The most widely used in vitro amplification technique is PCR. Described first by Cetus scientist Kary Mullis in 1983, PCR has developed into a mainstay technique in many biological laboratories. PCR technique is an important breakthrough that

received wide acclaim in the 1980s. PCR technique is designed to amplify a nucleic acid target sequence with oligonucleotide primers whose sequence is synthesized to be complementary to the target DNA. A typical PCR protocol comprises 30 to 50 thermal cycles, each time a cycle is completed; there is a theoretical doubling of the target sequence. Thus repeating the thermal cycle results in a geometric accumulation of amplified target sequence. Many technical improvements have been made to enhance the analytical sensitivity and specificity of PCR. Important improvements include the use of thermo stable DNA Polymerase isolated from a thermophilic bacterium, *Thermus aquaticus* the second was the development of programmable thermal cycler.

The PCR reaction is done with gene specific forward and reverse primers, each of the four deoxynucleotide triphosphate, reaction buffer, and the DNA template. The DNA sequences targeted in PCR reactions are highly conserved for each organism so that only the desired DNA is amplified.

Primers for the detection of *C. trachomatis* from clinical specimens are derived from 3 different *C. trachomatis* specific DNA sequences-the gene coding for the major outer membrane protein (MOMP), the 16S ribosomal RNA (16S r RNA) gene and the 7.5 kb plasmid. Several authors have described PCR for the detection of *C. trachomatis* with primers against the MOMP gene (Holland et al, 1990; Bobo et al, 1990; Palmer et al, 1991; Taylor-Robinson et al, 1992; Ossewaarde et al, 1992) and highly conserved endogenous plasmid (Class et al, 1990; Ostergaard et al 1990; Bailey et al, 1994; Elnifro et al, 1997) and the highly conserved 16S r RNA gene (Pollard et al, 1989; Williams et al, 1992; Fan et al, 1993; Roosendal et al, 1993). Among these three sets of primers the plasmid primer are the most sensitive in the PCR method and detects at least 0.1 infectious unit of *C. trachomatis* (Holland et al,1990; Mahony et al, 1993; Roosendal et al,1993; Satpathy et al,1998). PCR for the detection of Adenoviruses are designed with primers targeted against the hexon

gene sequences Saitoh-inagawa et al, 1996; Dalapathy et al, 1998; Saitoh-inagawa et al, 2001), fiber gene (Adhikary et al, 2001). Nested primers enhance the sensitivity of PCR. Here two sets of primers are used. In a typical nested amplification, a first round of amplification is performed with a single primer for 15-30 cycles. The first round amplified products are transferred to a new PCR reaction by using a second primer pair that is specific for the internal sequence amplified by the first primer pair (Lan et al., 1995; Saitoh-inagawa et al; Dalapathy et al, 1998). The PCR authenticity is determined by any one of the following methods (White, 1993).

1. The amplified products are run on a agarose gel electrophoresis and the amplified products are verified for the expected size of the PCR product along with a ladder of molecular weight consisting of DNA fragments of known base pair size.

2. The amplified products can be confirmed of the position of a single restriction site within the amplified DNA.

3. Nucleic acid blotting technique can also be applied. Here a probe whose sequences are complementary to the target DNA amplified is added after transferring the denatured DNA onto a nitrocellulose membrane. The other approach is to separate the DNA on agarose or polyacrylamide gel and transferred to nitrocellulose membrane, by use of capillary transfer and vacuum transfer and the specific probe with a suitable detector system are added.

Multiplex PCR

Multiplex PCR is a variant of PCR in which two or more DNA targets are simultaneously amplified in the same reaction. Chamberlain et al first described multiplex PCR in 1988(Chamberlain et al, 1988). Several parameters are required to

be optimized for a successful multiplex PCR assay. Following critical parameters need to be taken into account while standardizing Multiplex PCR.

1. The relative concentrations, length G, C content, amplification target sequence size.
2. Concentrations of the PCR buffer.
3. Cycling temperature.
4. Number of cycles for amplification.
5. Balance between the concentrations of $MgCl_2$ and deoxynucleotide triphosphates (dNTPs).

Co amplification of more than one DNA target sequences serves several purposes.

More than one organism can be detected simultaneously from the clinical specimen (Persing, 1993; Elnifro, 2000; Dabiel 2001;O' Neill et al, 2002). Serotyping of clinical isolates can be done by including several sets of primers targeted against the regions, which are specific for the serovars.

Reverse transcriptase PCR (RT-PCR)

Reverse transcriptase PCR is used for determining the MIC of antibiotics against *C. trachomatis* (Cross et al,1999). RT-PCR is technique where RNA is converted into cDNA by reverse transcriptase enzyme. cDNA is then amplified by specific primers thereby indicates that the organism is alive. *C. trachomatis* (standard inoculum) is exposed to varied concentration of antibiotics and RNA is extracted from each of these concentrations and RNA is converted into cDNA by reverse-transcriptase enzyme. Amplification will not be seen at a concentration where the growth of the organism is inhibited by the drug (MIC).

Molecular methods for Characterization of *C. trachomatis* and adenovirus

Immunofluorescence staining with monoclonal or polyclonal antibodies specific for the MOMP serovar specific domains were used serotyping *C. trachomatis* isolates (Wang et al., 1985; Wang et al, 1991; Ossewaarde et al, 1994). The antisera used for typing the isolates cross-react among themselves. Another major disadvantage of this method is that the serovar epidemiology has relied predominantly on isolates that grew well in culture (Frost et al, 1993). Adenoviruses are characterized by reproducing well in specific cell lines, and serotypes have been determined on the fact that immune sera specifically inhibit their reproduction (Fife et al, 1985; Wigand et al, 1987). Adenoviruses are classified into 6 subgenera based on physical, biochemical and biological properties of the virus (Table 1). However differences in virus reproduction rates in cultured cells and ease of adsorption and penetration of host cells create problems in attempting to identify the virus serotypes. In addition, determination of neutralization and determination of virus titer endpoints depend on the evaluation of cytopathic effect (CPE) in cell lines. The antisera cannot always be expected to perform neutralization under stable reproducible conditions. Therefore molecular methods were standardized to characterize the organism. The molecular typing is based on the physical characterization of the molecule produced by the organism (Matar, 1993). Cloning was the method used earlier for studying the genomic constitution and the corresponding amino acid sequences of bacteria. From the amino acid sequences the protein sequences were deduced and the secondary, tertiary structure was studied. The MOMP protein gene sequence of *C. trachomatis* was studied by isolating the entire genomic DNA and was digested with *Bam* HI and ligated to lambda phage 1059 DNA. DNA sequencing was done after restriction digestion (RE) and southern blotting after nick translation (Stephens et al, 1987). Adenovirus isolates were earlier typed by isolating the genomic DNA followed by restriction digest with combination of RE (Kemp et al, 1986). Later PCR based RE technique was introduced and successfully applied for typing the organisms. Here

DNA sequences, which account for serovar variation, are amplified as such with a set of primer specific for the conserved regions. The PCR amplified products are RE digested and number, size of the fragments of DNA are analyzed to identify the serotypes. Variations in the array of fragments generated by a specific RE are called restriction fragment length polymorphism (RFLP). The selection of RE For use in RFLP analysis is based on two important criteria. First the restriction fragments must be suitable for analysis in terms of size and frequency. Best results are obtained with restriction fragments of 1,000 to 15,000 bp. Second the fragments in this size range should not be too numerous, to avoid overlapping bands that obscure differences. PCR-based RFLP are targeted against the hexon gene. (Chang et al, 2001; Adikary et al, 2001). *Chlamydia trachomatis* clinical isolates are typed by performing RFLP with MOMP amplified products (Frost et al, 1991; Rodreguez et al, 1991; Lan et al, 1993; Frost et al, 1993; Mittal et al, 1998). Amplified fragment length polymorphism (AFLP) is another typing method employed to identify the serotype of *C. trachomatis.* The isolates of *C. trachomatis* are RE digested with *EcoRI* and *MseI.* Different primer combinations are used to amplify the fragments generated by the RE digestion. The products are run on Polacrylamide gel electrophoresis (PAGE) and the AFLP patterns are analyzed (Kemp et al, 1986; Koelman et al, 1998, Meijer et al, 1999; Morre et al, 2000). *C. trachomatis* isolates have been identified by other typing methods which include rRNA spacer analysis (Meijer et al, 1997), arbitrary primer PCR (Lan et al, 1995). Ribosomal DNA RFLP analysis is another method used for typing bacteria. The technique involves the finger printing of genomic DNA restriction fragments that contain all or part of the genes coding for the 16S and 23S rRNA. Pulse field gel electrophoresis (PFGE) is used to generate simplified chromosomal restriction fragment patterns without having resort to other methods. In this method, RE that cut DNA infrequently is used to generate large fragments of chromosomal DNA, which are then separated by PFGE. After RE digestion of the genome PFGE is run where the electrical field is periodically inverted in both forward and reverse direction to separate the fragments.

DNA sequencing of isolates is the best method to identify the serotypes and is the best means of quantitatively determining whether two strains are similar or different. The combination of PCR to amplify the genomic DNA fragments and an automated DNA sequencing procedure involving fluorescent dye labeled terminators to directly sequence the DNA are used (Peterson et al, 1990; Dean et al, 1991; Dean et al, 1995; Frost et al, 1995). The MOMP gene sequencing has been done to genotype *C. trachomatis* isolates from different parts of the world (Takour et al, 2001; Pedersen et al, 2000; Stothard et al, 1998; Sylvan et al, 2002). For adenovirus isolates the hexon gene amplified products are sequenced to genotype the organisms (Takeuchi et al, 1999;Saitoh-Inagawa et al, 2001). DNA sequencing methods used currently are the enzymatic method of Sanger et al, (1977) and the chemical degradation method of Maxam and Gilbert. In Sanger's deoxy-mediated chain termination method specific primers for extension by DNA Polymerase, dNTPs and in appropriate proportion deoxynucleotide triphosphates (ddNTPs) which lake a 3'hydroxyl residue are added. The absence of 3'hydroxyl residue prevents formation of a phosphodiester bond with the succeeding dNTP. Further extension of the growing DNA chain is therefore becomes impossible. Thus when a small amount of one ddNTP is included with the four conventional dNTPs in a reaction mixture there is competition between extension of the chain and infrequent but specific termination. By using four different ddNTps in four separate enzymatic reaction, populations of oligonucleotides are generated that terminate at positions occupied by every A, C, G or T in the template strand (Sambrook et al, 1989b).

Maxam-Gilbert chemical degradation of DNA method

Maxam-Gilbert method involves chemical degradation of the original DNA. In this procedure a fragment of DNA radio labeled at one end is partially cleaved in five separate chemical reactions, each of which are specific for a particular base or type of base. This generates five populations of radio labeled molecules that extend from a common point to the site of chemical cleavage. These populations are then separated by electrophoresis through polyacrylamide gels, and the ends labeled molecules are detected by autoradiography (Sambrook et al, 1989b).

Materials and Methods

MATERIALS AND METHODS

Standard strains

1. Adenovirus type 2, American type culture collection (ATCC, VR-846) – obtained from National Institute of Virology (NIV), Pune.

2. *C. trachomatis* A (ATCC VR-347) – obtained from Center for Disease Control (CDC), Atlanta, USA.

3. *C. trachomatis* B (ATCC VR-347) obtained from CDC, Atlanta, USA.

4. *C. trachomatis* Ba (ATCC VR-347) obtained from CDC, Atlanta, USA.

5. *C. trachomatis* C (ATCC VR-347) obtained from CDC, Atlanta, USA.

Cell lines

1. McCoy cell line (National Center for Cell science [NCCS], Pune, India) used for isolation and cultivation of *C. trachomatis.*

2. Vero cell line (National Center for Cell science [NCCS], Pune, India) used for the isolation and cultivation of Adenoviruses.

Patients and specimens

Number of conjunctival specimens processed for *C. trachomatis*: 486 (Collected from 347 patients)

Number of conjunctival specimens processed for adenovirus: 85 (Collected from 62 patients)

Number of endocervical and urethral swabs processed for *C. trachomatis*: 100 (collected from 97 patients)

Number of nasopharyngeal aspirates for *C. trachomatis*: 45 (Collected from 45 children).

Number of nasopharyngeal aspirates for Adenovirus: 45 (Collected from 45 children)

Total number of specimens processed	:	726
Number of patients	:	530
Period of study	:	1999-2003

Place of study

Ocular specimens were collected from Government Ophthalmic Hospital (GOH), Chennai and Sankara Nethralaya, Medical Research Foundation (MRF), Chennai.

Genital specimens were collected from out patient department, Sexually transmitted diseases clinic at Government general hospital (GH), Chennai, India.

Nasopharyngeal aspirates received at clinical microbiology laboratory, Sankara Nethralaya MRF, Chennai, from children admitted in Child Trust hospital, Chennai, for detection of Adenovirus and *C. trachomatis* were included for the study.

I. Basic protocols

Collection and processing of clinical specimens

Conjunctival swabs

Sterile swabs were used to rub over the lower conjunctival sac from medial to lateral side and back again with out touching the lid margin and placed in 1.0ml of Hanks balanced salt solution (HBSS) solution in a screw capped transport vial and were stored at -70^0C until processed for the isolation of adenoviruses and *C.*

trachomatis. For direct antigen detection smears were made on clean microscopic glass slide and fixed in methanol for 10 minutes.

Urethral, endocervical and nasopharyngeal aspirates were collected by the respective clinical consultants.

Urethral swabs

Two urethral swabs were collected from each patient. Sterile cotton swab was inserted into the endourethra and gently rotated. First collected swab tip was broken and put in 2M sucrose phosphate buffer (2SP - pH 7.0) and transported to the laboratory. In the laboratory, 500µl of specimen was stored at -70^{0}C until further processed for PCR. The remaining specimen was decontaminated for isolation purpose. For decontamination the specimen was mixed with gentamicin 10µg /ml, amphotericin B 10µg/ml and incubated at 37^{0}C for 1 hour. At the end of 1 hour the specimen was centrifuged at 1000rpm for 10 minutes and the supernatant was preserved for isolation. With the second collected swab, smears were made on clean microscopic glass slide for the direct antigen detection.

Endocervical swabs

Endocervical swabs were collected using a sterile speculum. Cervical mucus was cleared with a sterile swab and a second swab was inserted about 1cm into cervical canal, rotated and then kept in place for a few seconds to absorb secretions. If any lesion is noticed they were also swabbed. Swab tip was placed in 2SP (pH 7.0) and transported to the laboratory. Similarly specimen was collected with another swab to prepare smears for the direct antigen detection. Cells sticking to the swabs were removed by vortexing the transport medium with the swab in a cyclomixer and squeezing it against the wall of the container. Swab stick was then discarded into disinfectant.

Nasopharyngeal aspirates

Nasopharyngeal aspirates (NPA) were collected by using a bulb syringe with 3 to 7ml of buffered saline. The saline was squirted in to the nose by squeezing the bulb and aspirated back by releasing the bulb. The specimen in the bulb was transported to the laboratory. In the laboratory smears were made with NPA by crush preparations of the solid material containing cells in the aspirated specimen and fixed in methanol for direct antigen detection. Rest of the specimen was diluted with 1.0ml of HBSS. The diluted specimens in 500µl amount were stored at -20^0C for PCR. The remaining specimen was decontaminated with gentamicin 10µg /ml, amphotericin B 10µg/ml and incubated at 37^0C for 1 hour. At the end of 1 hour the specimen was centrifuged at 1000rpm for 10 minutes and the supernatant was preserved for isolation.

Conventional methods

Fluorescent antibody test (FAT)

1. Primary antibody for Herpes simplex virus (HSV)- Polyclonal rabbit anti-HSV type-1 antisera (DAKO A/S, Denmark) or Polyclonal rabbit anti-HSV type-2 antisera (DAKO A/S, Denmark). Primary antibody for Adenovirus - rabbit anti-adenovirus antiserum (ATCC, USA).

2. Secondary antibody for HSV and adenovirus - Fluorescein isothiocynate (FITC) conjugated anti-rabbit immunoglobulin raised in swine against HSV antisera (DAKO, A/S, Denmark) diluted 1.15.

The above optimal dilutions of primary and secondary antibody were determined by titration against the respective viral antigen.

Antisera for C. trachomatis

Monoclonal antibodies raised against the major outer membrane protein (MOMP) of *C. trachomatis* containing Evan's blue (Orion Diagnostics, Finland) was used.

Procedure

Smears for Adenovirus and HSV antigen were fixed in methanol for 10 minutes. Then smears were incubated with respective primary antiserum for 45 minutes at room temperature in a moist chamber followed by incubation with secondary antibody for another 45 minutes. At the end of every incubation period slides were washed gently thrice with Phosphate buffered saline containing Tween 80 (PBST). Smears were then counter stained with 0.05% Evan's blue for 1 minute.

For *C. trachomatis* antigen detection, methanol fixed smears were incubated with the antiserum at room temperature in a moist chamber for 15 minutes.

Smears were carefully blot dried and mounted with 0.95% glycerol saline and observed under the fluorescence microscope (Optiphot, Nikon, Japan) with blue filter (excitation at 495nm, emission at 525nm-Minnich et al, 1988). Adenovirus infected cells showed bright apple green fluorescence, negative cells took the counter stain and appeared brick red in color. *C. trachomatis* infected epithelial cells showed bright green elementary bodies measuring around 300nm in diameter and reticulate bodies.

II. Conventional tube culture method for adenovirus isolation

Two hundred µls of conjunctival swab specimen and NPA (decontaminated) specimen in the HBSS were inoculated onto monolayers of Vero cell line grown in growth medium (GM) containing 10% fetal calf serum ([FCS], Hi-Media, Mumbai, India.), 200mM L-glutamine, gentamicin 50µg/ml, Ciprofloxacin 10µg/ml in Eagle's

minimum essential medium (EMEM) over glass tubes (Borosil, India) after aspirating out the growth medium (GM). All the reagents/antibiotics were supplied by Hi-Media, Mumbai, India. Cells with inoculated specimen were kept for rocking on a rocking machine (10 rocking per minute) at room temperature for 30 minutes and Maintenance medium (MM) containing one percent FCS, 200mM L-glutamine, gentamicin 50µg/ml, Ciprofloxacin 10µg/ml was added in 800 µl amounts. Inoculated cultures were incubated at 37^{0}C and were observed every day under phase contrast microscope (Nikon, Japan) for the presence of Cytopathic effect (CPE). The growth of virus was confirmed by FAT as described above (Smith, 1989). Uninfected cells as negative control and adenovirus, *C. trachomatis* as positive control were included to verify the quality of staining.

Rapid shell vial technique for *C. trachomatis* isolation

McCoy cell culture was grown over 8.0ml glass vials (Borosil, India), a modified shell vial with out cover slip in GM with out Ciprofloxacin. Monolayer of cells in shell vials was pretreated with cycloheximide (Hi-Media, Mumbai, India) 1µg/ml overnight. Specimens brought to room temperature were inoculated in 200µl amount and centrifuged at 3,000rpm for 1 hour at room temperature. At the end of 1 hour MM containing 10% FCS, 200mM L-glutamine, cycloheximide 1 µg/ml, 0.5% Glucose (Hi- Media, Mumbai, India) was added in 800µl amounts. Shell vial cultures from each specimen were performed in duplicates and were incubated at 37^{0}C incubator for 48 hours. At the end of incubation period the MM from one shell vial was aspirated out and the cells were gently scraped and harvested onto a sterile storage vial and smears were made with 1.0 µl of culture harvest. Growth was confirmed by FAT as described above. If the staining is negative the cells in the duplicate shell vial were passed again and results were recorded. If staining is positive the cells were serially passed to increase the titer.

Molecular biological methods

(I) DNA extraction methods

Reagents

1. Equilibrated phenol

- Phenol was liquefied at 68^{0}C and hydroxyquinolone was added to a final concentration of 0.1%.
- To this liquefied phenol, equal volume of 0.5M Tris-Cl (pH 8.0) was added and stirred on a magnetic stirrer for 15 minutes.
- By allowing the mixture to stand at room temperature for 10 minutes, two phases were separated.
- The upper aqueous phase containing the acidified buffer was aspirated and discarded.
- To the lower phenol layer, equal volume of 0.1M Tris-Cl (pH8.0) was added and stirred on a magnetic stirrer for 15 minutes. This was allowed to stand at room temperature for 10 minutes. The aqueous phase was discarded. This was repeated till the pH of the phenol layer was greater than 7.8
- The equilibrated phenol solution was stored under 0.1M Tris-Cl (PH 8.0) in a dark bottle at 4^{0}C.

Method I (Lakeman et al, 1995)

Two hundred µl of conjunctival swab specimen deposit (obtained by centrifuging 500 µl of conjunctival specimen at 10,000rpm for 10 minutes) was boiled for 10 minutes in a sterile 1.5ml microfuge tube and snap-cooled in ice.

Method II (Modified protocol of Saitoh-inagawa et al, 1996)

- Two hundred µl of specimen deposit collected in transport medium was mixed with 20.0µl of proteinase K (20mg/ml) in 200µl of lysis buffer (0.1M Tris-Cl [pH8.0], 0.01 Ethylene diamine tetra acetic acid (EDTA), 1.0% Sodium Dodecyl Sulphate [SDS]). The mixture was vortexed well.

- This mixture was incubated at 56^0C for 1 hour.

- Equal volume of phenol-chloroform, isoamyl alcohol (25:24:1) was added to the lysate and vortexed followed by centrifugation at 15,000 rpm for 20 minutes.

- DNA in the aqueous phase was precipitated by adding $1/10^{th}$ volume of 5M sodium chloride[NaCl], and twice the volume of chilled absolute ethanol.

- This was incubated at -20^0C for 1 hour, followed by centrifugation at

  15,000 rpm for 15 minutes to bring down the precipitated DNA.

- DNA pellet was washed thrice with 500 µl of 75% ethanol by centrifugation at 10,000rpm for 10 minutes.

- The supernatant was discarded and the DNA pellet was dried at 37^0C and dissolved in 20 µl of sterile Milli Q water.

Method III (Guanidine thiocyanate [GTC] method)-Modified protocol of Harris (1996)

- Two hundred µl of specimen (in case of NPA 50 µl was taken) was placed in a 1.5ml microfuge tube and treated with 20µl of Proteinase K (20mg/ml) at 56^0C for 1 hour.

- DNA was extracted by addition of 150 µl each of 10.0M GTC, 0.1M EDTA pH 7.0, 7.5mM ammonium acetate and 450 µl of chloroform: isoamyl alcohol 24:1 V/V.

- Separation of aqueous phase was obtained by centrifugation at 14,000 rpm for 15 minutes.

- Aqueous phase was transferred onto another microfuge tube and equal volume of isopropanol was added and left at -20^0C for 1 hour. This step would precipitate the DNA present in the aqueous phase.

- This was followed by centrifugation at 14,000 rpm for 15minutes to pellet down the precipitated DNA.

- DNA was washed thrice with 80% ethanol by centrifuging at 10,000 rpm for 10 minutes. DNA was dried and reconstituted with 20µl of autoclaved Milli Q water.

(II) Primer dilution

Primers obtained in lyophilized form were reconstituted with 100 µl of Milli Q water. From this 1 µl was made up to 1.0 ml with Milli Q water and the optical density (OD) was measured in a spectrophotometer at 260nm. One OD corresponds

to 33 µg/ml of primer. The concentration of primer was calculated with the OD value and the molecular weight of the oligonucleotide. Required working concentration of the primer was calculated and diluted with Milli Q water

(III) Precautions for PCR

The DNA extraction and PCR cocktail preparation, addition of sample and positive control DNA, detection of amplified products were done in physically separated rooms under clean air laminar flow work bench to prevent amplicon contamination. To prevent amplicon carry over, dedicated pipettes, filter guarded tips for adding DNA and aliquots of single use PCR reagents were used.

(IV) Agarose gel Electrophoresis

Reagents

1. 10X Tris Boric acid EDTA buffer (TBE, pH 8.0)

TBE buffer was prepared by dissolving 54.1gm of Tris base, 3.65gm of EDTA and 27.8gm of boric acid in Milli Q water. The salts were dissolved in 300ml of Milli Q water with the aid of magnetic stirrer and the pH was adjusted to 8.0 and the final volume was made up to 500ml. Working TBE buffer (1X) was made by diluting 10X buffer 10 times with Milli Q water.

2. Bromophenol solution (Tracking dye):

Bromophenol blue at concentration of 0.1% was made with 1X TBE buffer and mixed with equal volumes of 40% sucrose solution made with Milli Q water.

3. Ethidium bromide(2mg/ml):

0.2 gram of ethidium bromide was dissolved in 100 ml of Milli Q water. This stock solution was added to 2.0% agarose gel at a concentration of 0.5 µg/ml.

Procedure

Agarose at concentration of 2.0% in 1X TBE was boiled into a solution and 0.5 µg/ml ethidium bromide was added and mixed gently. Boiled agarose was poured onto a gel tray and combs were placed. The gel was allowed to solidify at room temperature. Amplified products (10µl) were loaded after mixing with the tracking dye, bromophenol blue onto the lanes. Two µl of molecular weight marker (ϕ x 174 DNA */Hinf* I digest) mixed with 8µl of 1X TBE buffer, tracking dye was loaded every time to verify the base pair size of the amplified product. Electrophoresis was carried out at 100 volts and the products were visualized under an UV transilluminator at 302nm (Pharmacia, Biotech). Positive results were documented in the gel documentation system, Image master Visual Documentation System Version 1.1(Pharmacia, Biotech, Amersham, USA).

(V) Polyacrylamide gel electrophoresis (PAGE) (Sambrook et al, 1989a)

Reagents

1. 30% Acrylamide

Acrylamide	–	29.2 gm
Bis acrylamide	-	0.8 gm.

The above reagents were dissolved in 30 ml of Milli Q water and made up to 100 ml of Milli Q water. This was filtered and refrigerated in a brown bottle.

2. Tris-Cl (pH 8.8)

Eighteen grams of Tris was dissolved in 40 ml of Milli Q water. The pH was adjusted to 8.8 with 1N HCl. The final volume was made up to 100ml with Milli Q water.

3. Ammonium persulphate

Hundred milligram of Ammonium persulphate was dissolved in 1.0 ml of Milli Q water. This was prepared every time and should be used within one day and stored at 25^0C.

Procedure

- The glass plates were assembled with spacers in between.
- Then 1.0% agarose prepared in Milli Q water was poured onto the casting slots and the assembled glass plates were placed on the casting slot to make them leak proof.
- This was left undisturbed at room temperature (approximately 15 minutes) for the agarose to solidify.
- A 12.0% polyacrylamide separating gel was prepared by mixing 4.0ml of 30% acrylamide, 2.0ml of Tris-Cl buffer (pH 8.8), 6.0ml of Milli Q water, 50µl of Ammonium persulphate, 5µl of N, N, N- tetramethylene diamine. This mixture was poured in between the glass plates and left undisturbed at room temperature (approximately 45 minutes) for the acrylamide to polymerize.
- Once polymerization occurred stacking gel made with 1.3 ml of 30% acrylamide, 2.5 ml of buffer (pH 6.0), 6.2 ml of Milli Q water, 50µl of Ammonium persulphate and 5µl of N,N,N- tetramethylene diamine (TEMED) was poured in between the glass plates over the separating gel.
- Then combs were placed in between the glass plates.

- After the stacking gel gets solidified, the restriction digested and undigested amplified products mixed with tracking dye were loaded and the electrophoresis was carried out at 100 volts with IX TBE buffer.

Silver staining technique

The polyacrylamide gel with the separated restriction enzyme (RE) digested products was fixed in 10.0 % methanol (made with Milli Q water) containing 500 µl of acetic acid for 3 minutes. At the end of 3 minutes the gel was placed in fresh 10 % methanol fixative for 3 minutes. At the end of 3 minutes, the gel was placed in 0.1 % silver nitrate solution prepared in Milli Q water to stain the bands for 15 minutes followed by 3 washes with Milli Q water. The gel was then differentiated with 1.5 % sodium hydroxide containing 0.1 % formalin till the bands attain satisfactory intensity. The differentiated bands were fixed in 0.75 % sodium carbonate for 10 minutes. The results were documented in the gel documentation system (Image master Visual Documentation System Version 1.1 [Pharmacia Biotech, Amersham, USA]).

Statistical Method

McNemar test was used for analyzing the results (Rosner, 1990).

A. Conventional cell culture method in growing *C. trachomatis Evaluation of the susceptibility of McCoy, HeLa, BHK-21, Hep-2, Vero and A549 cell lines for the growth of C. trachomatis*

Standard strains: *C. trachomatis* A, B, Ba and C McCoy, HeLa, BHK-21, HEp-2, Vero and A549 cell lines were grown over sterile cover slips placed inside glass shell vials. Cells grown over the cover slips were observed under phase contrast microscope to verify their healthy growth. The cells were then pretreated with MM

containing Cycloheximide at concentrations of 1µg/ml for 24 hours. At the end of 24 hours standard inoculum (150 IFU/ml) of the original stock of the above 4 standard strains obtained from Centre for Disease Control (CDC), Atlanta was inoculated in 100µl amounts. The shell vials were centrifuged at 3,000 rpm for 1 hour and 900 µl of MM was added at the end of 1 hour. Cultures were incubated at 37^{0}C for 48 hours. At the end of 48 hours, MM was aspirated and the cover slips were fixed with methanol. The fixed cover slips were stained by IF staining as described before in page number 69 under section basic protocols. The experiment was carried out at the same time with all the cell lines with a single aliquot of inoculum and MM. The cover slip preparations were observed under fluorescence microscope with a blue filter (Optiphot, Nikon, Japan). The growth was graded according to the number of elementary bodies present under 20X objective. The grading is shown in Table 2.

B. Evaluation of PCR using plasmid primer with MOMP primer for the detection of *C. trachomatis* in primary conjunctivitis

Eighty conjunctival swabs collected from 55 consecutive patients with clinical diagnosis of primary conjunctivitis attending the GOH, Chennai during the period November 2000 to January 2001 were included in this study. DNA extracted from the specimens was amplified with both plasmid and MOMP primers and the results were compared DNA extraction from conjunctival specimens were done in the beginning of study period by Method I, II, III and during the later period of the study only method III (modified protocol of Harris et al, 1996) was followed. When DNA extraction was done from conjunctival specimens, buffer extraction control was included in order to rule out the presence of amplicon contaminants in the DNA extraction reagent.

TABLE – 2

Showing the grading made based on the EBs count of *C. trachomatis* in different types of cell lines

EBs count under 20X	Grade	Growth rate
65-50	++++	Very good growth
49-35	+++	Good growth
34-20	++	Moderate growth
19-1	+	Scanty

Standardization of PCR with MOMP and plasmid primers

PCR was standardized with plasmid primers targeted against the plasmid gene (Bailey et al, 1994) and MOMP primers targeted against the MOMP gene of *C. trachomatis* (Talley et al,1992). The primers were custom synthesized by Bangalore genei pvt Ltd, India.

Following were the primers sequences: -

Plasmid primers (Bailey et al, 1994)

Forward primer 5' TTC TCA TCA TAA AAA CAT ATT CAT AGT AT-3'
Reverse primer: 5' CTG ACT GTG AGA ATA TAT CAT AAA TAG AC-3'

MOMP primers (Talley et al, 1992)

Forward primer CT1: 5'CCT GTG GGG AAT CCT GCT GAA-3 '

Reverse primer: CT2: 5'GTC GAA AAC AAA GTC ACC ATA GTA-3.'

Sensitivity and specificity of MOMP and plasmid primers

Analytical sensitivity of MOMP and plasmid DNA was calculated with serial ten fold dilutions of DNA extracted from *C. trachomatis* Ba (ATCC-VR-347). Each dilution was kept for amplification. The corresponding number of EBs present in the culture harvest taken for DNA extraction was enumerated by counting the same under fluorescence microscope in a smear stained with antisera.

Specificity of the primers were found against DNA extracted from Adenovirus type 2 (ATCC-VR-846), Herpes Simplex Virus (ATCC 733 VR), *Staphylococcus aureus* (ATCC 25293) *Haemophilus influenzae* (ATCC 35056), *Mycobacterium tuberculosis* (H37RV) and laboratory isolates of *Streptococcus pyogenes, Propionibacterium acnes,* DNA extracted from human blood.

PCR amplification

PCR reaction was carried out in a 50 µl reaction volume consisted of 5 µl of 10X PCR buffer (500mM Potassium chloride, 100mM Tris-chloride, 15mM Magnesium chloride, gelatin 0.1% pH 8.3) 100 pmoles of MOMP primers and 50picomoles of plasmid primers 200 mM deoxyribonucleotide triphosphate (dNTPs) and 1.5 units of *Taq* Polymerase for the MOMP PCR and 1.0 unit of *Taq* Polymerase for the plasmid reaction. The volume was made up to 45.0 µl except for PCR reaction control, which was made up to 50 µl with autoclaved Milli Q water. PCR reaction control was included in batch of reaction to rule out the presence of amplicon contamination in the reagents. Five µl each of extracted DNA was added one each in MOMP and plasmid PCR reaction. Positive control DNA extracted from cell culture harvest of *C. trachomatis* Ba (ATCC VR-347) was added in 2.0 µl amounts. Extraction control was also included in order to rule out the presence of amplicon contamination in the DNA extraction control.

Thermal profile

The PCR amplification was carried out in Perkin Elmer 2400, (Cetus, CA, U.S.A) automatic thermocycler.

MOMP PCR- The first cycle consisted initial denaturation at of 95^{0}C for 5 minutes, followed by 48 cycles each consisting of Denaturation at 95^{0}C for 1 minute, annealing at 45^{0}C for 2 minutes, extension at 72^{0}C for 2.0 minutes. This was followed by a final extension at 72^{0}C for 5 minutes.

Plasmid PCR- The first cycle consisted initial denaturation at of 95^{0}C for 5 minutes, followed by 48 cycles each consisting of Denaturation at 95^{0}C for 1 minute, annealing at 45^{0}C for 2 minutes, extension at 72^{0}C for 2.0 minutes. This was followed by a final extension at 72^{0}C for 5 minutes.

Criterion for PCR positivity

MOMP PCR	–	144 bp product
Plasmid primer	-	317 bp product

C. Evaluation of conventional methods against plasmid PCR in detection of *C. trachomatis* DNA in conjunctivitis cases.

Patients and specimens

Adult patients with primary conjunctivitis attending the outpatient departments of the Regional government ophthalmic hospital and Sankara Nethralaya ophthalmic hospital, Chennai were included in this study during a period of 15 months - November 2000 to January 2002. Conjunctival swabs were collected from patients with primary conjunctivitis which was defined for the purpose of the study as a primary inflammation of conjunctiva not related to any adnexal disease or intraocular inflammation or trauma or resulting from application of topical medication. Four hundred and eighty six (486) conjunctival swabs were collected from 347 (both eyes - 139 and one eye only -208) patients. Conjunctival swab from a 10 day-old infant with ophthalmia neonatorum was also included in the study. Conjunctival swabs placed in 2M-sucrose phosphate (2SP) buffer were stored at -80^0C until processed for culture and DNA extraction for PCR. Culture and PCR were performed within 48 hours of collection of the specimen.

Smears of the conjunctival specimens were prepared using another sterile swab for the direct antigen detection of *C. trachomatis,* Adenovirus, HSV. Smears were stained by FAT method (procedure described under basic protocols in page number 69). Smears were also made for Gram staining and Giemsa staining. Gram staining was done after heat fixing the smear to detect the presence of bacterial pathogens causing conjunctivitis. Giemsa staining was done to detect the presence of

inclusions of *C. trachomatis,* Adenovirus, Herpes simplex virus (HSV). Isolation of *C. trachomatis* was done by rapid shell vial technique as mentioned above (page number 70) . Specimens were subjected to conventional culture technique for Adenovirus and HSV using Vero cell line as described earlier (page number 69, 70). At the end of 7 days of incubation the cultures were scraped and stained by FAT technique as described earlier.

Specimens were also processed for bacterial culture by semi quantitative method (Folkens et al, 1996) where 100 µl of conjunctival swabs collected in HBSS were inoculated onto Chocolate agar (incubated at 37^0C CO_2 incubator- Forma scientific, USA), Blood agar (incubated at 37^0C) and Brucella blood agar (incubated in anaerobic work station (Don Whitely, India). The plates were incubated for 48 hours and readings were taken daily. Significant bacterial colony forming unit (CFU/ml) and the threshold value was determined according to Folkens et al, (1996). Significant bacterial pathogen isolated were identified to species level by standard methods (Collee et al, 1994)

Polymerase chain reaction

DNA extraction from the conjunctival specimen was done as mentioned above (modified protocol of Harris, 1996). Amplification of DNA was done with plasmid primers as mentioned above.

D. Evaluation of conventional methods for the detection of *C. trachomatis* and HSV in males with urethritis and females with cervicitis against PCR

Patients and collection of specimen

Twenty five endocervical swabs from 25 female patients with clinical diagnosis of mucopurulent cervicitis and 75 urethral swabs from 72 male patients with clinical diagnosis of urethritis were included in this study. The specimens were

collected between July –August 2002 from Sexually transmitted diseases out patient clinic attending Government general hospital (GH), Chennai. Specimens were collected three days a week during the study period. One female and 3 male patients were seropositive for human immunodeficiency virus (HIV) infection. Collection of specimen is described in page number 67 under section basic protocols. Smears made with the specimens, fixed in methanol were stained as described in page number 69. Cultivation of *C. trachomatis* and HSV were carried out with decontaminated specimens as described under section basic protocols, page number 69, 70.

Polymerase chain reaction

PCR was standardized with DNA extracted from *C. trachomatis* serotype Ba (ATCC VR-341) grown on McCoy cell cultures. DNA extraction from the clinical specimen and standard strains were done by GTC method.

Primers:

The primers were custom synthesized by Invitrogen Life Technologies, Grand Island, New York, USA. The primers were specific for the plasmid DNA of *C. trachomatis* (Lan et al, 1995).

Sequences of the primer are **CTP1**: 5'-TAG TAA CTG CCA CTT CAT CA-3' **CTP2**: 5'-TCC CCT TGT AAT TCG TTG C3-3'.

***Specificity of the primer*:**

The specificity of the primer was tested against DNA extracted from HSV-2 (SP 753167), *Staphylococcus aureus* (ATCC 25293), *Staphylococcus epidermidis* (ATCC 12228), *Klebsiella pneumoniae* (ATCC 138880) and laboratory strains of Propioibacterium *acnes*, beta-haemolytic streptococci, *Corynebacterium xerosis*, *Candida albicans* and DNA extracted from human blood.

Sensitivity of the primer

The analytical sensitivity of the primer was determined by performing PCR with 10-fold dilutions of DNA extracted from cell culture harvest of *C. trachomatis* Ba (ATCC VR-341). Each dilution was kept for amplification and the amount of DNA present in each dilution was estimated spectrophotometrically. The corresponding number of EBs that would be present was enumerated by counting the same in the pinhead smear made with 1µl of cell culture harvest.

Amplification profile

The DNA was amplified in a thermal cycler (Perkin –Elmer, Model No.2400, USA) for 40 cycles with 50 pico moles of plasmid primers; 200 µM dNTPs, 10X PCR buffer, 1.5 units of *Taq* Polymerase. The amplification profile is denaturation at 95^0C for 1 minute, annealing at 53^0C for 1.5 minute and extension at 72^0C for 1.5 minutes. The amplified products were run on a 2.0 per cent agarose gel incorporating 0.5 µg/ml ethidium bromide and visualized under ultra violet UV (302) transilluminator (Pharmacia, USA).

Criterion for positivity:

Amplified product of 217bp

E. To evaluate PCR with plasmid primers against direct antigen detection and isolation of *C. trachomatis* in interstitial pneumonia

Patients and specimens

Forty five (45) NPA collected from 45 children were included for the study.

Specimen collection and processing was done according to procedure mentioned under basic protocols. Smears were stained for *C. trachomatis* by IF technique and isolation was done by rapid shell vial technique mentioned under basic protocols. PCR was carried out with DNA extracted from NPA by Harris, 1996. PCR amplification was done with primers specific for plasmid DNA (Lan et al, 1995). The PCR amplification profile, reaction settings mentioned for cervical specimens were followed.

F. Evaluation of Polymerase chain reaction (PCR) against the conventional virological methods of antigen detection and virus isolation for the detection of adenovirus conjunctivitis

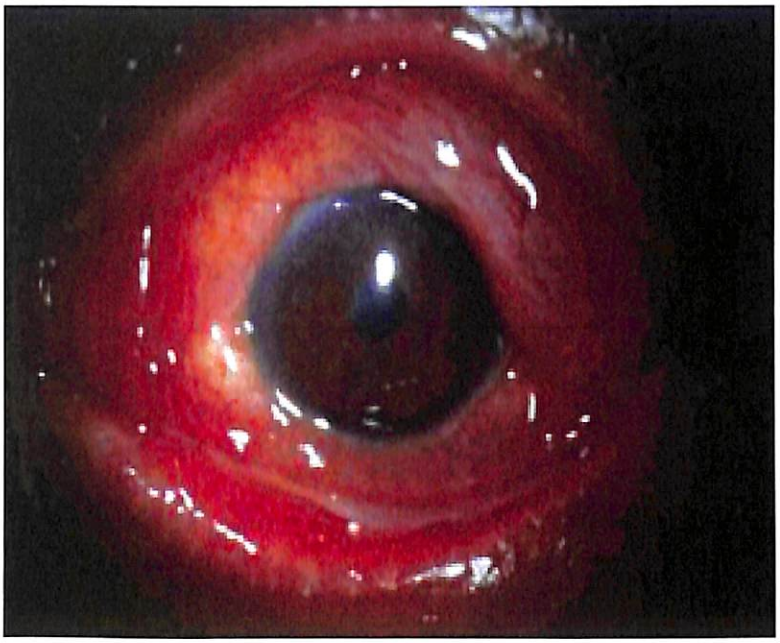
Patients and specimen

During the epidemic in August – December 2002, 85 conjunctival swabs from 62 patients were collected as described under basic protocols. This included 39 unilateral and 23 bilateral specimens. The clinical photograph of a patient's eye with adenovirus conjunctivitis is shown in Fig.1. Two swabs were collected from each patient. The swab collected first was placed in HBSS for virus isolation and DNA extraction. Second swab was used for preparation of smears for direct antigen detection. Staining was done for direct adenovirus detection by FAT technique is described earlier. Adenovirus isolation was done by technique described earlier.

DNA extraction for the detection of adenovirus was done by method described earlier (Harris, 1996). DNA extracted from adenovirus serotype 2 grown in HEp-2 cells was included as positive control. Amplification of DNA was done by a nested PCR with two rounds of primers targeted against conserved region of the hexon gene of adenovirus 2 (Dalapathy et al, 1998). The analytical sensitivity of the primers was earlier determined to be 0.0032 fg which corresponds to $8.3X\ 10^{-3}$ virus particles.

Figure - 1

Clinical picture showing epidemic conjunctivitis caused by adenovirus



The primers analytical specificity was earlier determined with DNA extracted against viruses other than Adenovirus and bacteria known to cause conjunctivitis (Dalapathy et al, 1998). Amplification of Adenoviral DNA was carried out using Fastype conjunctivitis adenovirus SSP detection kit (provided by Alcon laboratory, USA), which was prepared based on the method described by Dalapathy et al, (1998). The first round amplification kit contained 10X PCR buffer, 0.5 µM concentration each of forward primers in a lyophilized form in high quality micro strip tubes.

Specificity of the kit were found against DNA extracted from Adenovirus type 2 (ATCC-VR-846), Herpes Simplex Virus (ATCC 733 VR), *Staphylococcus aureus* (ATCC 25293) *Haemophilus influenzae* (ATCC 35056), *Mycobacterium tuberculosis* (H37RV) and laboratory isolates of *Streptococcus pyogenes, Propionibacterium acnes,* DNA extracted from human blood. Sensitivity was determined with serial 10 fold dilutions of DNA extracted from Adenovirus 2(ATCC-VR-846).

Ten µl reaction volume was set by adding 8µl of sterile autoclaved Milli Q water, 1µl of 10X dye solution (provided in the kit) and 0.25 unit of *Taq* Polymerase. DNA extracted from conjunctival specimens were added in 5.0µl volumes and amplified in a thermal cycler (Perkin Elmer, 2400) using the following amplification profile initial denaturation at 96^{0}C for 1.0 minute followed by 10 cycles of amplification with denaturation at 94^{0}C for 20 seconds annealing at 65^{0}C for 60 seconds. This was followed by 20 cycles of amplification with denaturation at 94^{0}C for 20 seconds, annealing at 61^{0}C for 50 seconds, extension at 72^{0}C for 30 seconds and a final extension at 72^{0}C for 5 minutes.

PCR reagent control, buffer extraction was also added every time to rule out the presence of amplicon contamination.

For the second round 1.0µl of first round product was added to the second round Fastype conjunctivitis adenovirus SSP detection kit containing 0.5µM each of second round primers. The reaction was set as described for the first round with the inner set of primer. Amplification profile mentioned for the 1st round was followed.

Primer sequences:

First round

Forward primer : TU 7 -5' GCC ACC TTC TTC CCC ATG GC-3'

Reverse primer : TU 4 - 5' GTA GCG TTG CCG GCC GAG AA-3'

Second round

Forward primer : AdnUS '-5' TTC CCC ATG GCN CAC AAC AC-3'

Reverse primer : AdnUA-5' GCC TCG ATG ACG CCG CGG TG-3'.

Criterion for positivity

First round : Amplified product of 1006 bp.

Second round : Amplified product of 960bp.

G. Evaluation of PCR against conventional methods of antigen detection and virus isolation for the detection of adenovirus in interstitial pneumonia.

Forty five (45) NPA collected from 43 children were included for the study. Specimen collection and processing was done according to procedure mentioned under basic protocols. Smears were stained for adenovirus antigen by IF technique according to the procedure mentioned under basic protocol. PCR amplification with DNA extracted (Harris, 1996) from NPA was done by method mentioned above.

H. Application of reverse transcriptase Polymerase Chain Reaction (RT-PCR) to determine the minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of drugs against *C. trachomatis.*

Standard strains: *C. trachomatis* A, B, Ba and C

Clinical isolates: three clinical isolates from 2 patients (one from a case of adult inclusion conjunctivitis, and the other two from both the eyes of a 10-day old child with ophthalmia neonatorum).

Drugs tested: Ciprofloxacin, Erythromycin (Hi-Media, Mumbai, India), and Sparfolxacin and Roxithromycin (Cadila pharmaceuticals, Ahmedabad, India). Stock solutions of all the drugs except Sparfloxacin was prepared in autoclaved deionized water (10mg/ml). Sparfloxacin was dissolved in dimethyl sulfoxide (DMSO) to make the stock solution (10mg/ml). From the stock antibiotics solutions a 1in10 working dilution was made with EMEM without fetal calf serum and antibiotics, pH 7.0). Required volume of this working solution was added to the MM (after inoculating the organism) to attain the concentrations given below.

Following are the concentrations of the drugs (μg ml) used for the study-

Erythromycin

0.2, 0.4, 0.6, 0.8, 10,20,30,40,50,60,70,80,90,100,120,140,160,170,180,190

Roxithromycin

0.005,0.010,0.0125,0.025,0.05,0.075,0.1

Ciprofloxacin

0.05,0.06,0.125,0.25,0.5,1.0,1.5,2.0,2.5,3.0,4.0

Sparfloxacin

0.000625,0.00125,0.0025,0.005,0.01

Determination of MIC, MBC of drugs by Micro immunofluorescence (MIF) method

The standard strains of *C. trachomatis* causing trachoma and the 3 laboratory clinical isolates were grown on stationary cycloheximide pretreated McCoy cell cultures in shell vial and at the end of 48 hours of incubation, the infected cells were harvested and stored at -70^{0}C. One µl of the culture harvests were placed on microscopic slides, stained with monoclonal antibodies raised against the major outer membrane protein (Orion Diagnostics, Finland) of *C. trachomatis* and number of inclusion forming units (IFU) was counted under fluorescent microscope (Optiphot, Nikon, Japan) with a blue filter. The harvests were diluted to adjust the count to approximately 150 IFU/ ml for use in the drug sensitivity tests. Determination of MIC, MBC were done by shell vial technique with out cover slip. In this technique McCoy cell cultures were grown on a sterile shell vial without cover slip and cells were pretreated with Cycloheximide (1µg/ml). Standard strains and isolates were inoculated (approximately 150 IFU/ml) onto the shell vials, which were centrifuged at 3,000 rpm for 1 hour. At the end of 1-hour 950 µls maintenance medium (MM) containing varied concentrations of Ciprofloxacin, Sparfloxacin, Erythromycin, Roxithromycin were added. Stock and working concentration of erythromycin, roxithromycin and sparfloxacin were prepared as described by Andrews et al, (2001). At the end of 48 hours of incubation, MM was aspirated out from the shell vials and the cells were scraped using rubber policemen or sterile disposable bacteriology loop. From each concentration, 10µls of cell suspension was placed on microscopic slides to make the small smears of about 2-3 mm diameter. Smears were fixed in methanol for 10 minutes and stained with monoclonal antibodies raised against the major outer

membrane protein of *C. trachomatis.* Concentration of antibiotics at which 90-100% reduction in growth is observed on comparison with control is considered as the MIC. Cultures were passed twice to know the viability of growth. To determine the MBC the culture harvest of the organism exposed to varied concentrations were passed again and were stained by IF at the end of 48 hours of incubation.

RT-PCR to determine MIC and MBC of antibiotics

Culture harvest in 500 µl amount from each concentration of the drugs were taken for RNA extraction. Total RNA was extracted with Tri-reagent (Sigma Chemicals, U.S.A). Poly A tail formation and amplification of cDNA was done with RT-PCR kit supplied by Bangalore genei pvt Ltd, Bangalore, India. Poly A tail formation was carried out by incubating the extracted RNA with oligo dT primers at 65^0C for 10 minutes then it is incubated at room temperature for 2 minutes. *In vitro* complementary DNA synthesis was carried out in a total of 20 µl reaction volume by adding Rnase inhibitor 1µl, Dithiothretiol 1µl, dNTPs (30 mM) 2 µl, and 5 X Assay buffer, reverse transcriptase 4 µl to the poly A tail formed. The reaction mixture was made upto 20 µl reaction volume with RNAse free water. Finally AMV Reverse transcriptase enzyme was added in 0.4 µl amounts and the reaction was carried out in Perkin Elmer 2700 (Cetus, CA, USA). The reaction mixture was gently mixed, pulse centrifuged and incubated at 42^0 C for 60 minutes. Then it was incubated at 95^0 C during which the RNA-DNA hybrids formed were denatured. The cDNA was amplified using primers directed against the Dna K protein gene of *C .trachomatis* . The primer sequences used in the test were-

Forward primer : 5'- CCTGCAAAACGTCAAGCAGT 3',

Reverse primer : 5' – ATGCGTCCAGCATCTTTTG 3'.

Primers were added at a concentration of 100 pico moles. PCR reagents and primers were added to the 20µl cDNA product and amplified in a thermal cycler (Perkin Elmer 2700, Cetus, CA, USA). The thermal profile followed was a modified protocol of Cross et al., 1999 with Denaturation at 94^{0}C for 60 seconds, annealing at 60^{0}C for 60 seconds and extension at 72^{0}C for 60 seconds for a total of 44 cycles. Final extension was carried at 72^{0}C for 6 minutes. An amplified product of 318bp was obtained at the end of 44 cycles. Products were visualized under the UV transilluminator (Pharmacia, Amershem USA) at 302 nm. Dedicated pipettes and RNAse treated filter guarded tips were used through out the work. Eppendorf vials used for setting up the reaction were treated with 1.0 % solution of diethyl pyrocarbonate (DEPC) Sigma chemicals, U.S.A) overnight and autoclaved and dried.

Controls: RNA extracted from four trachoma ATCC standard strains and the three clinical isolates grown in McCoy cell cultures with out exposure to the drugs were used as the positive controls. RNA extracted from each concentration was exposed to direct DNA amplification to rule out the false positive result due to DNA contamination. Reagent controls were used to rule out the presence of amplicon contamination.

I. Determination of MIC, MBC of antibiotics in direct clinical specimen with *C. trachomatis.*

The influence of clinical specimen on the MIC and MBC of antibiotics were studied by spiking the least amount of *C. trachomatis* Ba in 5 clinical specimens. These specimens were negative for IF and were positive for PCR. Among these 5 specimens two specimens were exposed to varied concentrations of Ciprofloxacin. Two other specimens were exposed to varied concentrations of erythromycin. One specimen was exposed to different concentration of erythromycin. The stock and working solutions were prepared as mentioned above for determination of MIC and

MBC against ocular isolates. The spiked clinical specimens were taken in 200 µls amount and the required working concentrations of the antibiotics were added. Following are the concentrations of antibiotics used to determine the MIC and MBC.

Ciprofloxacin - 0.25- 2.5µg/ml

Erythromycin - 0.5-4.0 µg/ml

Roxithromycin - 0.025-0.25 µg/ml

This was then incubated at 37^{0}C for 1 hour. At the end of 1 hour, they were inoculated onto McCoy cell cultures after aspirating out the growth medium and then were centrifuged at 3000 rpm for 1 hour. At the end of 1 hour MM was added in 800 µls amounts. The cultures were incubated at 37^{0}C for 48 hours. The harvesting and determination of MIC and MBC were done as mentioned above.

J. Sequencing of *C. trachomatis* plasmid and MOMP amplified products to determine the serotype

Isolates and amplified products

Two *C. trachomatis* isolates from a child with ophthalmia neonatorum were isolated on McCoy cell cultures.

One *C. trachomatis* isolate from a case of inclusion conjunctivitis was isolated from an adult patient with inclusion conjunctivitis.

Plasmid-amplified products of the 3 laboratory isolates, 3 genital specimens and 5 ocular specimens were subjected to DNA sequencing. For the purpose of plasmid sequencing, 3 genital specimens positive by PCR with plasmid primer (Lan et al, 1995) were reamplified with primers used for amplifying the ocular specimens (Bailey et al, 1994). The location of the primer in the plasmid sequence is 1708 (Forward primer) and 2105 at the 5' end (Reverse primer).

Controls:

C. trachomatis A, B, Ba, C grown on McCoy cell cultures *C. trachomatis* isolates recovered from both the eyes of a child with ophthalmia neonatorum standard strains of *C. trachomatis* A, B, Ba and C were genotyped by sequencing. Sanger dideoxy sequencing protocol (PE Applied Biosystems Automated DNA sequencing-A division of Perkin Elmer, Chemistry guide), using fluorescent labeled 3'- dye labeled dideoxynucleotide triphosphate (ddNTPs) was followed. DNA from the culture harvest was extracted by GTC method and was amplified with primers targeted against the MOMP protein gene of *C. trachomatis*. These primers with *C. trachomatis* DNA gave an amplified product of 1020 bp. This primer set covered the variable domains of the MOMP gene. Following are the primer sequences. Forward primer: 5'- TCC TTG CAA GCT CTG CCT GTG GGG AAT CCT 3' Reverse primer:5' CCG CAA GAT TTT TCT AGA TTT C-3' (Pederson et al, 2000). A total of 50µl reaction volume with 200mM dNTPs solutions, 1X PCR buffer solution, 25picomoles each of forward and reverse primers, and 2 units of *Taq* DNA Polymerase. The reaction volume was made up to 50 µls with Milli Q water. The products were kept for DNA amplification in a thermal cycler by using a temperature cycle of initial denaturation at 95^{0}C for 5 minutes followed by 49 cycles of amplification with denaturation at 95^{0}C for 1 minute, annealing at 50^{0}C for 1.5 minutes and extension at 72^{0}C for 2 minutes. At the end of 49 cycles the products were kept for primer extension at 72^{0}C for 5 minutes. The entire amplified products were run on a 2.0 % agarose gel and the products were cut under UV transilluminator.

Amplified DNA was eluted out of the gel using QIAEX II gel elution kit. Elution of DNA was done according to the manufacturer's instructions. The eluted DNA was then kept for cycle sequencing with both forward and reverse primer. Cycle sequencing reaction was set with 2.0 µls of eluted DNA, Ready reaction (RR) mixture

2.0 µls. 5X buffer 3.0 µls. forward primer/reverse primer 3.5 pM/µl. sterile Milli Q water (3.0/µl). The reaction mixture was amplified for a total of 24 cycles. The reaction profile is 96^{0}C for 10 seconds. annealing at 50^{0}C for 5 seconds, and extension at 60^{0}C for 1.0 minute. The amplified products were purified by adding 10 µls of 3 M sodium acetate (pH 8.0) 80 µls of Milli Q water, 250 µls of 95 % ethanol. The mixture was then kept in ice for 20 minutes. It was then centrifuged at 10,000 rpm for 20 minutes. The supernatant was then discarded and 250 µls of 70 % ethanol was added. Following this centrifugation was done at 10,000 rpm for 5 minute and this step was repeated twice and the pellet was air-dried. The purified and dried DNA was resuspended in 20 µl of Template suppression Reagent (TSR) and it was denatured at 90^{0}C for three minutes. This was loaded in ABI PRISM 310 Genetic Analyzer, PE Applied Biosystems, USA) for analysis. . The sequenced data was then compared with the plasmid and MOMP reference sequence using the sequence analysis software.

K. Analysis of Adenovirus serotypes by neutralization test using type specific polyclonal anti-adenovirus antisera.

Patients and specimens

Ten adenovirus isolates from patients diagnosed to have epidemic conjunctivitis were subjected to neutralization test with adenovirus type specific antisera 4.

Neutralization test

Vero cell culture was grown over a 24-well tissue culture plate. The isolates were diluted 1 in 10. Fifty µl amounts were mixed with equal volume of type specific adenovirus 4 antiserum and incubated at room temperature for 1 hour for the antisera

to neutralize the pathogenicity of the virus. The test was performed in duplicates and a virus control. cell control were included in the test. At the end of incubation period the virus antiserum mixture were added in duplicates and kept for rocking at room temperature for 30 minutes to facilitate infection. At the end of 30 minutes 900µl of MM was added and incubated at 37^0C carbon di-oxide (10%) incubator. The results were read every 12 hours under phase contrast microscope. Wells inoculated with virus serum mixture showing no CPE were considered to be positive for neutralization.

L. Multiplex PCR for the subgenus typing of Adenovirus isolates

No. of isolates: Ten Adenoviruses isolated from 7 patients with epidemic conjunctivitis were subjected to subgenus typing.

DNA extraction:

DNA extraction from the isolates were done using Bangalore Genei DNA extraction kit.

Extraction was done according to the manufacturer instruction.

Multiplex PCR

Primers included in the multiplex PCR were targeted against the loop 14 region (Pring-Akerblom et al, 1999), which carries the subgenus specific determinants in the hexon gene of adenovirus. Three sets of primers specific for subgenus B, C and E were used, as the serotypes included in these 3 subgenus are the common causative agent of conjunctivitis.

Primer sequences for each of the subgenus and the corresponding amplified product size are as follows (Pring-Akerblom et al, 1999):

Subgenus	Primer	Amplified product length (bp)
B1	Hsg B1 5'- TCT ATT CCC TAC CTG GAT -3'	465
B2	Hsg B2 5'-ACT CTT AAC GGC AGT AG-3'	
C1	Hsg C1 5'-ACC TTT GAC TCT TCT GT-3'	269
C2	Hsg C2 5'-TCC TTG TAT TTA GTA TC-3'	
E1	Hsg E1 5'-GAC TCT TCC GTC AGC TGG-3'	399
E2	Hsg E2 5'-GCT GGT AAC GGC GC CT-3'	

Primers specific for subgenus B amplifies Adenovirus 3, 7, 11, 14, 16, 34, 35. Subgenus C primers amplifies Adenovirus 1,2,5 6 and subgenus E amplifies only serotype 4.

For subgenus typing a 50µl reaction volume containing 5µl of the DNA extracted from the cell cultures, 5µl of 10X PCR buffer, each dNTP at 200 µM concentrations, 10µM concentrations of each primer and 1 unit of *Taq* DNA Polymerase. The amplification was carried out in a thermal cycler (Perkin Elmer 2700, Cetus, CA, USA) for a total of 40 cycles. A cycle consisted of Denaturation at 91^{0}C for 40 seconds, annealing at 40^{0}C for 30 seconds, and primer extension at 72^{0}C for 40 seconds. Final extension was carried out at 72^{0}C for 7 minutes. At the end of amplification 10µl of the amplified product was analyzed in a 2.0% agarose gel stained with Ethidium bromide.

While performing PCR assay measures were taken to prevent the risk of amplicon contamination.

M. Typing of adenovirus isolated from conjunctival specimen by restriction fragment polymorphism

Isolates and Patients **Ten adenovirus isolates from 7 patients diagnosed to have epidemic conjunctivitis were subjected to RFLP with *Hae* III enzyme.**

DNA extraction from the isolates were done using Bangalore Genei DNA extraction kit.

The DNA was amplified using adenovirus primers Ad TU 7,4 (sequences mentioned above under page number 109,110). PCR amplifications were carried out in 50µl volumes containing 10mM Tris (pH 8.3), 50mM KCl, 1.5Mm $MgCl_2$, 200µM each deoxynucleotide triphosphate, 0.5µM each of first round primers were added. The PCR was carried out with a thermal profile consisting of 94^{0}C for 1 minute, annealing at 50^{0}C for 1 minute and primer extension at 72^{0}C for 2 minute for 36 cycles. After the first round of amplification the products were loaded on a 2.0% agarose gel and the product was cut under UV transilluminator. The products were then purified using QIAGEN II gel elution kit according to the manufacturer's instructions. The purified product was then kept for restriction digestion with *Hae* III restriction enzyme. A 25µl restriction reaction was set containing 10 units of restriction enzyme and 2.5µl of the restriction buffer (For buffer give the breakup). The reaction mixture was incubated at 37^{0}C for 1 hour in a thermal cycler (Perkin Elmer 2700, Cetus, CA, USA). At the end of 1 hour the digested products were run on 12.0% PAGE (procedure described under basic protocols) along with undigested products and molecular weight marker *Hae* III digest. Silver nitrate staining was done (0.1%) to visualize the restriction pattern. The digested products were analyzed with digested pattern of standard strains.

M. Typing of adenovirus detected in interstitial pneumonia by restriction fragment polymorphism

Three NPAs positive for PCR were restriction digested with *Hae* III enzyme. Methods and procedures described for typing adenovirus ocular isolates was followed.

Results

RESULTS

Susceptibility of McCoy, HeLa, Vero, BHK-21, HEp-2, and A549 cell lines for growing C. trachomatis

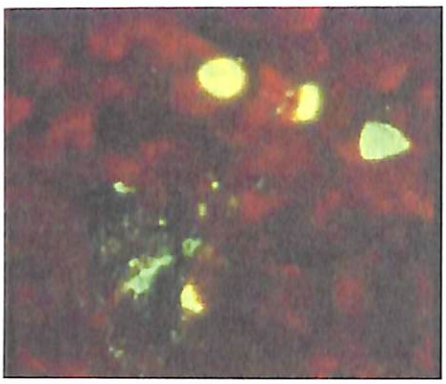
The growth of *C. trachomatis* in different cell line was graded based on EBs counted under 20X objective. The count and the corresponding grade are shown in Table-2 under Material and Methods. The results on growth of *C. trachomatis* in different cell lines are shown in Table -3. None of the serotypes showed growth in A549 cell lines. Overall *C. trachomatis* serotype C had shown a luxuriant growth (graded ++++) in all the cell lines except A549 cell line. Growth of serotype B was moderate (graded ++) in BHK-21 and Vero cell lines. The growth of serotype B was graded +++ in McCoy and HeLa cell lines. Scanty growth (+) of serotype B was observed in HEp-2 cell line. No growth of serotype B was observed in Vero cell line. Serotype Ba had shown good growth (+++) in McCoy and HeLa cell lines. Moderate growth of serotype Ba was observed in HEp-2 cell line, the growth was graded (++). Scanty growth of serotype Ba was seen in Vero and BHK-21 cell lines, growth graded +. Serotype A had grown very well in McCoy; HeLa and BHK-21 cell lines (graded ++++). Moderate growth (++) of serotype A was observed in HEp-2 cell line and Vero cell lines. Overall the growth of *C. trachomatis* was good in McCoy and HeLa cell lines. Next to these two cell lines Vero cell lines had supported the growth *C. trachomatis* better. BHK-21 and HEp-2 cell lines were found to be not very supportive for the growth of *C. trachomatis.* A 549-cell line does not support the growth of *C. trachomatis.* The growth of serotypes A, B and C are shown in Fig.2.

TABLE - 3

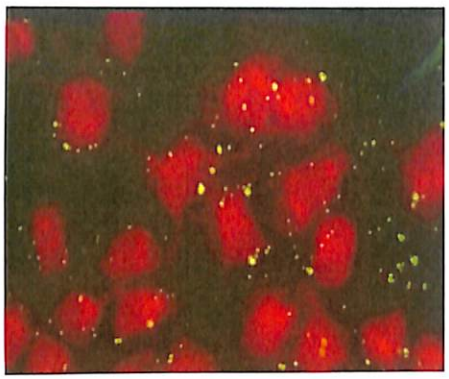
Showing the results on the growth of *C. trachomatis* A,B, Ba and C in McCoy, HeLa, Vero, BHK-21, HEp-2 , and A549 cell lines

Cell lines	Grading on the growth of *C. trachomatis* serotypes			
	Serotype A	**Serotype B**	**Serotype Ba**	**Serotype C**
McCoy	++++ (Very good growth)	+++ (Good growth)	+++ (Good growth)	++++ (Very good growth)
HeLa	++++ (Very good growth)	+++ (Good growth	+++ (Good growth	++++ (Very good growth)
BHK-21	+++ (Good growth	++ (Moderate growth)	+ (Scanty growth)	++++ (Very good growth)
Vero	++ (Moderate growth)	++ (Moderate growth)	+ (Scanty growth)	++++ (Very good growth)
HEp-2	+++ (Good growth)	+ (Scanty growth)	++ (Moderate	+++ (Good growth)
A549	No growth	No growth	No growth	No growth

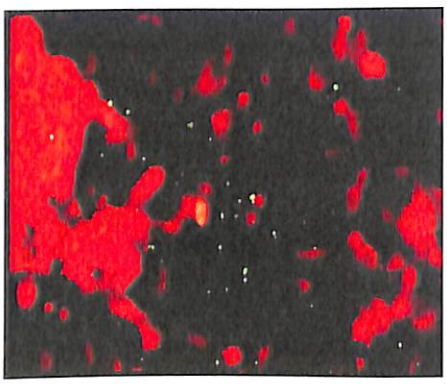
Figure - 2



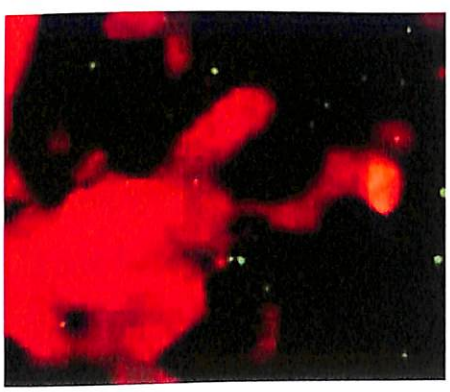
Growth of *C. trachomatis* C in McCoy cell line Growth (++++)



Growth of *C. trachomatis* Ba in McCoy cell line Growth (+++)



Growth of *C. trachomatis* A in HEp-2 cell line Growth (++)



Growth of *C. trachomatis* B in HEp-2 cell line Growth (+)

Effective in house DNA extraction method to amplify *C. trachomatis* from clinical specimen.

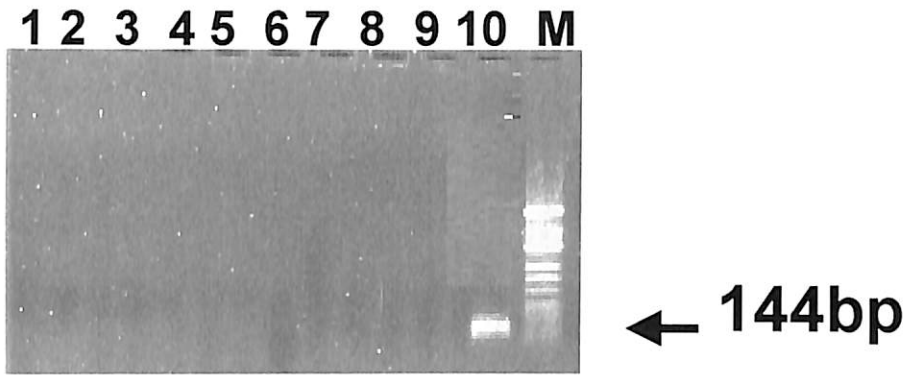
Sixteen specimens were subjected to DNA extraction by methods I (boiling), II (proteinase K digestion, followed by phenol chloroform extraction) and III (GTC method). Two sets of plasmid primers – primer I (Bailey et al, 1994) and Primer II (Lan et al, 1995) were used for DNA amplification to verify the effectiveness of the DNA extraction methods. Of the sixteen specimens processed, 11 specimens extracted by method III (GTC method) was amplified by the two sets of primers. These 11 specimens, included 5 PCR positive specimens in which DNA was also extracted by method II and 2 specimens in which DNA was also extracted by method I. Compared to Method I and II, additional six specimens, extracted by Method III were positive for PCR with both the sets of primers. So boiling (method I) was effective to amplify the DNA from only two specimens. When compared to Method I, Method II was more effective in DNA extraction as shown by the positivity of additional 3 specimens for PCR with two sets of primers. DNA extracted by method I was not stable and the DNA underwent degradation on storage. So method III (GTC) was found to be an effective DNA extraction method for amplification of DNA by PCR. A comparative result on effective in house DNA extraction method to amplify *C. trachomatis* from clinical specimen is shown in Table 4.

Evaluation of Plasmid and MOMP primers for the detection of *C. trachomatis* in conjunctivitis

Both plasmid and MOMP primers were absolutely specific for the detection of *C. trachomatis* DNA. The specificity of MOMP and plasmid primers are shown in Fig 3 and 4 respectively. The analytical sensitivity of plasmid primers was estimated to be 2ng (Fig.5), which was equivalent to 10 EBs of *C. trachomatis*. The analytical sensitivity of MOMP primers was 6ng (Fig.6), which was equivalent to 30 EBs of

Figure - 3

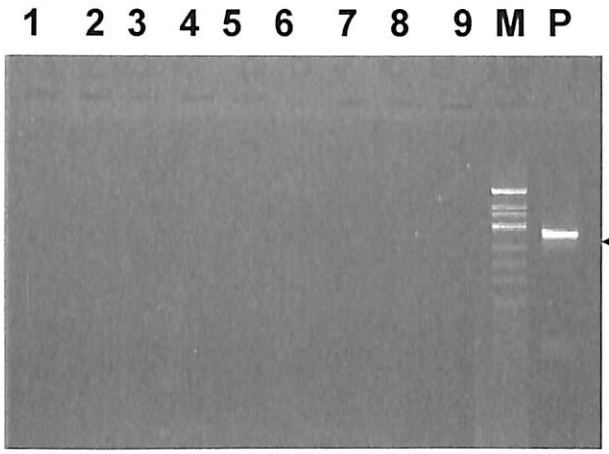
Agarose gel electrophoretogram showing the specificity of MOMP primers



1: Negative reagent control
2: HSV-1
3: *S. aureus*
4: *H. influenzae*
5: *M.tuberculosis*(H37RV)
6.*S pyogenes*
7: *P.acne*
8: Human DNA
9:Adenovirus type 2
10: positive (*C.trachomatis* Ba)control
M: PHI X 174 DNA/ *Hinf* I digest)

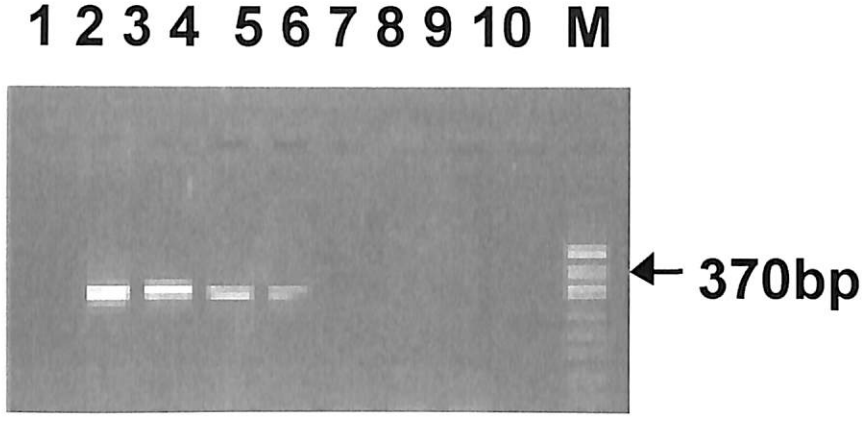
Figure - 4

Agarose gel electrophoretogram showing specificity of plasmid primer (Bailey et al,1994)



1: Negative reagent control
2: HSV-1
3: *S. aureus*
4: *H. influenzae*
5: *M.tuberculosis*(H37RV)
6: *S. pyogenes*
7: *P.acne*
8: Human DNA
9:Adenovirus type
M: PHI X 174 DNA/ *Hinf* I digest)
p: positive(*C.trachomatis* Ba)control

Figure - 5
Agarose gel electrophoretogram showing the sensitivity of plasmid primer (Bailey et al,1994)



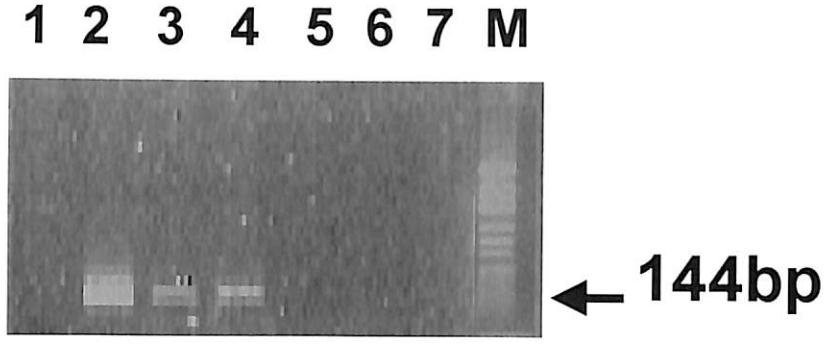
1: Negative reagent control

2-10:serial 10-fold dilutions of *C. trachomatis* Ba DNA PCR positivity seen up to 10^{-5} (sensitivity 2ng)

M:DNA ladder PHI X 174 DNA/ *Hinf* I digest)

Figure - 6

Agarose gel electrophoretogram showing the sensitivity of MOMP primers



1: Negative control

2: 1-7: serial 10 fold dilutions of

C. trachomatis Ba DNA (sensitivity limit 6ng)

M : DNA ladder PHI XDNA/ *Hinf* I digest

TABLE - 4

Comparison of three DNA extraction methods for amplification by Polymerase chain reaction (PCR) using plasmid Primer I and II.

Total no of specimens	DNA extraction method	No of specimens positive by PCR with	
		Primer I	Primer II
16	Method I	2	2
	Method II	5	5
	Method III	11	11

- Method I: Lakeman et al, 1995-Boiling and snap-cooling of the clinical specimen.
- Method II: Saitoh-inagawa et al, 1996- Specimen treated with 20.0µl of Proteinase K (20mg/ml) in 200µl of lysis buffer (0.1M Tris-Cl [pH8.0], 0.01 EDTA, 1.0% Sodium Dodecyl Sulphate [SDS]). This was followed by standard Phenol-Chloroform extraction.
- Method III: (Guanidine thiocyanate [GTC] method)-Modified protocol of Pitcher et al (1989)-Specimen treated with Proteinase K followed by lysis with 150 µls each of 5.0M GTC, 0.1M EDTA pH 7.0, 7.5 M ammonium acetate and 450 µl of chloroform: isoamyl alcohol 24:1V/V. DNA precipitated with isopropanol and washed with 80% ethanol.

C. trachomatis. Of the 80 specimens subjected for this study, 11 specimens (13.8%) from 10 patients (12.5%) were positive by PCR with plasmid (Fig. 7) primers whereas only 3 specimens (3.8%) from 2 (2.5%) were positive by PCR with MOMP primers. The time duration of thermal profile for PCR with the plasmid primer was 180 minutes whereas the thermal profile for PCR with MOMP primer was 270 minutes. Thus PCR with plasmid primer was more rapid and sensitive for the detection of *C. trachomatis* from conjunctivitis specimens than with MOMP primers. This difference was statistically significant (McNemar test $P<0.01$). Fig. 8 shows PCR positivity on conjunctival specimens with MOMP primers. Comparative results of PCR with MOMP and plasmid primer are shown in Table 5.

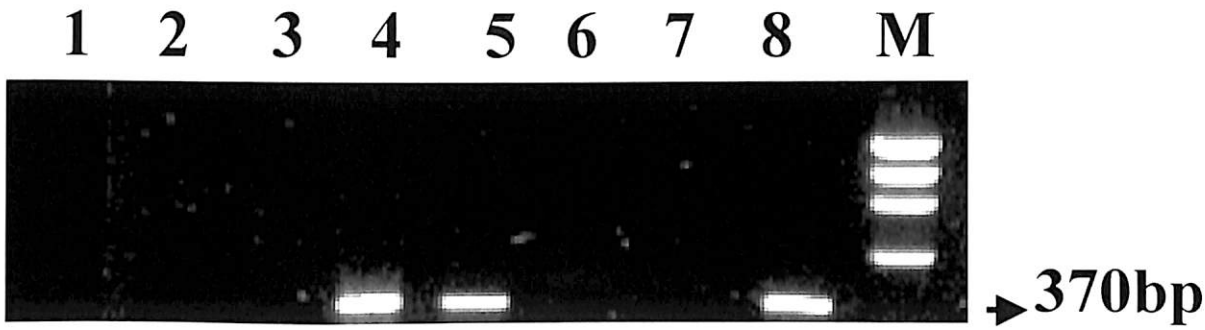
The sensitivity of MOMP primers against plasmid primers was calculated and determined to be 27.3 per cent. The efficiency of PCR with MOMP primer was 90 per cent.

Evaluation of PCR with plasmid primer against conventional method for the detection of *C. trachomatis* in conjunctivitis.

A total of 486 specimens collected from 347 patients with clinical diagnosis of primary conjunctivitis were processed for the detection of *C. trachomatis* by PCR, IF and culture. Among these 486 specimens, 27 (5.6%) specimens from 22 (6.3%) patients were positive for *C. trachomatis* by PCR with plasmid primer. Among these 27 specimens, 3 (0.6%) specimens collected from 2 (0.57%) patients were positive for IF (Fig.9), and culture (Fig.10). Fig.11 shows the EBs of *C. trachomatis* in the McCoy cell cultures, isolated from the case of Ophthalmia neonatorum (Giemsa stained) observed under dark ground microscope. Among these three specimens, two specimens were collected from both the eyes of a 10-day old infant diagnosed to have Ophthalmia neonatorum. The other specimen collected from a 13 year old girl with conjunctivitis. Thus PCR has increased the clinical sensitivity by 5.0 per cent in

Figure - 7

Agarose electrophoretogram showing the plasmid amplified products of *C. trachomatis* from conjunctival swabs



1: Negative control

2: Extraction control

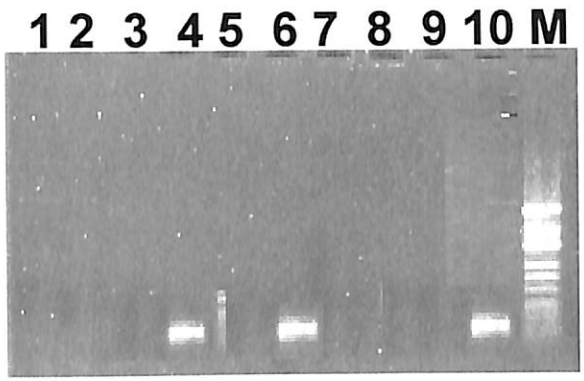
4,5 :conjunctival swabs positive for PCR

3,6,7 :conjunctival swabs negative for PCR

8:positive control : *C. trachomatis* Ba DNA M: PHI X 174 DNA/ *Hinf* I digest)

Figure - 8

Agarose gel electrophoretogram showing the amplified products of MOMP primers



1: Negative reagent control

2: extraction control

4 & 6 : specimens positive for C. trachomatis

3,5,8,9 Specimens negative for C. trachomatis

10: positive (*C.trachomatis* Ba)control

M: PHI X 174 DNA/ *Hinf* I digest)

Figure - 9
Conjunctival swab stained with immunofluorescence stain showing elementary bodies of *C. trachomatis* (20X Magnification)

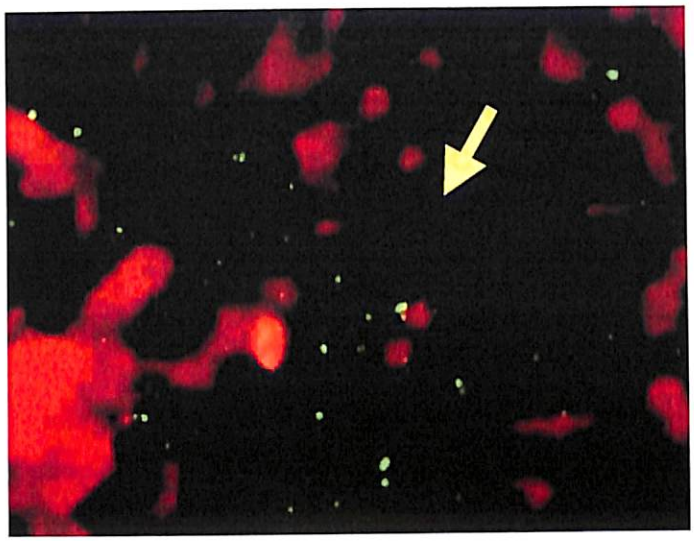


Figure - 10
Immunofluorescence staining showing Reticulate (white arrow) and Elementary bodies (yellow arrow) of *Chlamydia trachomatis* isolated from a case of ophthalmia neonatorum in McCoy cell culture (40x Magnification)

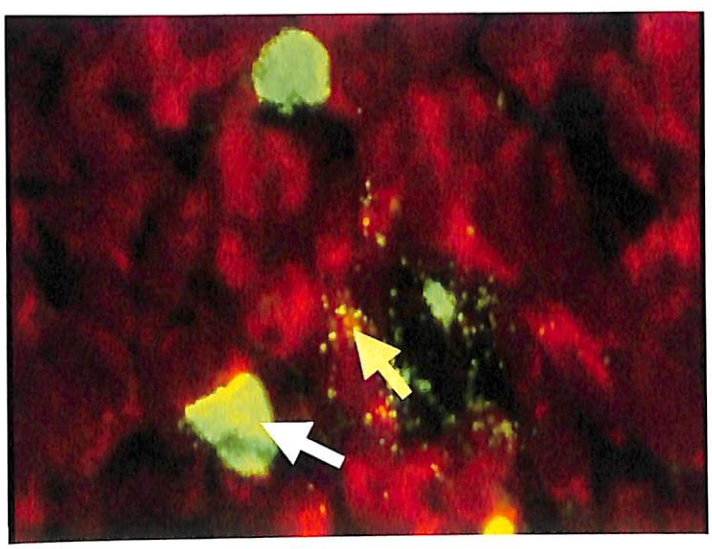


Figure - 11
Darkground microscopic picture showing elementary bodies of *C.trachomatis*(Grown in McCoy cell line) in a Geimsa stained slide (20X Magnification).

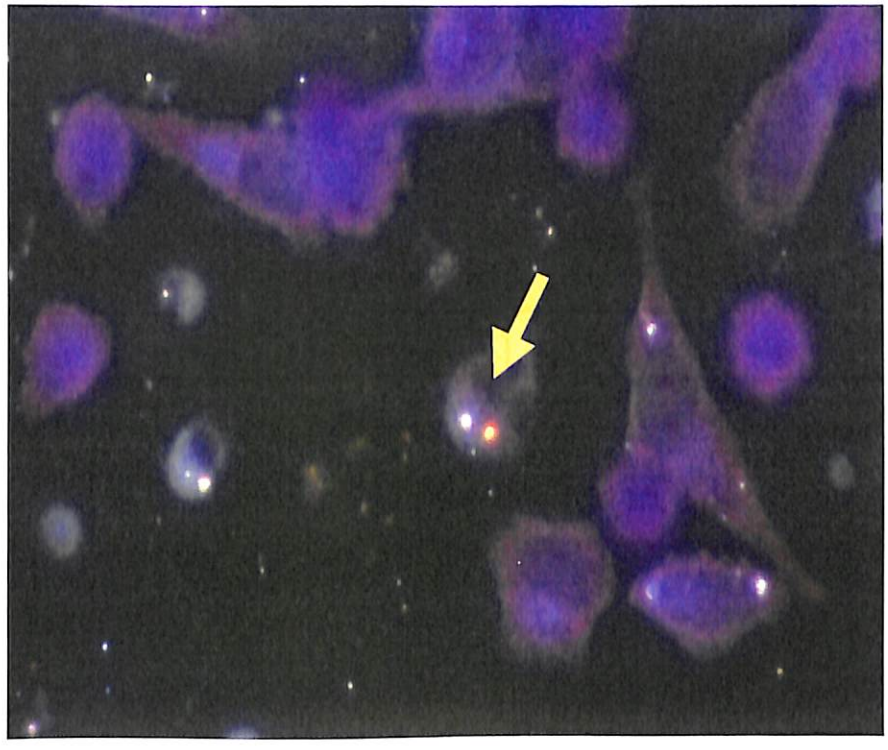


TABLE - 5

Results on Polymerase chain reaction (PCR) with MOMP and plasmid primers for the detection of *C. trachomatis* in conjunctivitis

Total no of specimens	Total no of specimens	PCR positive with plasmid primer		PCR positive with MOMP primer	
		No of specimens	No of patients	No of specimen	No of specimen
80	55	11(13.8%)	10(12.8%)	3*(3.8%)	2(2.5%)

* Three specimens positive by MOMP PCR were also positive for PCR with plasmid primer.

TABLE - 6

Comparative results of Immunofluorescence (IF), culture and PCR with plasmid primer on *C .trachomatis* positive conjunctivitis specimens.

Total no of specimens	Total no of patients	IF positive		Culture		Plasmid PCR	
		No. of specimen	No. of patients	No. of specimen	No of patients	No of specimen	No of patients
487	347	3* (0.6%)	2(0.6%)	3* (0.91%)	2(0.6%)	27(5.6%)	22 (6.3%)

* 3 specimens positive by smear and culture were also positive by PCR

TABLE - 7

Significant bacterial pathogens isolated from the conjunctivitis cases.

Bacterial pathogens isolated	No of specimens
Haemophilus influenzae	(1.6%)
Staphylococcus aureus	28 (5.8%)
Streptococcus pneumoniae	3 (0.6%)
Streptococcus viridans	2 (0.41%)

detecting *C. trachomatis* in conjunctivitis. When the result of PCR was compared with results of the conventional method IF, the difference in results was statistically significant (McNemar $P<0.01$). Similarly when the results of PCR and culture were compared the difference was statistically significant (McNemar test $P<0.01$). The sensitivity of IF, culture technique was 11.1 per cent. The specificity was 100 per cent. The efficiency of IF and culture technique was calculated to be 95 per cent. Comparative results of PCR, IF and culture on the detection of *C. trachomatis* from conjunctivitis is shown in Table 6. In 4 specimens (0.8%) collected from three patients, adenovirus antigen was detected by IF staining. Adenovirus was not isolated in any of the specimens. Culture and IF staining for HSV were negative for all the specimens. In 4 specimens collected from 2 patients eosinophils were seen by Geimsa staining. The significant bacterial pathogens isolated from the conjunctival specimens are shown in Table 7. The significant bacterial pathogens isolated from these cases were *Haemophilus influenzae* in 8 (1.6%) specimens, *Viridians Streptococcus* in 2 (0.41%) and *Staphylococcus aureus* in 28 (5.8%) and *Streptococcus pneumoniae* in 3(0.61%) specimens.

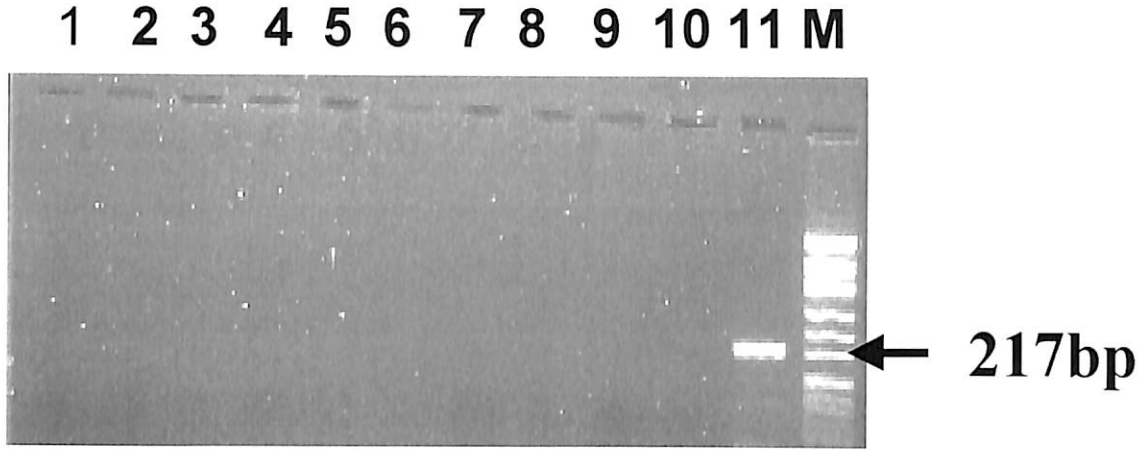
Evaluation of conventional methods for the detection of C. trachomatis and HSV in males with urethritis and females with cervicitis against PCR.

Analytical sensitivity and specificity of the plasmid primers

The plasmid primers were absolutely specific for *C. trachomatis* plasmid DNA. No amplification was observed with DNA of other microorganisms and human DNA tested was observed. The specificity of plasmid primers is shown in Fig 12. The analytical sensitivity of the primer was 0.2 ng equivalent to 10 EBs of *C. trachomatis.* Fig. 13 shows the PCR results on the analytical sensitivity of the plasmid primer.

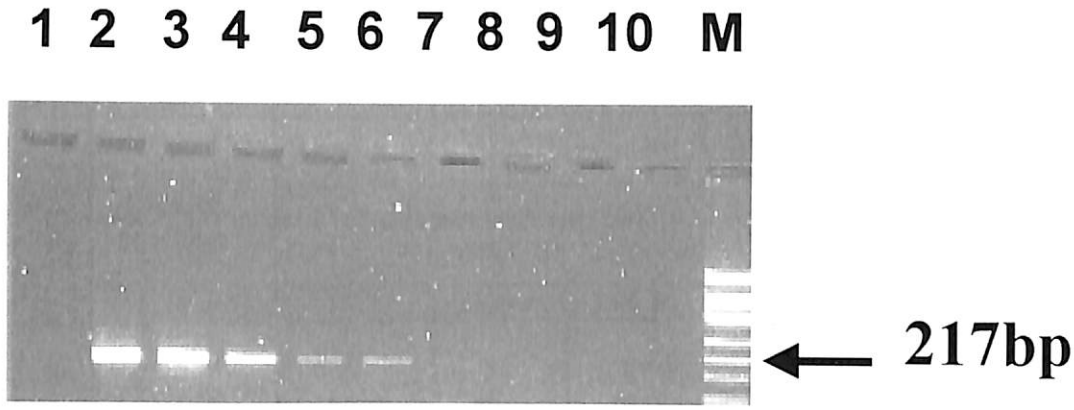
Figure - 12

Agarose gel electrophoretogram showing the specificity of plasmid primer (Lan et al, 1995)



1: Negative reagent control
2: *C.albicans*
3: S. *aureus*
4: S. *epidermidis*
5: *C. xerosis*
6: *K.pneumoniae*
7: *P.acne*
8: Human DNA
9:HSV- 2
10: *S. pyogenes*
11: positive (*C.trachomatis* Ba)control
M: PHI X 174 DNA/ *Hinf* I digest)

Figure - 13
Agarose gel electrophoretogram showing the sensitivity of plasmid primers (Lan et al,1995)



1: Negative reagent control

2-10: serial 10-fold dilutions of *C. trachomatis* Ba DNA PCR positivity seen up to 10^{-6} dilutions (limit of sensitivity 2ng)

M :DNA ladder PHI X 174 DNA/ *Hinf* I digest)

TABLE - 8

Comparative results of microbiological investigations and Polymerase chain reaction (PCR) for detection of *Chlamydia trachomatis* and herpes simplex virus (HSV)

No. of patients	No. of specimens	Specimens positive for ·					
		C. trachomatis no.(%) [95%CI]			HSV no.(%) [95%CI]		
		FAT	Culture	PCR	FAT	Culture	PCR
25 females with mucopurulent cervicitis	25(cervical swabs)	none	none	1(4.0%) [0.0-11.7%]	none	none	3(12.0%) [0.0-24.7%]
72 males with urethritis	75 (urethral swabs)	3(4.0%)* [0.0-8.4%]	none	9(12.0%) [4.6-19.3%]	none	none	5(6.6%) [1.0-12.4%]

CI-95% confidence interval; FAT, Fluorescent antibody test;* 3specimens positive by FAT were also positive by PCR.

Results of PCR and microbiological investigations on detection of C. trachomatis and HSV

Of the 25 endocervical swabs (from 25 women) tested, 1 (4.0%) was positive for *C. trachomatis* by PCR. None of the specimens were positive by smear and culture for *C. trachomatis.* Among the 75 urethral swabs from 72 male patients processed, PCR detected *C. trachomatis* plasmid DNA in 9 (12.0%) specimens. Among these, 3(4.0%) were positive by direct smear showing more than 10 EBs in direct smear. One of these patients was serologically positive for HIV infection. Thus overall PCR detected *C. trachomatis* in 7 additional (includes both endo cervical and urethral specimens) as compared to IF, which detected in 3 (3.0%) of the specimens. Fig 14 shows the *C. trachomatis* plasmid PCR amplified products of urethral specimen.

Thus the detection of *C. trachomatis* by PCR was found to be statistically more significant compared to IF (McNemar test, $P<0.05$) and culture (McNemar test, $P<0.01$).

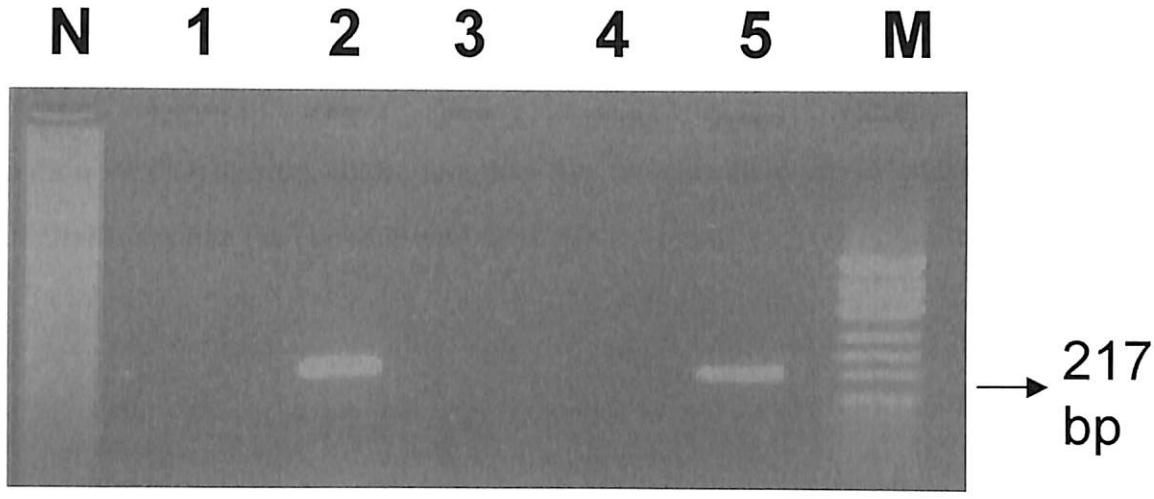
HSV DNA was detected in 3 endocervical specimens (12.0%) by PCR. Five (6.6%) urethral swabs were positive for HSV PCR. Smear and culture were negative for all the specimens. Thus PCR detected HSV in 8 (8.0%) specimens compared to none by smear and culture (McNemar test $P<0.01$). Comparative results of PCR, IF and culture on detection of *C. trachomatis* and HSV in cervicitis and urethritis is shown in Table 8.

Evaluation of Polymerase chain reaction for the detection of *C. trachomatis* in interstitial pneumonia against the conventional methods.

A total of 45 NPA specimens with clinical diagnosis of interstitial pneumonia was processed for the detection of *C. trachomatis* by PCR, IF and culture. Among the 45 specimens, 7 (15.6%) specimens were positive for *C. trachomatis* by PCR. Among

Figure - 14

Agarose gel electrophoretogram showing the PCR amplified products of the cryptic plasmid gene of *Chlamydia trachomatis*



N - Negative control

1 - Extraction control

2 - genital swab positive for *C .trachomatis*

3 & 4 -genital swab negative for *C .trachomatis*

5: Positive control (*C. .trachomatis* Ba DNA)

M - DNA ladder PHI 174DNA/*Hinf* I digest

these 7 specimens. 1 (2.2%) specimen was positive for IF technique. Culture remained negative for all the specimens. Among the 6 specimens positive for *C. trachomatis* by PCR, 2 of them were positive for adenovirus also. Thus PCR has increased the clinical sensitivity by 13.4%. Statistical calculation was not possible as the sample size was small. In none of the specimens PCR inhibitors were encountered.

The comparative results of PCR, IF and culture in detecting *C. trachomatis* from interstitial pneumonia is tabulated (Table 9).

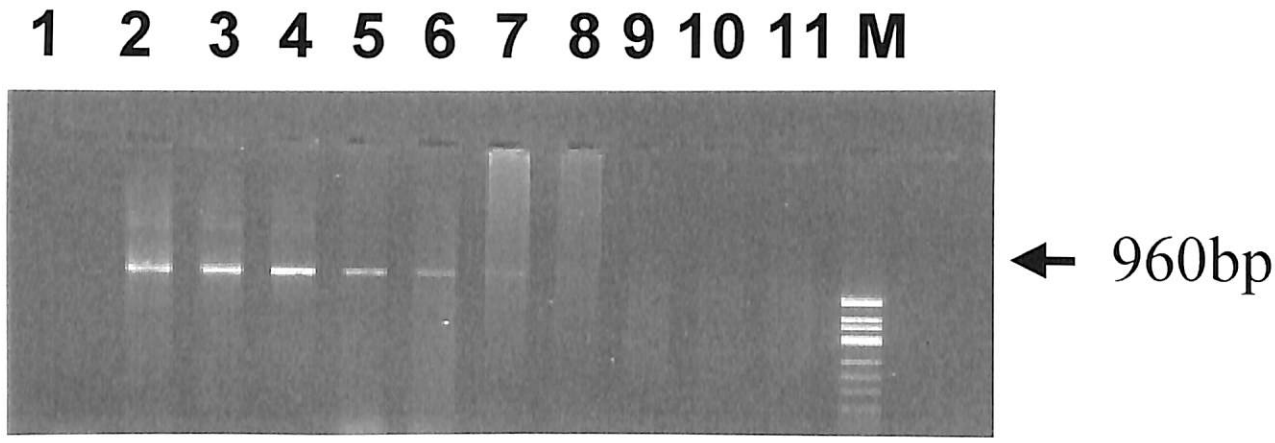
Evaluation of Polymerase chain reaction for the detection of Adenovirus in conjunctivitis against the conventional methods.

The analytical sensitivity after second round of amplification with the Fast type SSP adenovirus detection kit was estimated to be 0.0030 fg. The kit was absolutely specific for the adenovirus DNA. Fig.15 shows the analytical sensitivity of the Fast type SSP adenovirus detection kit. Fig. 16 shows the specificity of the kit.

Of the 85 conjunctival swabs processed, 77 (90.0%) specimens were positive by PCR (Fig 21). Among these 77 specimens, in 63 (74.1%) specimens adenovirus antigen was detected by IF staining (Fig.17). Of these 63 specimens, in 10 (11.8%) specimens, adenovirus was isolated in Vero cell culture. The virus isolation was confirmed by IF staining (Fig 18). The virus was isolated with in 72 hours of inoculation. Fig. 19, Fig.20 shows the normal and adenovirus infected Vero cell line respectively. Comparative results of PCR, IF and culture are shown in Table 10. When the results of PCR were compared with IF results, the difference were statistically significant (McNemar $P<0.01$). The specificity of IF technique when compared to PCR was 82 per cent. The efficiency of IF technique in detecting adenovirus in conjunctivitis was 83 per cent. Similarly when the results of PCR were

Fig - 15

Agarose gel electrophoretogram showing the sensitivity of SSP Fast track adenovirus detection kit after the second round



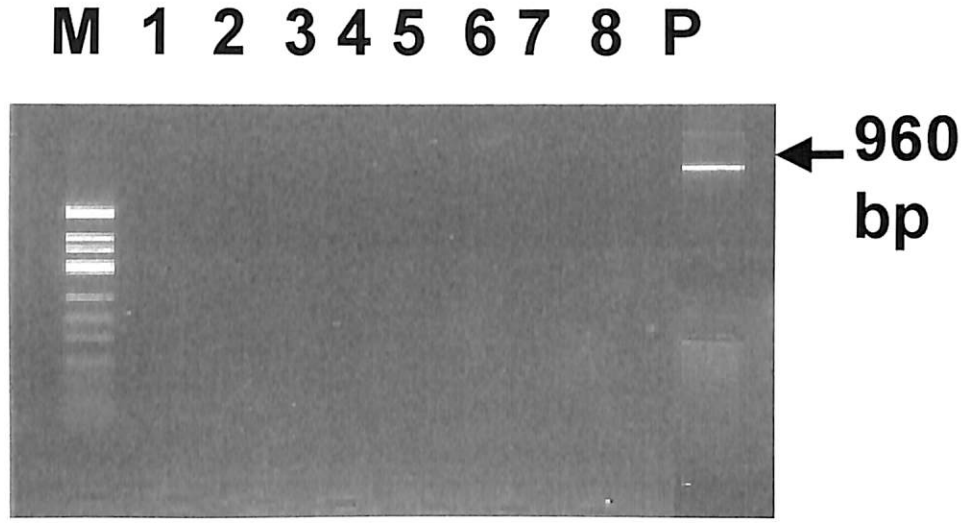
1:negative control

2-11 : serial 10 fold dilutions of Adenovirus DNA ,PCR positivity seen upto 10^{-7} dilutions (detection limit 0.0.0030fg)

M: DNA ladder PHI XDNA/ *Hinf* I digest

Figure - 16

Agarose gel electrophoretogram showing the specificity of SSP Fast track adenovirus detection kit



M : DNA ladder PHI X174 DNA/*Hinf* I digest

1: HSV-1 2: *S.aureus* 3: *H. influenzae*

4: *M.tuberculosis* 5: *S. pyogenes* 6: *P.acnes*

7:Human DNA 8 :unloaded

P : Positive control-adenovirus 2 DNA (ATCC-VR-846)

Figure - 17

Immunofluorescence staining stained smear showing conjunctival cells positive for adenovirus (20X magnification)

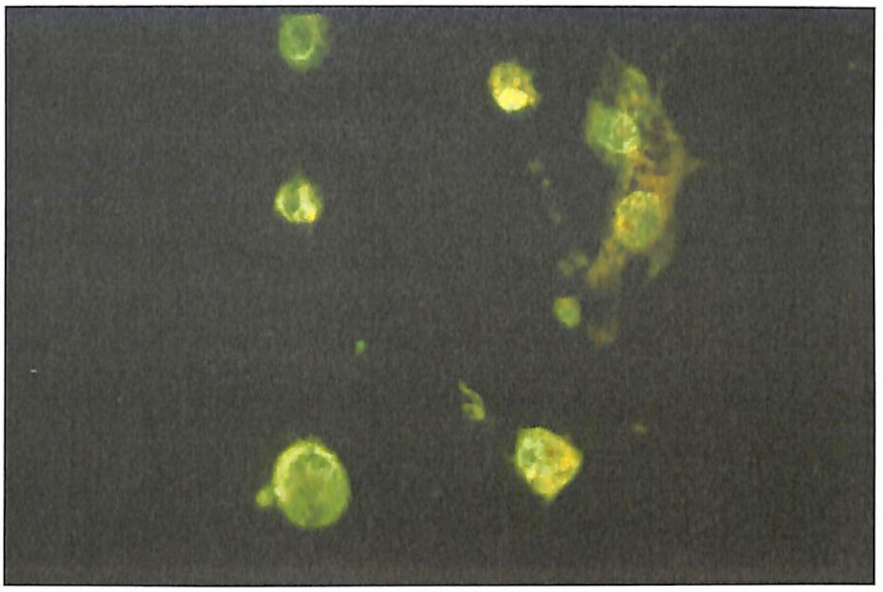


Figure - 18

Adenovirus grown in Vero cell line from a patient with clinical diagnosis of epidemic conjunctivitis (20x magnification)

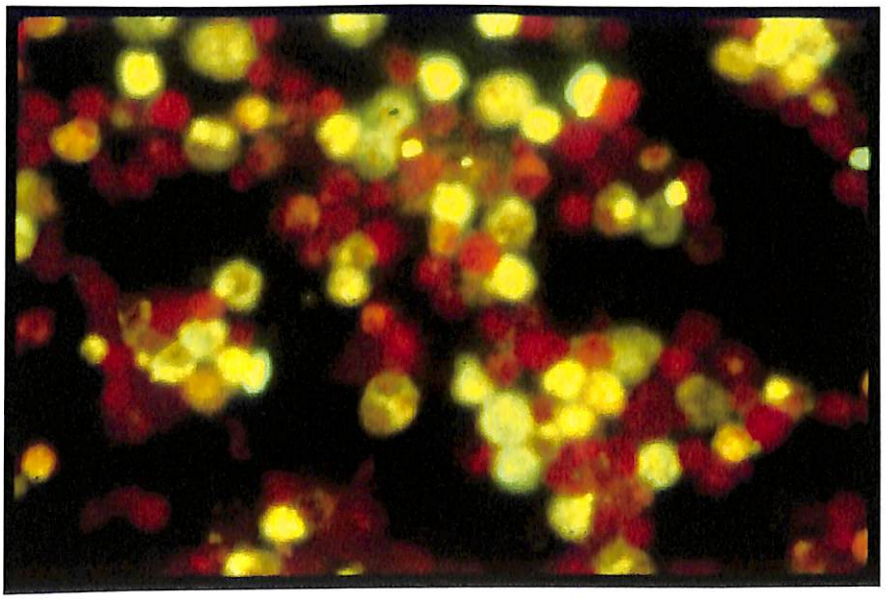


Figure - 19

Normal Vero cell line (20x magnification)

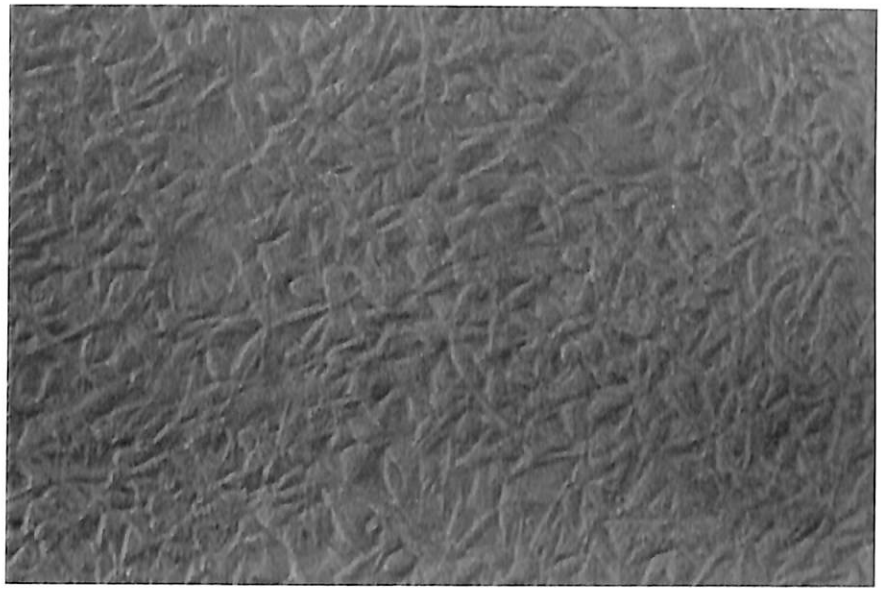
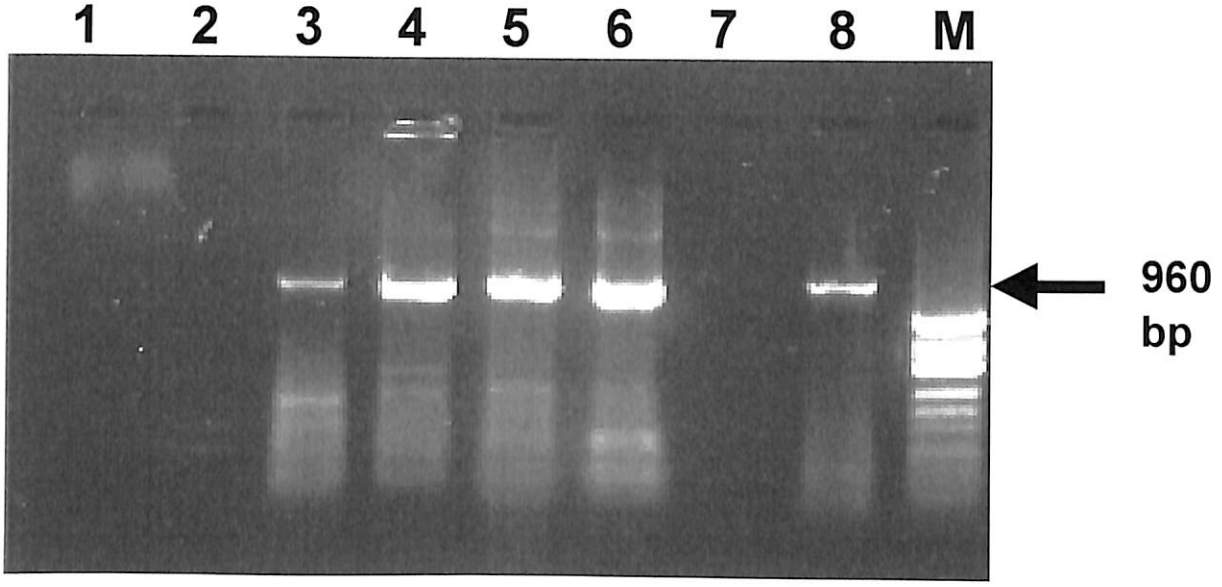


Figure - 20

Adenovirus Cytopathic effect in Vero cell line, isolated from a case of conjunctivitis (20x magnification)

Figure - 21

Agarose gel electrophoretogram showing amplified products of adenovirus from conjunctivitis specimens after second round of nested PCR



1 : Negative control

2 : DNA extraction control

3-6 : conjunctivitis specimens positive for adenovirus

7: conjunctivitis specimen negative for adenovirus

8 : positive control (Adenovirus 2 DNA)

M : DNA ladder PHI X DNA/*Hinf* 1 digest

TABLE - 9

Comparative results of PCR, IF and culture in detecting *C. trachomatis* from interstitial pneumonia

Total number of specimens	Total no of patients	Specimens positive for *C. trachomatis* by		
		PCR	IF	Culture
45	45	7(15.6%)	1*(2.2%)	none

* One specimen positive for IF was also positive by PCR.

TABLE - 10

Comparative results of PCR, IF and culture on detection of adenovirus from conjunctivitis cases.

Total number of specimens	Total no of patients	Specimens positive for *C. trachomatis* by		
		PCR	IF	Culture
85	62	77(90.0%)	63*(74.1%)	10#(11.8%)

* These 63 specimens positive by IF were also positive for PCR.

\# These 10 specimens positive by IF were also positive for PCR.

compared with culture results the difference was statistically significant (McNemar $P<0.01$). The specificity of culture was calculated to be 100.0 per cent. The efficiency of culture technique in detecting adenovirus from conjunctival specimen was calculated to be 19.3 per cent. Fig.21 shows 960bp PCR amplified products of adenovirus from conjunctivitis specimens after second round.

Evaluation of Polymerase chain reaction for the detection of Adenovirus in interstitial pneumonia against the conventional methods.

A total of 45 NPA specimens with clinical diagnosis of interstitial pneumonia were processed for the detection of adenovirus by PCR, IF and culture. Among these 45 specimens, 4 specimens were positive by PCR for adenovirus (8.8%). Of these 4 specimens, 1(2.2%) specimen was positive by IF technique. Statistical calculation is not possible as the sample size is very less. But the PCR technique has increased the clinical sensitivity by detecting adenovirus in 3(6.6%) additional specimens. Adenovirus was not isolated in any of the specimens. PCR inhibitors were not encountered in any of the specimens. Comparative results of PCR, IF and culture on the detection of adenovirus from NPA are shown in Table 11.

RT-PCR to determine the MBC of antibiotics against *C. trachomatis*

The results of the MIF and RT-PCR to determine the MBC of drugs tested against the standard strains and the clinical isolates of *C. trachomatis* shown in Table12. The MIC is defined, as the lowest antibiotic concentration required to inhibit development of Chlamydial inclusion after 48 hours of incubation. The Minimum bactericidal concentration (MBC) is defined as the lowest antibiotic concentration required to kill the organism. The MBC concentrations for all the antibiotics by IF technique was found by passing the culture harvests exposed at MIC to McCoy cell cultures. *C. trachomatis* exhibited varied susceptibility to the drugs that had been tested.

TABLE - 11

Comparative results of PCR, IF and culture on the detection of adenovirus from NPA

Total number of specimens	Total no of patients	Specimens positive for *C. trachomatis* by		
		PCR	IF	Culture
45	45	4(8.8%)	1*(2.2%)	none

* These 63 specimens positive by IF were also positive for PCR.

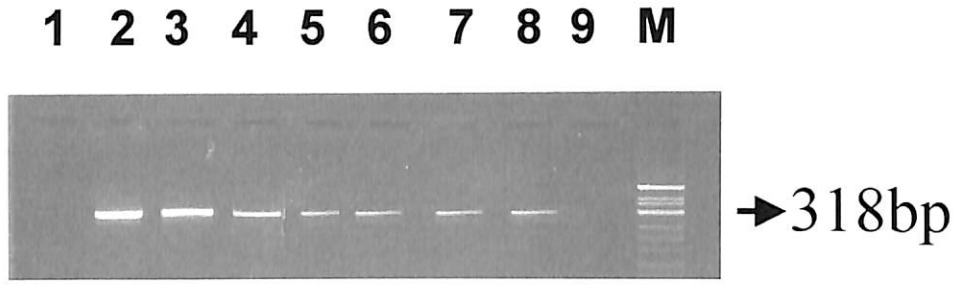
C. trachomatis serovar Ba exhibited profound resistance to erythromycin up to 80µg / ml by MIF and RT-PCR 160µg / ml. (Fig. 22) Further passing of the culture harvest exposed to 100µg / ml of erythromycin showed good growth of this serovar as observed by MIF staining. All other strains tested were sensitive in a broad range of 0.2-1.5 µg / ml of erythromycin. All the standard strains exhibited resistance upto Roxithromycin up to 0.10 µg / ml by RT-PCR (Fig. 24) whereas by MIF, the presence of organism was found only up to 0.025 µg / ml. Ciprofloxacin showed a range of MBC of 1.0-2.0 (Fig. 23) by RT-PCR. The MBC value for Ciprofloxacin against *C. trachoamtis* Ba by RT-PCR was 2.0 µg / ml. All the strains of *C. trachomatis* were sensitive to Sparfloxacin at a concentrations of 0.000625 –0.005µg / ml by MIF and RT-PCR (Fig.25). The MIC of all the drugs determined by MIF staining were three to six tube dilution or more lower than RT-PCR except for Ciprofloxacin against *C. trachomatis* Ba. All the results were reproducible. The MIC and MBC concentrations determined by IF were the same for all the antibiotics and all the organisms. Throughout the study DNA contamination and its amplification were not encountered with McCoy cell culture harvests in all the experiments. Difficulties were encountered in interpretation of MIC range for sparfloxacin by MIF as this drug showed auto fluorescence and observer variability was present in the interpretation of MIF results.

Determination of MIC of antibiotics in direct clinical specimen with C. *trachomatis*.

Five conjunctival swabs collected from individuals with out any clinical symptoms of conjunctivitis were spiked with *C. trachomatis* serotype Ba. The spiking was done in such a way that the specimens were negative by IF staining but positive for PCR. With two specimens spiked with *C. trachomatis* Ba MIC against Ciprofloxacin and erythromycin was determined. For one specimen MIC of Roxithromycin was determined. In one specimen the MIC of Ciprofloxacin by MIF

Figure - 22

Agarose gel electrophoretogram showing RT-PCR results on sensitivity of *C . trachomatis* to Erythromycin



1: Negative control 2: 40µg/ml 3: 60µg/ml

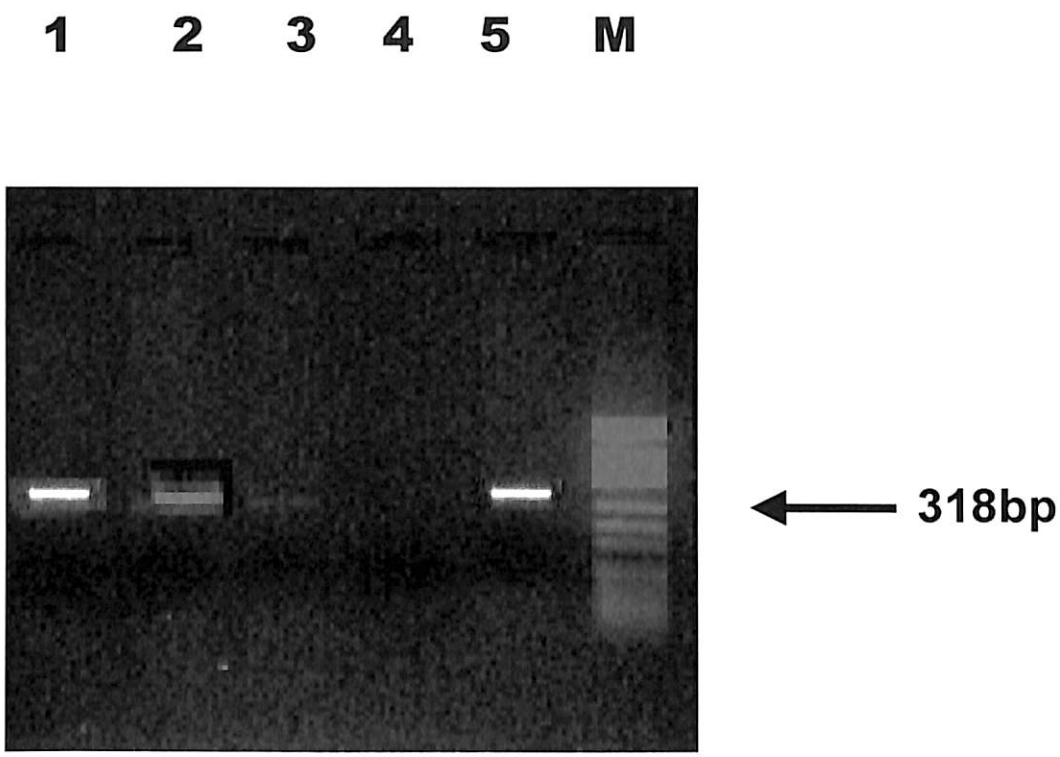
4 : 80 µg/ml 5: 100 µg/ml 6: 120 µg/ml

7:140 µg/ml 8:160 µg/ml 9: 170 µg/ml

M: DNA ladder, Hinf1 digest of Phi X DNA

MBC 170 µg/ml

Fig 23
Agarose gel electrophoretogram showing
RT-PCR results on sensitivity of *C.trachomatis*
Ba to ciprofloxacin



1 : 1.0 µg/ml 2 : 1 .5 µg/ml

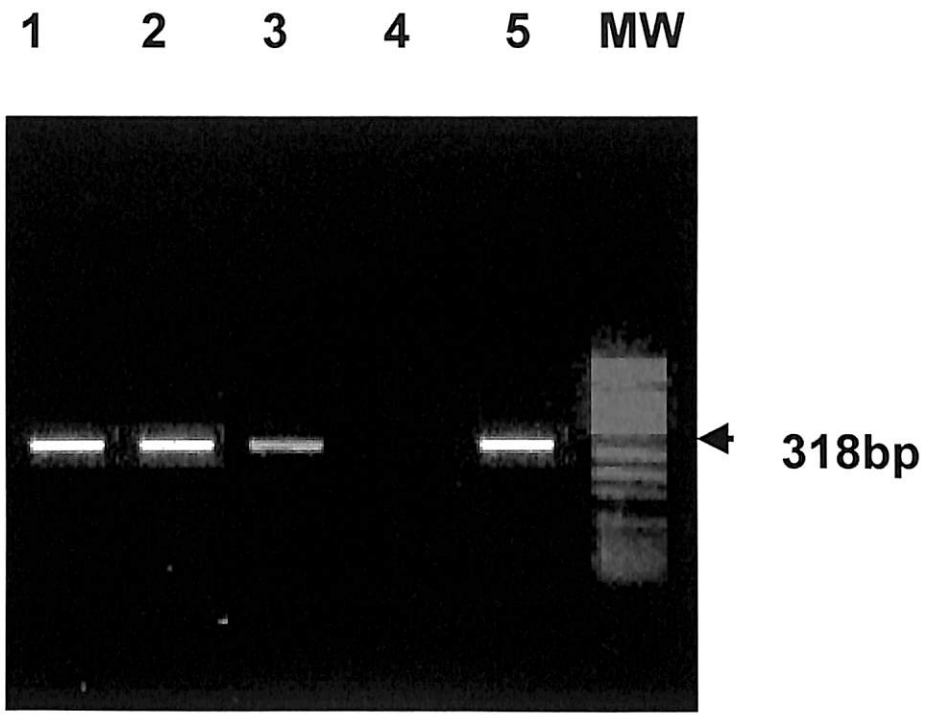
3 : 2. 0 µg/ml 4: 2.5µg/ml

5:positive control

M: *Hinf I* digest of Phi XDNA

MBC : 2. 0 µg/ml

Figure - 24
Agarose gel electrophoretogram showing RT-PCR results on sensitivity of *C.trachomatis* Ba to Roxithromycin

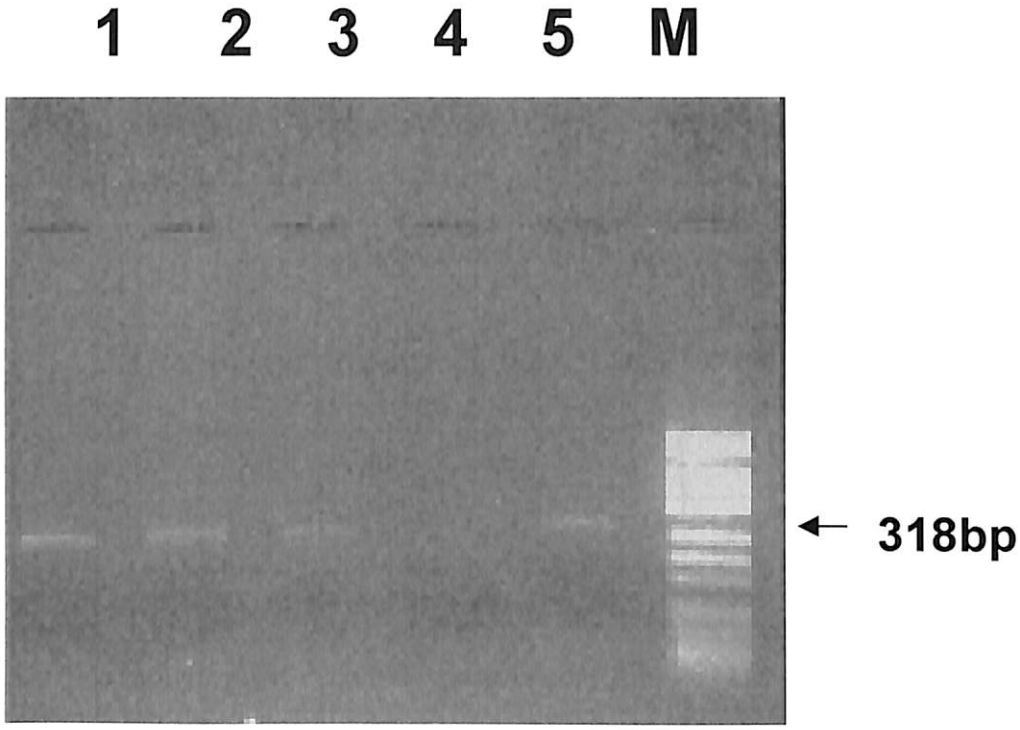


Lane 1: 0.025µg/ml
Lane 2 : 0 .050 µg/ml
Lane 3: 0.075 µg/ml
MBC:0.1 µg/ml

Lane 4: 0.1 µg/ml
Lane 5: positive control
MW: *Hinf I* digest Phi XDNA

Figure - 25

Agarose gel electrophoretogram showing RT-PCR results on sensitivity of *C.trachomatis* Ba to sparfloxacin



1: 0.000625 µg/ml 2: 0. 00125 µg/ml

3: 0. 0050 µg/ml 4 : 0. 0025 µg/ml

5: positive control

M: *Hinf I* digest of Phi xDNA

MBC: 0.0025µg/ml

TABLE - 12

Results of Microimmuofluorescence (IF) and Reverse Transciptase Polymerase Chain Reaction (RT-PCR) methods to determine the Minimum Bactericidal Concentrations (MBC) of drugs in µg/ml against *Chlamydia trachomatis*

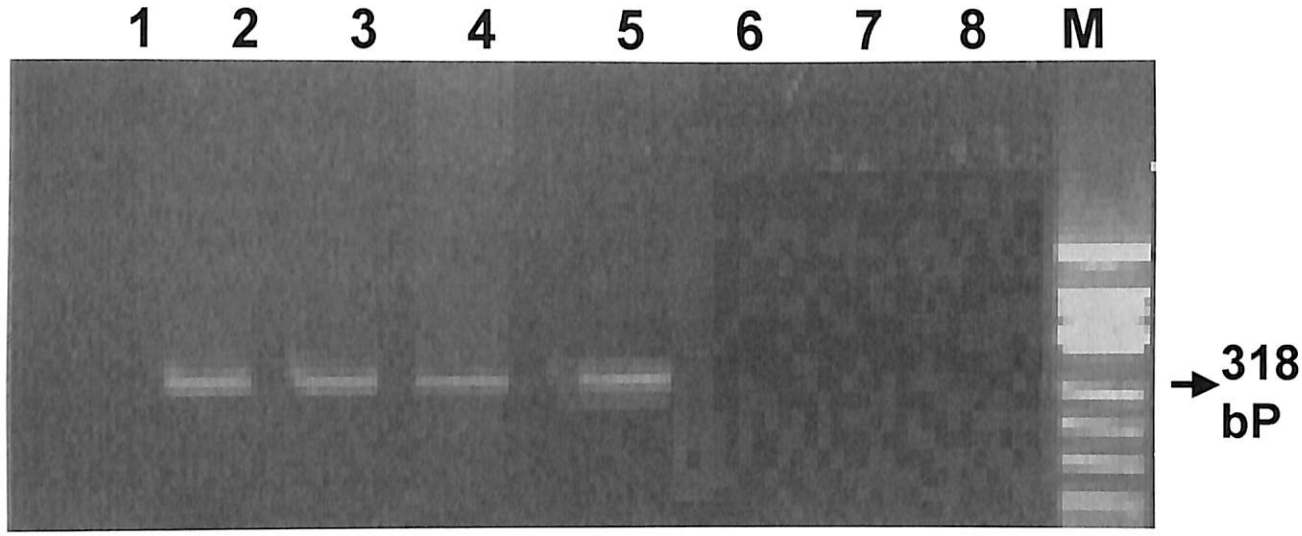
Strains of *Chlamydia trachomatis*	Minimum Bactericidal Concentrations (MBC) of drugs in µg/ml against *Chlamydia trachomatis* strains							
	Erythromycin		Roxithromycin		Ciprofloxacin		Sparfloxacin	
	IF	RT	IF	RT	IF	RT	IF	RT
ATCC A	0.4	1.0	0.05	0.1	1.0	2.0	0.000625	0.00125
ATCC B	0.2	0.4	0.025	0.075	0.5	1.0	0.000625	0.00125
ATCC Ba	80	170	0.05	0.1	4.0	2.0	0.00125	0.005
ATCC C	0.2	0.4	0.025	0.075	0.5	1.0	0.000625	0.00125
Isolate 1	0.25	2.0	0.025	0.04	0.06	0.25	0.000625	0.00125
Isolate 2	0.25	1.25	0.025	0.05	0.06	0.125	0.000625	0.00125
Isolate 3	0.5	1.5	0.025	0.05	0.125	0.25	0.000625	0.00125

was determined as 0.25µg/ml. The MBC value determined by RT-PCR was higher than that of MIC value obtained by IF technique (1.0µg/ml). *C. trachomatis* Ba which showed profound resistance to erythromycin, when spiked with the clinical specimen showed MIC value by both IF and culture within the acceptable range. The MIC value for erythromycin determined by IF was 2.5 µg/ml. The MIC value determined by RT-PCR technique was higher (3.5 µg/ml). The MIC value for roxithromycin was 0.05 µg/ml by IF and MBC was 0.20 by RT-PCR. With the second specimen the MIC of Ciprofloxacin was 0.5 µg/ml (determined by IF). The MBC value by RT-PCR (1.25 µg/ml) (Fig.26) was higher than that of IF value. The MIC of erythromycin with the second specimen was determined to be 0.5 and 3.0 µg/ml by IF and RT-PCR respectively. The MIC range of all the antibiotics were within the acceptable limit. The MIC value determined by IF was found to be the MBC value for all except for roxithromycin where the immediate higher concentration was determined to be the MBC (0.10 µg/ml). The MBC value was determined by passing the culture harvest exposed to antibiotics on to McCoy cell cultures.

Results on sequencing done with plasmid amplified products to genotype the *C. trachomatis*

Plasmid-amplified products of the 3 laboratory conjunctival isolates, 3 genital and 5 ocular PCR amplified products were sequenced to determine the genotype of *C. trachomatis*. The DNA sequencing done with forward plasmid primer showed a single nucleotide substitution at the 1939th position on comparison of sequence data with the reference sequence (Thymine was replaced by cytosine). This change was observed in both conjunctival and genital specimens (Fig 27). This substitution shows that our strains are trachoma biovars. This single nucleotide substitution does not have any effect as same aminoacid proline is coded. In another ocular strain nucleotide substitution was seen in (IC-21) the 1999th position. This replacement also does not make a significant result as CGT as well as CGC codes for Arginine. Otherwise no nucleotide variation was observed among strains.

Figure - 26
Agarose gel photograph showing the results of MBC against Ciprofloxacin onspiked Conjunctival swabs



1:Negative control **5: 1.0 µg/ml**

2: 0.25 µg/ml **6:1.25 µg/ml**

3: 0.5 µg/ml **7:1.5µg/ml**

4: 0.75 µg/ml **8: unloaded**

M : DNA ladder PHIX

DNA/*Hinf* I digest

MBC:1.25 µg/ml

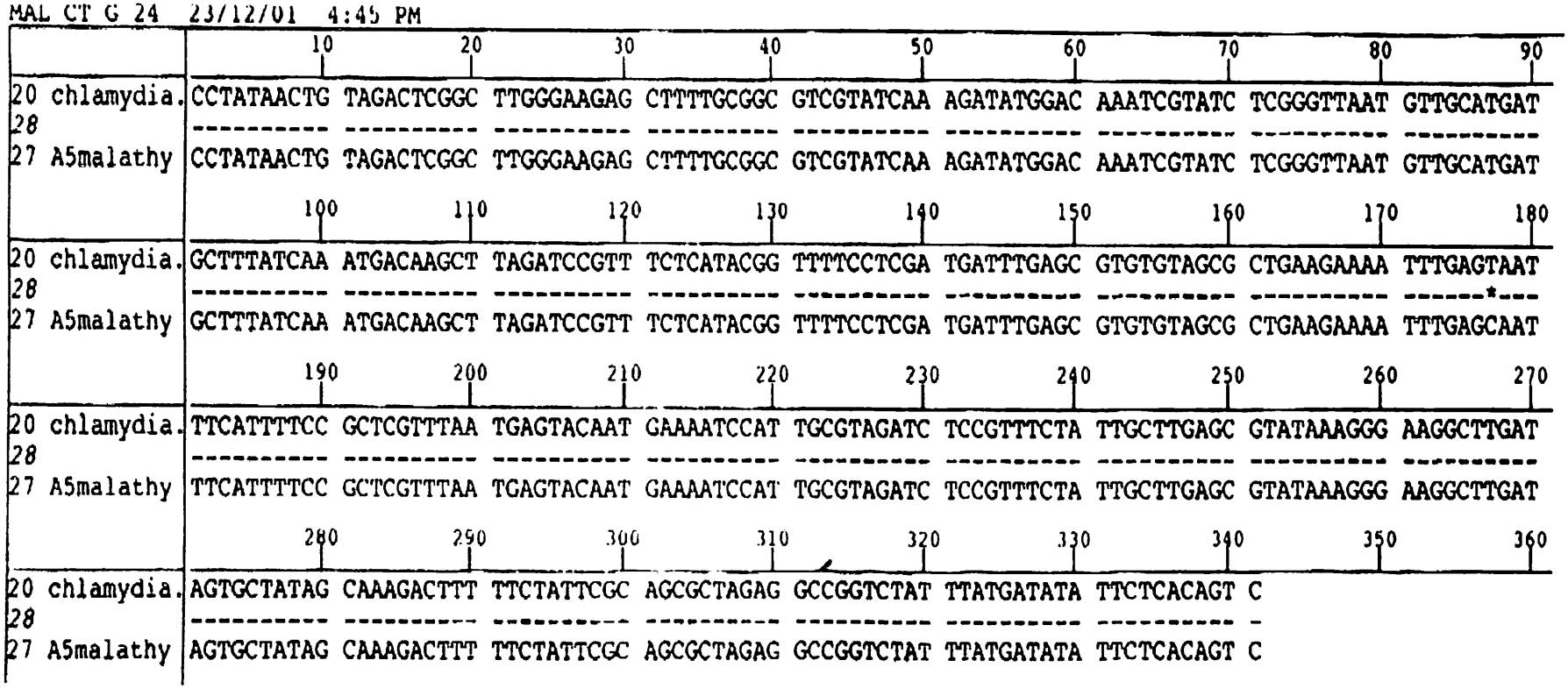
TABLE - 13

Results on MBC (µg/ml) of antibiotics in against *C. trachomatis* Ba spiked in conjunctival swab

Specimen	Antibiotic	MBC-IF	MBC-RT-PCR
1	Ciprofloxacin	0.25	1.0
2	Ciprofloxacin	0.5	1.25
3	Erythromycin	2.5	3.5
4	Erythromycin	1.5	3.0
5	Roxithromycin	0.05	0.20

Figure - 27

DNA sequencing done with plasmid amplified product of a urethral swab matched with the plasmid referral sequence



Chlamydia: Referral sequence (accession number-M 19487)

A5: Plasmid amplified sequence of a urethral specimen -a single nucleotide difference seen at the 177th position.

Figure - 28

Variable domains III and IV of *C. trachomatis* C(B6) and A (B8).

The * marks shows the differences

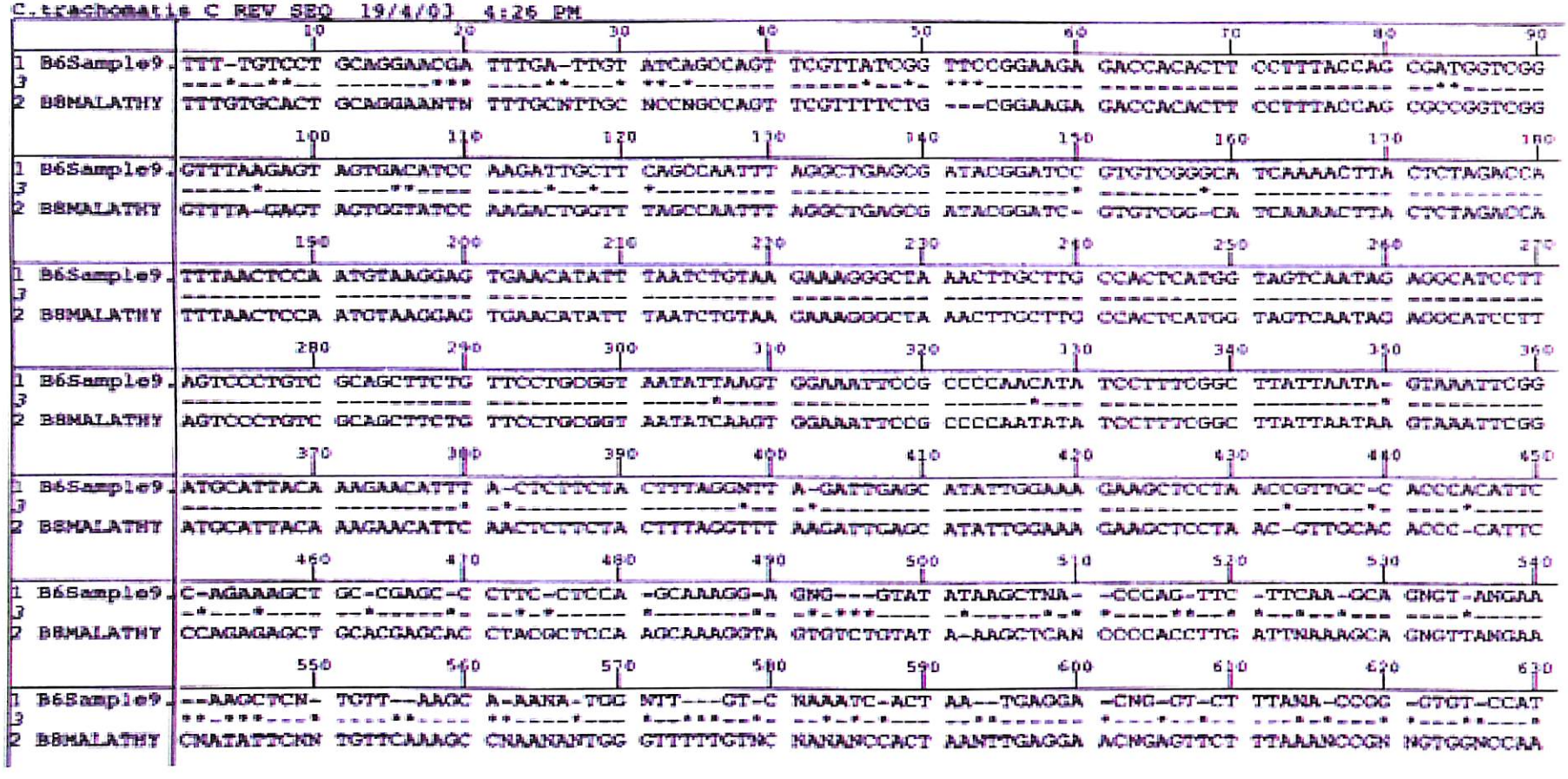


Figure - 29

Variable domain I of MOMP 1 gene of ocular isolate (*C. trachomatis* B) position : 141-230

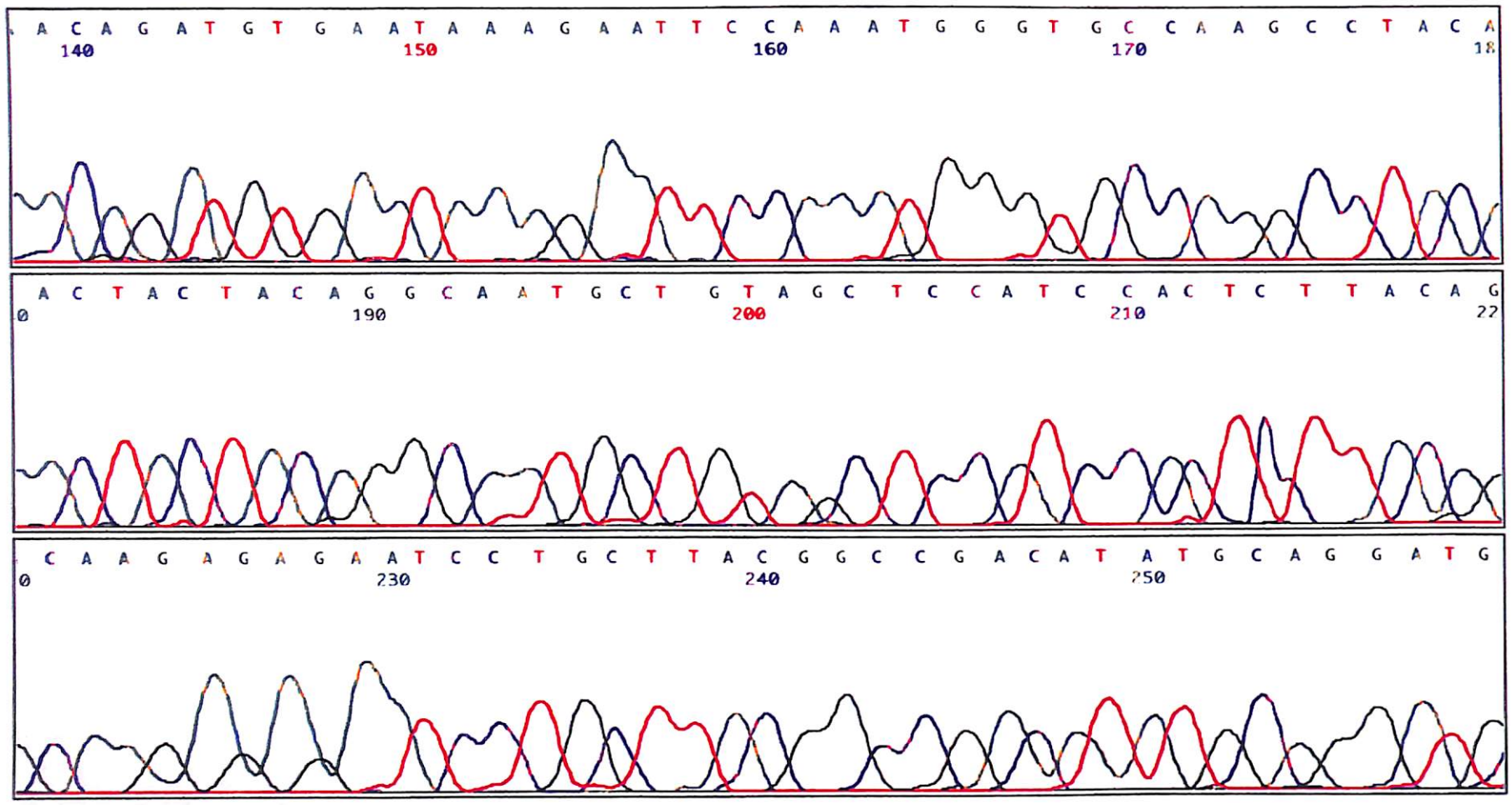


Figure - 30

Variable domain II of the ocular isolate of MOMP 1 gene of ocular isolate - *C. trachomatis* B) position 373- 460

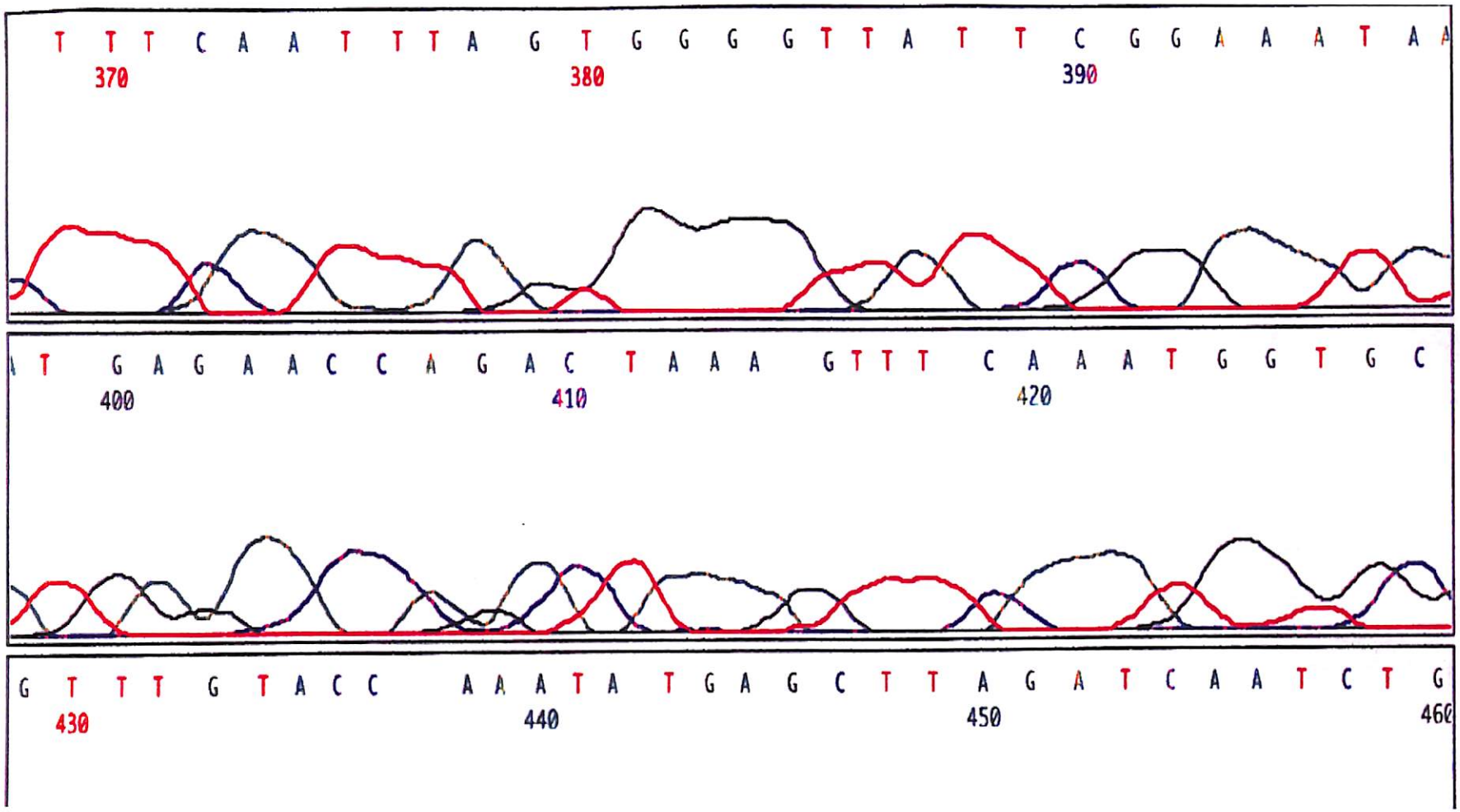


Figure - 31
Variable domain III of MOMP 1 gene of ocular isolate
(*C. trachomatis* B) position : 242-310

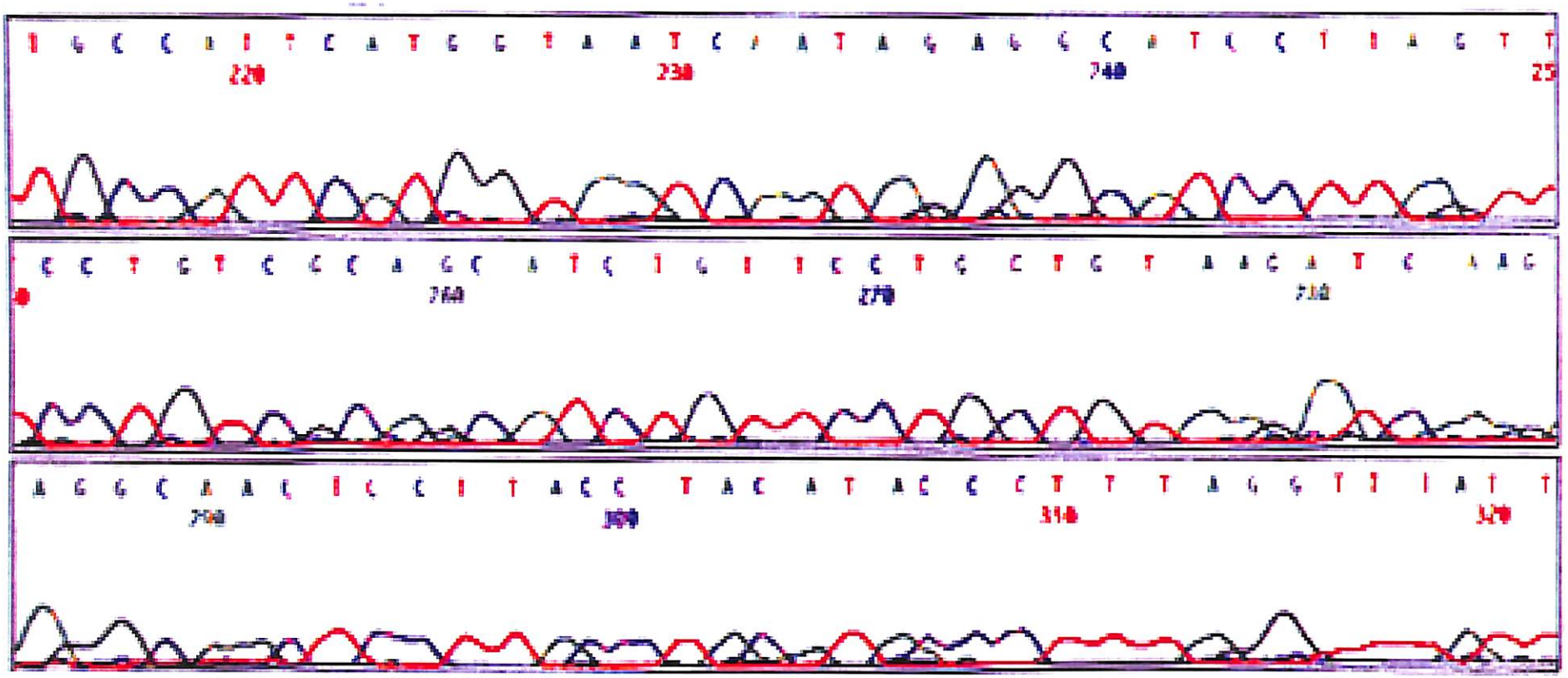


Figure - 32

Variable domain IV of MOMP 1 gene of ocular isolate

(*C. trachomatis* B) position : 41-143

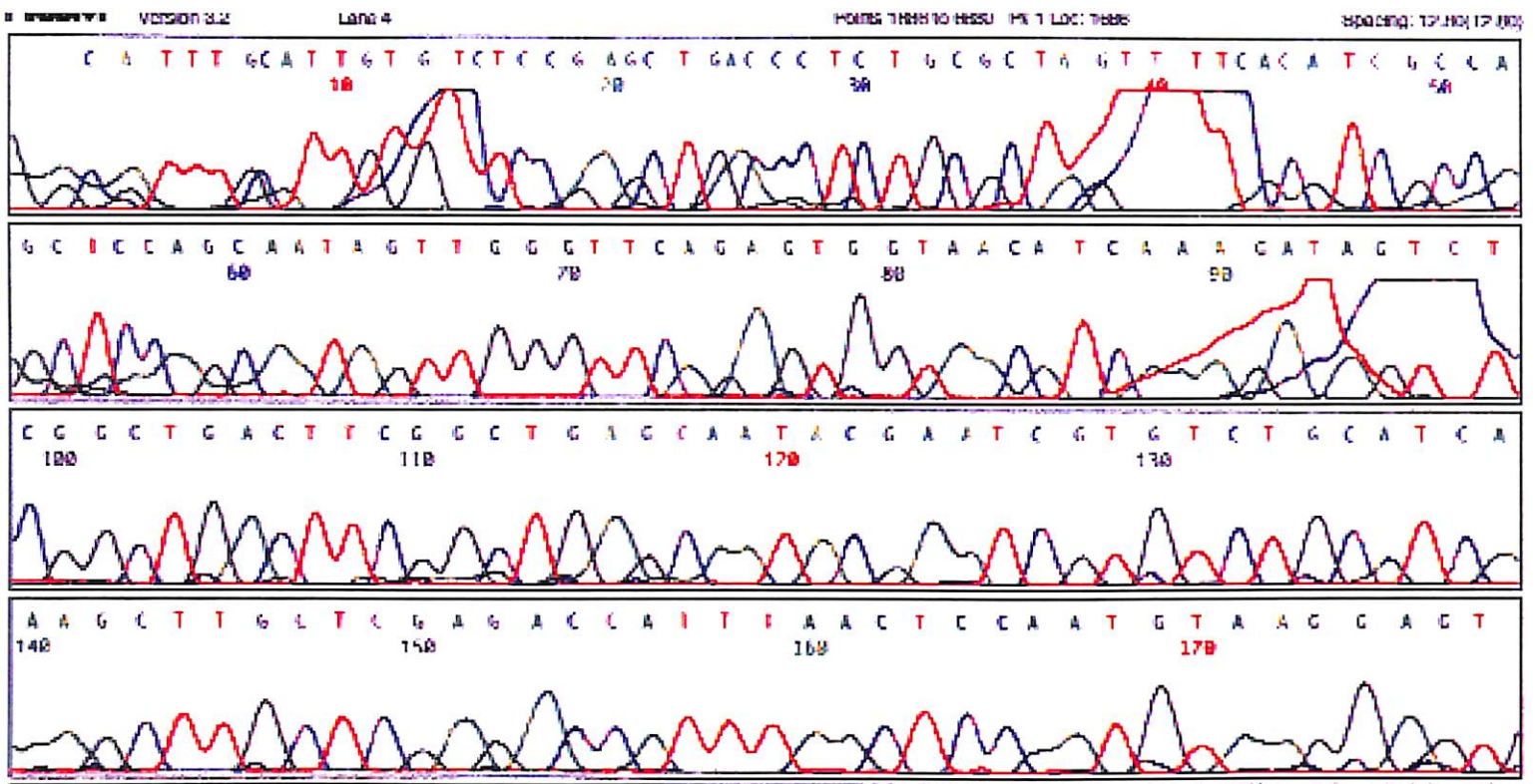


Figure - 33

VD I of MOMP gene of the ocular isolate (*C. trachomatis* B matched with the reference MOMP B sequence) position : 141-253. Sample : ocular isolate from a case of ophthalmia neonatorum. Momp ctb : reference sequence (Accession number M 33636)..

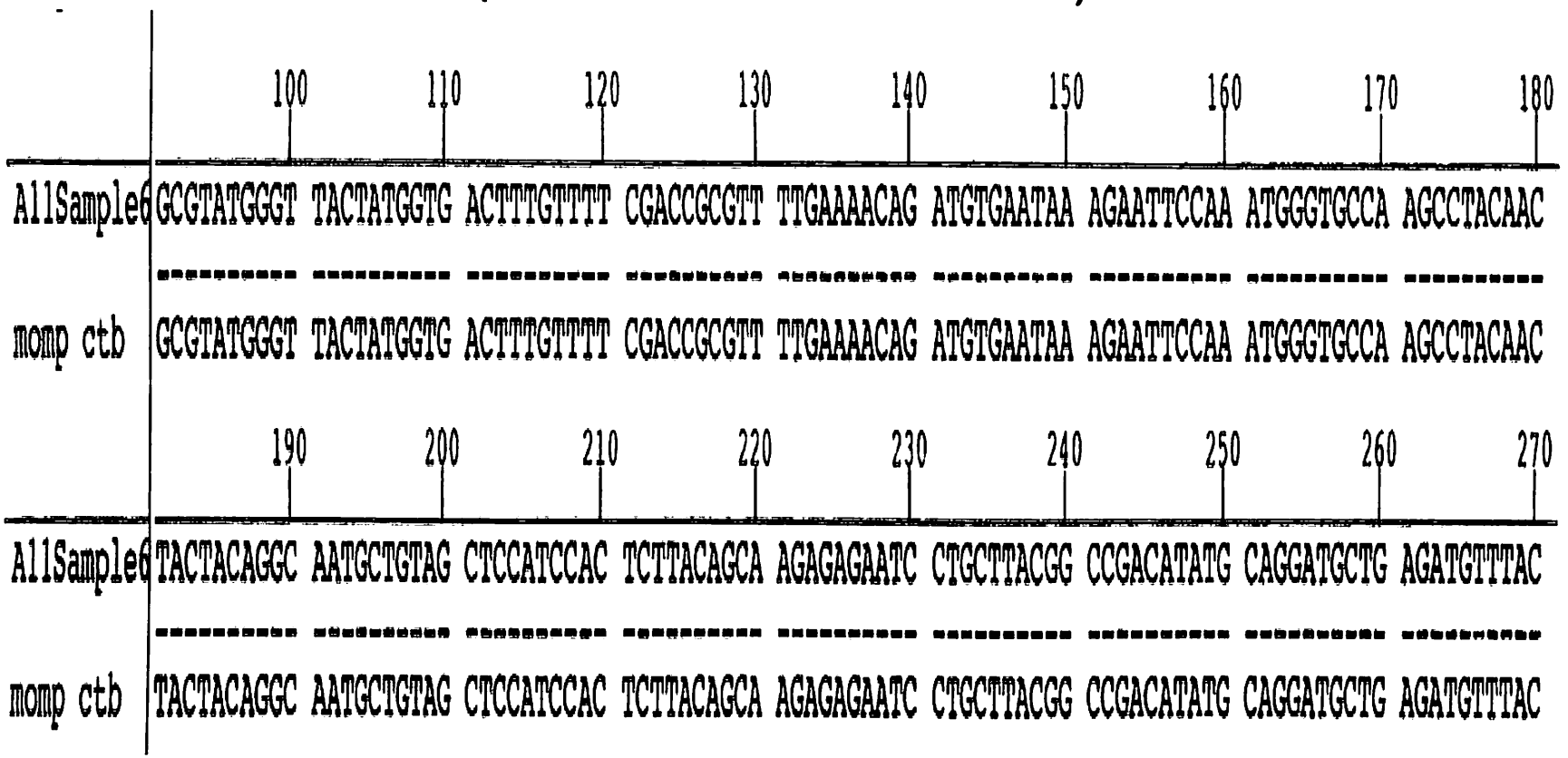


Figure - 34

VD II of MOMP gene of the ocular isolate (*C. trachomatis* B matched with the reference MOMP B sequence) position : 372- 460

Sample : ocular isolate from a case of ophthalmia neonatorum.

Momp ctb : reference sequence. A single nucleotide variation seen at the 422nd position (Accession number M 33636).

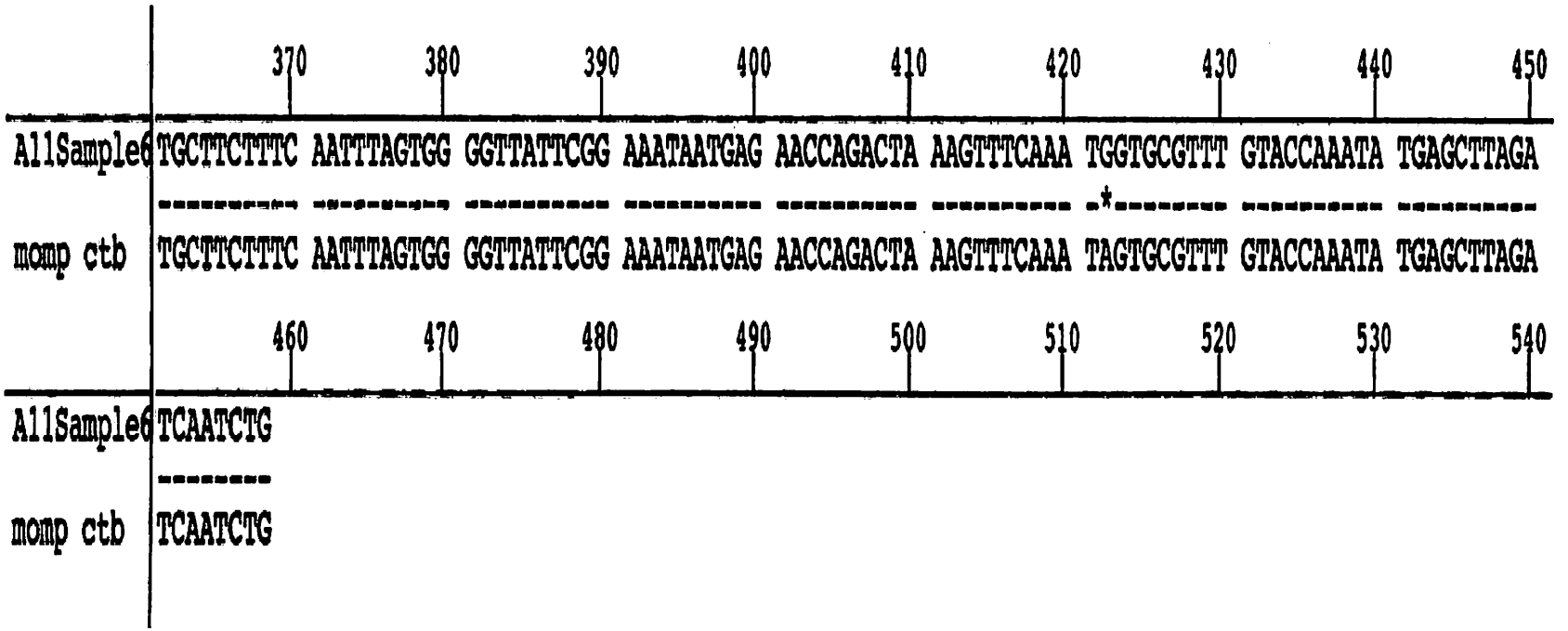


Figure - 35

VD III of MOMP gene of the ocular isolate (*C. trachomatis* B matched with the reference MOMP B sequence) position : 242- 310.

Sample : ocular isolate from a case of ophthalmia neonatorum.

Momp ctb : reference sequence (Accession number M 33636). A single nucleotide difference with the reference sequence at the 291th position.

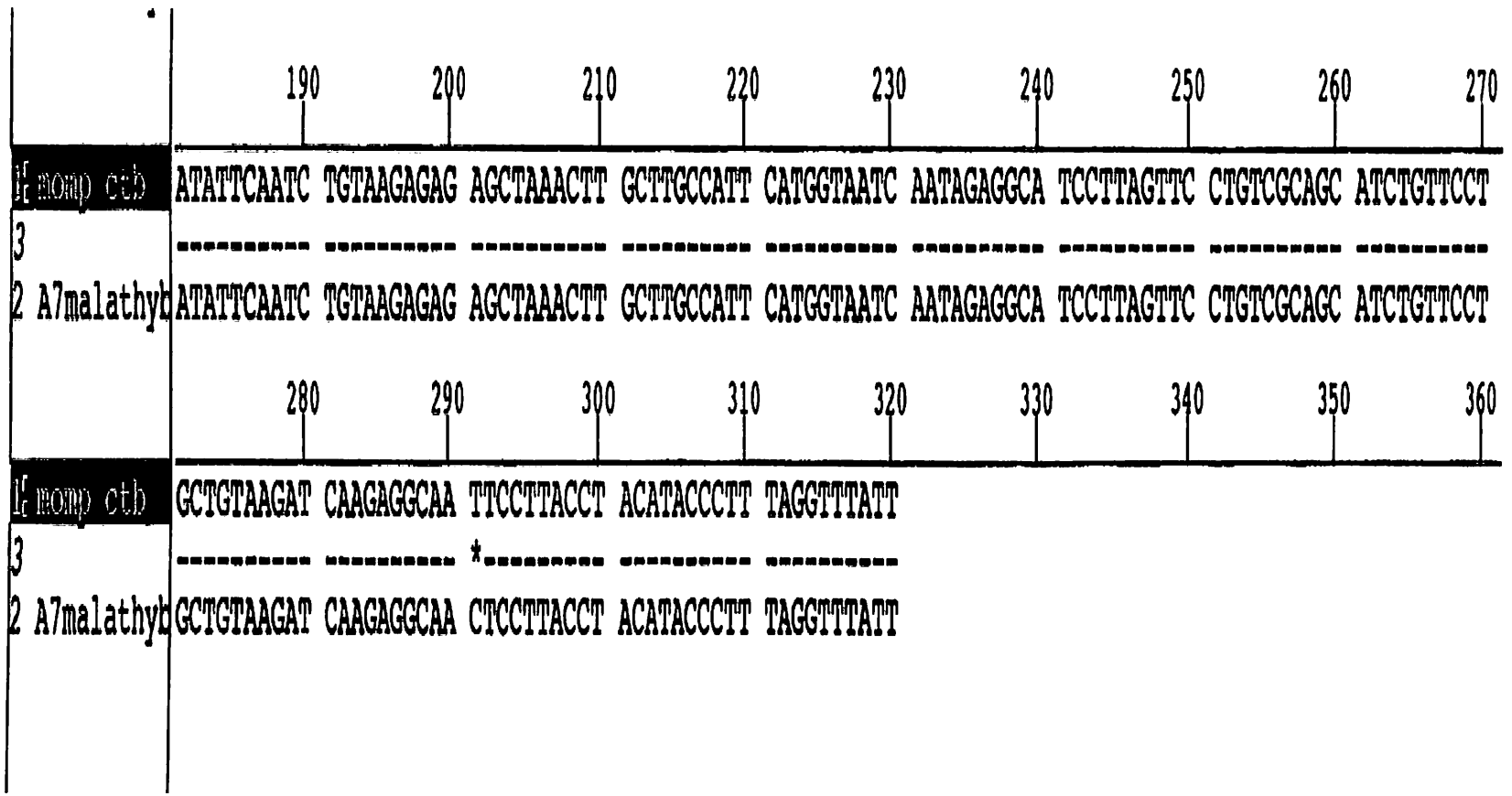
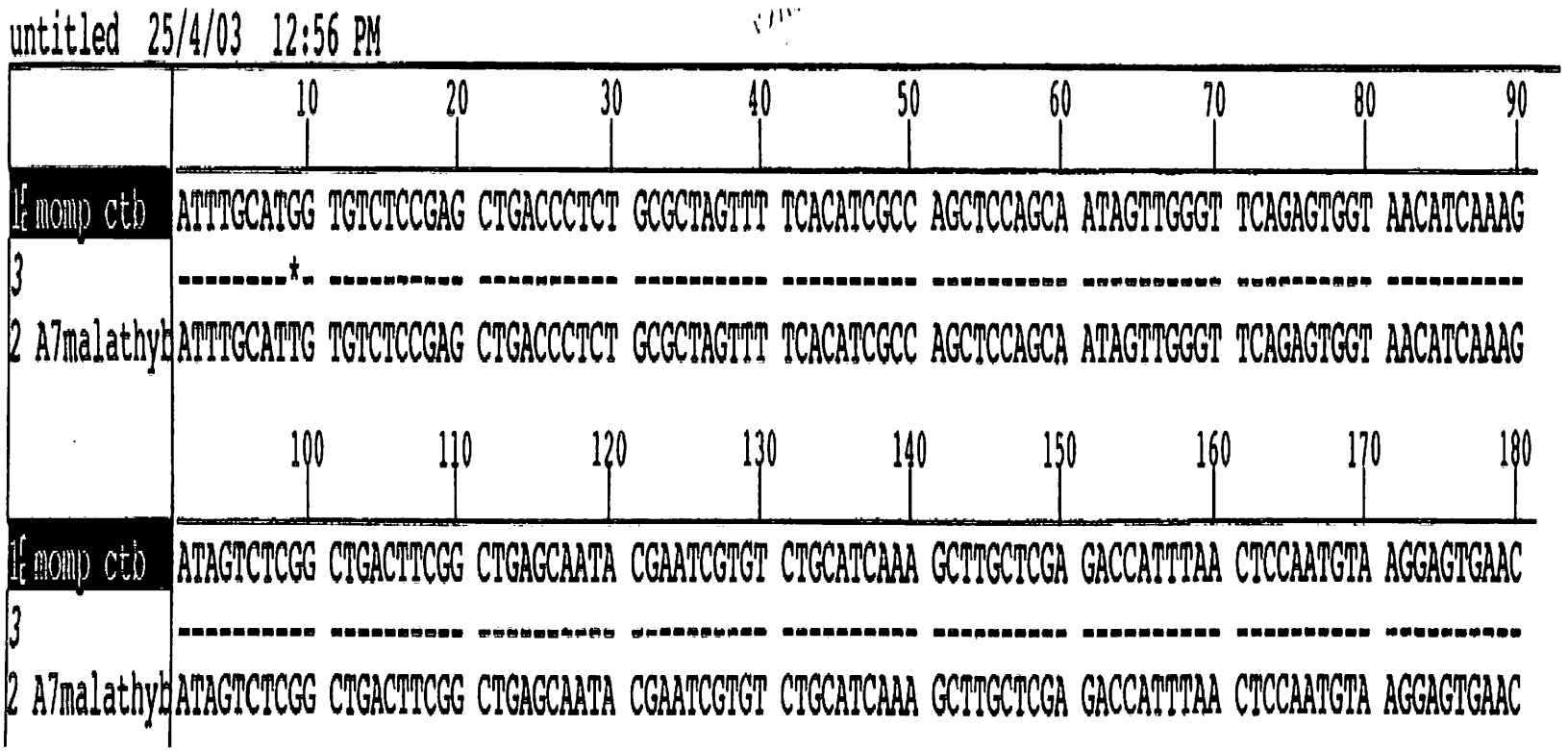


Figure - 36

VD IV of MOMP gene of the ocular isolate (*C. trachomatis* B matched with the reference MOMP B sequence) position : 41- 143. Sample : ocular isolate from a case of ophthalmia neonatorum. Momp ctb : reference sequence (Accession number M 33636)..



Results on DNA sequencing of MOMP gene to identify the genotype of *C. trachomatis*.

The DNA sequencing of MOMP amplified products were analyzed with reference DNA sequences of A (Accession Number M 33635), B (Accession Number M 33636), Ba (Accession Number AF063194) and C (Accession Number AF352789). All 4 variable domains of all the serovars were exactly matching with the sequences of variable regions published except for one nucleotide difference. Variable domain I of B group contained 60 nucleotides encoding 20 amino acids. A single nucleotide difference was observed at the 485th position. Thymine was replaced by adenine. Variable domain I of serovars C and A contained 66 nucleotides encoding 22 amino acids. Variable domain I of B serovar was very highly conserved. Variable domain II of A, C, Ba and B contained 66 nucleotides encoding 22 amino acids. More sequence variation among serovars was observed in variable domain II. Minimum sequence variation was observed among serotypes B and Ba. Variable domain III was the smallest domain with a total of 42 nucleotides encoding 14 amino acids. Variable domain III of serovars C and A had a single nucleotide difference at the 751th position. Serovar C had a thymine and serovar A had Guanine. Fig. 28 shows the sequence difference among serotypes A,C in variable domains.

Variable domain IV is the largest domain of the MOMP gene. There are 96 nucleotides encoding 32 amino acids in serovars C and A. Serovar B contained 99 nucleotides encoding 33 amino acids. No nucleotide variation was observed among servers B and Ba in variable domain VI. The MOMP sequence analysis of the two ocular isolates, recovered from a case of ophthalmia neonatorum showed perfect homology with the MOMP sequence of *C. trachomatis* B. This shows that *C. trachomatis* B can cause ophthalmia neonatorum (Fig 29,30, 31, 32, 33,34, 35, 36).

Analysis of adenovirus isolates by neutralization test using type specific adenovirus antiserum.

All the 10 isolates were neutralized with the anti-adenovirus type 4 antisera. The neutralization results were concordant with that of PCR results for subgenus typing (Fig.40).

PCR-RFLP to serotype adenovirus from conjunctivitis cases.

The PCR amplified products of the 10-adenovirus isolates, digested with *Hae* III enzyme gave six fragments of 325, 230, 158, 143, 100, 50bp. The fragments were calculated for their size with Image Master Visual Documentation System version 1.1 (Pharmacia, Biotech, Amersham, USA). The RFLP pattern was exactly matching with the RFLP pattern of the standard strain 4 of adenovirus. Hence the isolates from conjunctivitis cases were typed as Adenovirus type 4 (Fig 37).

PCR-RFLP to serotype adenovirus from interstitial pneumonia cases.

Three Adenovirus PCR amplified products from nasopharyngeal specimens, digested with *Hae* III enzyme gave six fragments of 325, 230, 158, 143, 100, 50bp. The fragments were calculated for their size with Image Master Visual Documentation System version 1.1 (Pharmacia, Biotech, Amershm, USA). The RFLP pattern was exactly matching with the RFLP pattern of the standard strain 4 of adenovirus. Hence they were typed as serotype 4 (Fig 37).

Multiplex PCR for subgenus typing of adenovirus isolates from conjunctivitis cases

The three sets of primers targeted against the hexon gene for the subgenus typing were absolutely specific for adenovirus DNA. The analytical sensitivity of the primers was calculated to be 20 nano gram of Adenovirus DNA (Fig 38). The

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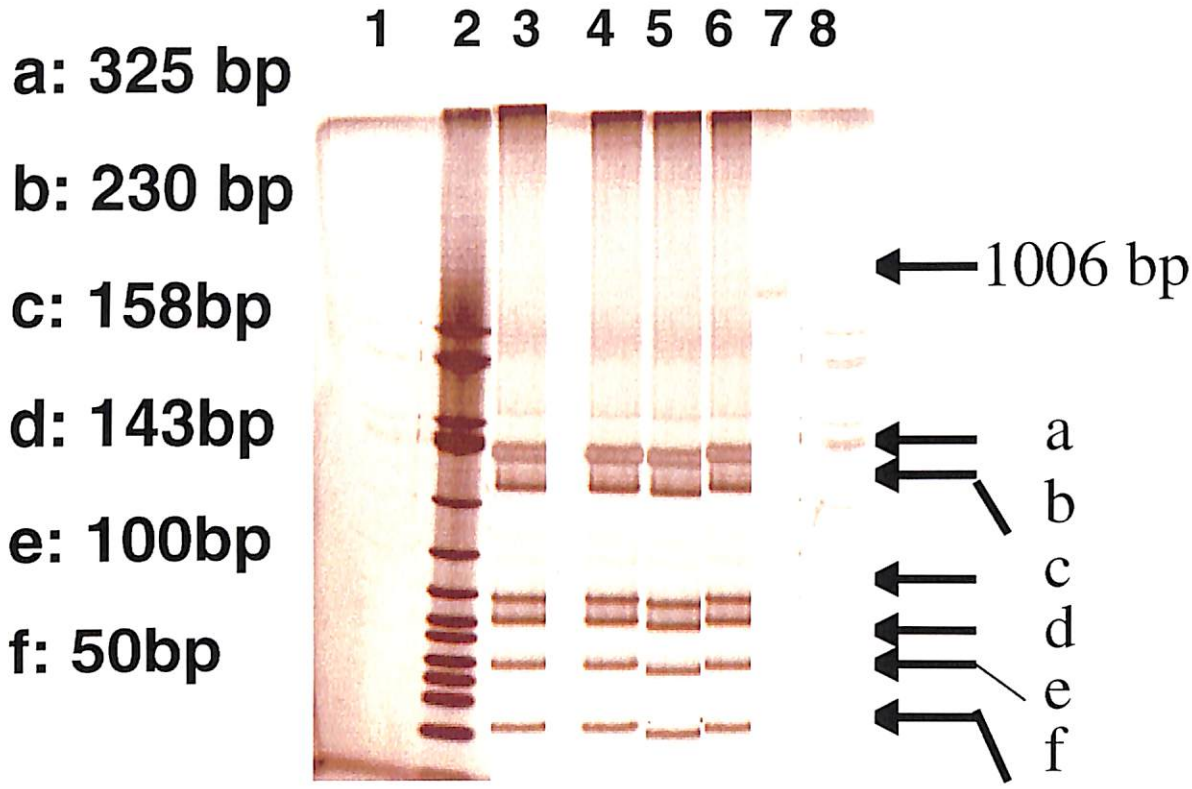
Figure - 37

PAGE showing PCR-RFLP products of adenovirus isolates with *Hae* III enzyme (Molecular weight calculated by gel documentation system)



1 : unloaded

2: DNA ladder PHIX DNA /*Hinf* I digest

3: Nasopharyngeal aspirate amplified product (digested)

4: Adenovirus 4 (Standard strain)

5: ocular isolate : digested pattern was identical with the standard strain

6:ocular isolate: digested pattern was identical with the standard strain

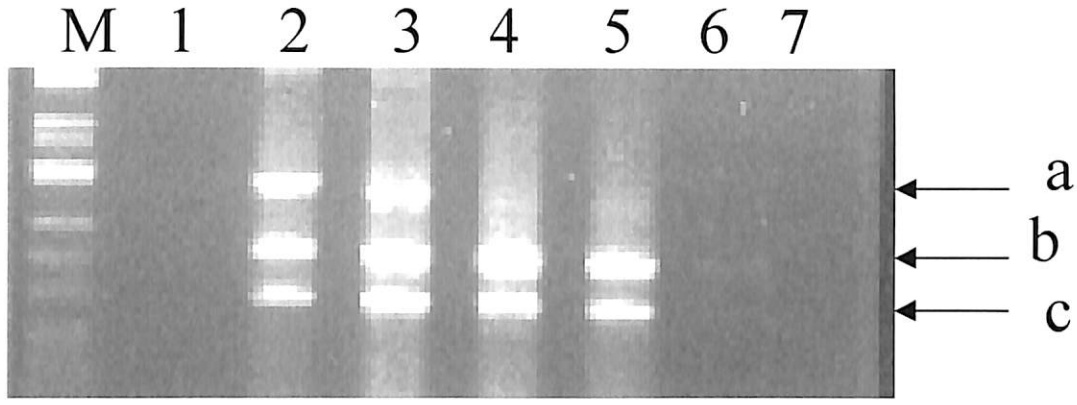
7:Undigested product

specificity result of the multiplex PCR is shown in Fig. 39. The primers could amplify only DNA extracted from the clinical isolates biologically amplified cell cultures. DNA extracted from the 10 adenovirus isolates from conjunctivitis cases on simultaneously amplification with the three sets of primers specific for hexon gene showed that they belonged to adenovirus subgenus E. All 10 adenovirus isolates gave an amplified product of 399 bp (Fig 40). The position of the amplified product was identical with the position of the amplified product of the adenovirus standard strain 4.

Based on PCR-RFLP and multiplex PCR for subgenus typing results it was concluded that the adenovirus serotype 4 is the causative agent of epidemic conjunctivitis that occurred during the period August – December 2002

Figure - 38

Agarose gel electrophoretogram showing the sensitivity of multiplex PCR (for subgenus typing)



M: DNA ladder PHI X 174DNA/*Hinf* I digest

1: negative control

2-7: serial 10 fold dilutions of adenovirus DNA (amplification seen till 10^{-5} dilutions, detection limit: 20 ng)

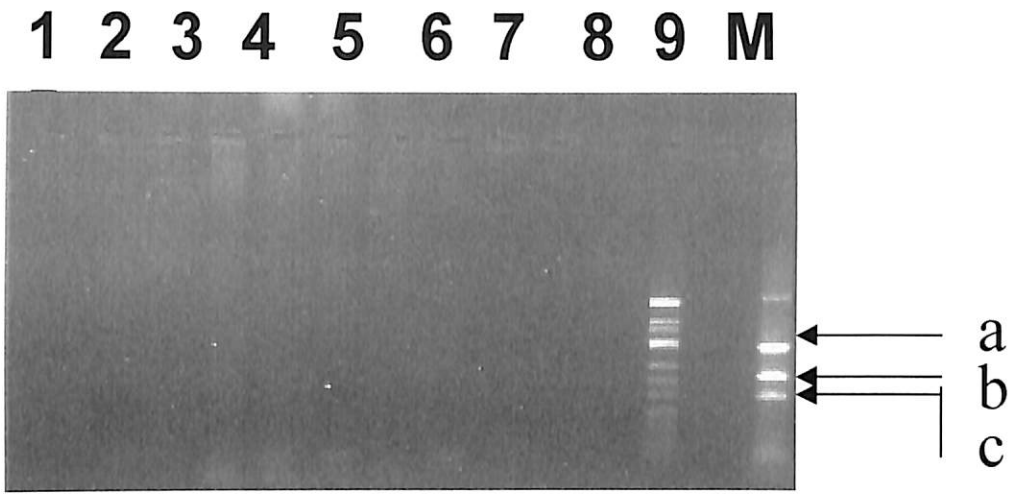
a: 465 bp-adenovirus 7 DNA (subgenus B)

b: 399 bp -adenovirus 4 DNA (subgenus E)

C:269bp--adenovirus 6 DNA (subgenus)

Figure - 39

Agarose gel electrophoretogram showing the specificity of Multiplex PCR for adenovirus subgenus typing



1 : negative control

2: HSV-1

3: *S.aureus*

4: *H. influenzae*

5: *M.tuberculosis*

6: *S. pyogenes*

7: *P.acnes*

7:Human DNA

P : a : 465 bp-adenovirus 7 DNA (subgenus B)

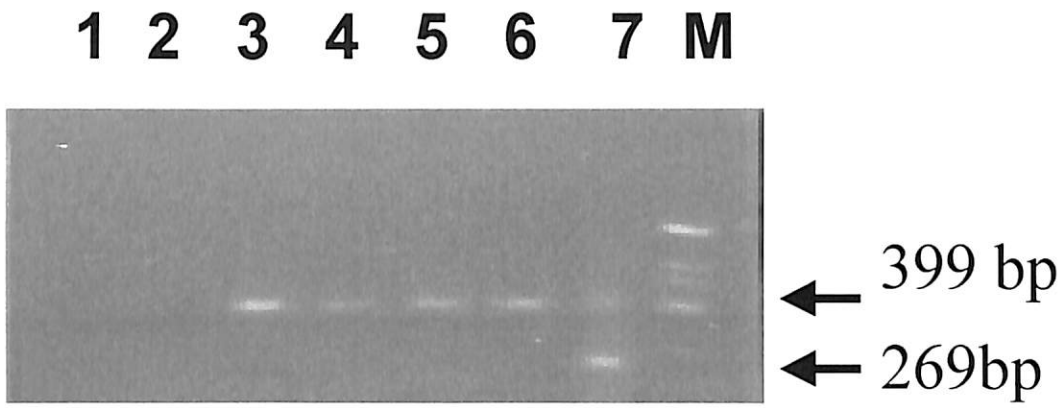
b: 399 bp -adenovirus 4 DNA (subgenus E)

C:269 bP--adenovirus 6 DNA (subgenus C)

M : DNA ladder PHI X 174DNA/*Hinf* I digest

Figure - 40

Agarose gel electrophoretogram showing the multiplex PCR results done on isolates of adenovirus



1: Negative control

2: DNA extraction control

3,4,5& 6: Adenovirus isolates

7:positive control (399bp-adenovirus 4) (269bp-adenovirus 6)

Discussion

DISCUSSION

C. trachomatis is the causative agent of a variety of diseases and syndromes, including trachoma, urogenital infections, chlamydial conjunctivitis infant pneumonia (Sturm-Ramirez et al, 2000). About 4 million *C. trachomatis* urogenital infections are reported to occur in the United States annually (Gaydos et al, 1998). It has been estimated that >50 million cases occur worldwide (Gaydos et al, 1998). Chlamydial infections occur primarily among young sexually active persons. Prevalence crosses all socioeconomic groups. Without antibiotic intervention, long-term *C. trachomatis* infection of the female genital tract results in chronic salpingitis and may eventually be complicated with tubal infertility and ectopic pregnancy (Lan et al, 1994). Approximately 10 to 201 infants born to infected mothers will develop pneumonia and of the cases of pneumonia among children hospitalized in the first six months of life, approximately one-third were shown to be due to *C. trachomatis* (Schachter et al, 1975). Infant pneumonia, if untreated tends to be chronic, with multiple exacerbations and remissions.

Antibiotic therapy does eliminate shedding of the organism and probably shortens the course of the illness. Although the number of children, who have had long-term follow-up after episode of chlamydial pneumonia in infancy is small, studies have shown that approximately one-third have developed asthma (Weiss et al, 1986). Hence timely diagnosis and treatment is necessary to prevent the sequelae of *C. trachomatis* infections. *C. trachomatis* infections have historically been diagnosed by isolation in cell culture, EIA, IF and DNA hybridization. PCR has been developed by several researchers and has shown to have sensitivity greater than that of culture, IF and EIA for a variety of clinical specimens. (Griffais and Thibon, 1989; Loeffelholz et al, 1992, Mohany et al, 1993, Williams et al, 1992).

Adenovirus is recognized as a significant pathogen in children following bone marrow transplantation and the virus is endemic in the general pediatric population (Wall et al, 2003). Adenovirus conjunctivitis, though is a self-limiting disease, does cause significant amount of morbidity. A rapid diagnostic method is important to differentially diagnose the etiological agent so the patient need not be put on antibiotics unnecessarily. PCR has been used to detect adenovirus DNA in clinical specimens and has been shown to amplify few copies of target DNA sequences (Allard et al, 1990; Hierholzer at al, 1993; Kinchington et al, 1995). Therefore, we applied PCR for the detection of *C. trachomatis* and Adenovirus from clinical specimens.

A. Effectiveness of In house DNA extraction methods to amplify *C. trachomatis* from clinical specimens.

DNA extraction method I of Boiling and snap cooling of specimen was not sensitive to amplify DNA from clinical specimen by PCR. The low sensitivity of this method may be due to the protein, which is known to inhibit the amplification of DNA. The DNA extracted by boiling method underwent rapid degradation; therefore it was not possible to store these DNA for a long time. DNA extracted by (heat denaturation) incubating the specimen at 97^0C had low sensitivity (Freise et al, 2001). Hence purification of extracted DNA is necessary to have increased PCR sensitivity. Phenol–chloroform extraction method is reported to remove the inhibitors from the clinical specimens (Toye et al, 1998). But in our study phenol-chloroform extraction method could amplify only 5 specimens whereas 11 specimens extracted by GTC method were amplified by two sets of primers. Hence GTC method was used for extracting DNA from clinical specimens.

B. Evaluation of the susceptibility of McCoy, HeLa, BHK-21, HEp-2, Vero and A549 cell lines for the growth of *C. trachomatis*

C.trachomatis trachoma strains A, B, Ba & C were grown in six continuous cell lines to determine the relative susceptibility of these cell lines. In this study McCoy and HeLa cell lines were determined to be the most sensitive cell lines as they gave good growth of all the four standard strains. Among the four standard strains serotype C had grown very well in McCoy, HeLa and Vero cell lines in spite of the fact that the inoculum was a egg yolk harvest. This standard strain had easily got adapted to the cell lines. None of the serotypes showed growth in A549 cell line. Next to serotype C, serotype A had shown a good growth in all the cell lines except A549. The relative sensitivity of BHK-21, CHO, HeLa S3, McCoy cell, WI-38, ST / BTC, owmp cell lines of mammalian origin were compared for their susceptibility to *C. trachomatis* already adopted in irradiated McCoy, BHK – 21, hister cell lines by Rota, (1977). They had reported higher infection rate in non-irradiated CHO cell lines than in irradiated CHO cells. Inclusion numbers was increased 300 times in HeLa S3 and up to 3 times in other cell types after treatment with DEAE dextran. Similar study conducted by Croy et al, in 1975 evaluated eleven mammalian cell lines HeLa 229, HeLa M, HEp-2, FT, Vero MK-2, L-Wo5A2, McCoy & L-929 for susceptibility to infection with trachoma strains and serotype E. Cell lines were pretreated with DEAE dextran and the inoculum was centrifuged onto the cell layers. HeLa 229 was found to be the most sensitive to infection followed by MK-4, HEP-2, McCoy and HeLa M. Vero and other cells had shown much lesser susceptibility in contrast to one study where Vero cell line also showed good growth of trachoma strains. There are studies where HEp – 2 cells were used to grow *C. trachomatis* from clinical specimens (Kuipers et al, 1995). But mostly McCoy cell cultures are used for *C.trachomatis* (Rodreiguez et al, 1991; Schachter et al, 1994; Mouton et al, 1997).

Kuo et al, (1975), had compared the relative sensitivity of yolk sac of embryonated eggs and HeLa 229 cells pretreated with DEAE dextran to determine the growth of 32 trachoma strains which were both yolk sac and cell culture established. Their study showed that the cell culture was of more sensitive than yolk sac culture. The 32 trachoma strains were isolated from the eye and genital tract in cell cultures and the ocular strains had shown better growth in cell culture. Similar study was conducted by Kuo et al, (1975) and similar observations were reported. Kuo et al, 1988 has also shown the enhanced growth of TWAR strain of *C. trachomatis* in HeLa cells pretreated with DEAE dextran. Cultivation of *C. trachomatis* in cycloheximide treated McCoy cells have also been reported to yield greater number of inclusion forming units than irradiation (Ripa et al, and Mardh, 1977). The application of centrifugation force at the time of inoculation had enhanced growth 22 to 36 times. Rapid shell vial technique is more sensitive in growing *C. trachomatis* and the result can be obtained in 48 hours of incubation (Madhavan et al, 1994). In our study pre treating the McCoy cell lines with cycloheximide and centrifuging the specimen at 3,000 rpm for 1 hour and incubating the inoculated cell cultures at 37^0C in maintenance medium containing 10% fetal calf serum with out any antibiotic was observed to give good growth of *C. trachomatis.*

C. Evaluation of PCR using plasmid primer with MOMP primer for the detection of C. *trachomatis* in primary conjunctivitis

PCR, a significant advancement in molecular biology, has the capacity to amplify genomic sequences from an infectious agent over a million times and yield results with in several hours. Therefore, PCR has found wide application for the detection of several infectious agents from different clinical specimen. PCR was compared with other methods of *C. trachomatis* and is reported to have more sensitivity. PCR for the detection of *C. trachomatis* is employed with primers targeted against MOMP 1 gene, plasmid or 16S ribosomal RNA (rRNA) gene.

Among these 3 sets of primers PCR with plasmid primers are reported to have greater sensitivity than the other two sets of primers. The enhanced sensitivity of plasmid primers is due to presence of 7-10 copies of plasmid in *C. trachomatis.* Satpathy et al, (1999) evaluated plasmid and MOMP primers in detection of follicular conjunctivitis caused by *C. trachomatis.* In their study a total of 71 specimens were processed for PCR assays with both plasmid and MOMP primers. Among these 71 specimens plasmid PCR was positive in 52 (73,23%) and MOMP PCR was positive in 43(60.56%) specimens. Plasmid PCR assay was reported to be more sensitive by Bailey et al, 1994. Roosendaal et al, 1993 studied the relative sensitivity of plasmid, MOMP and 16Sr ribosomal RNA for the detection of *C. trachomatis.* They have reported that plasmid primers were the most sensitive in the PCR method and detected 0.1 infectious unit of *C. trachomatis* in the presence of a superfluous amount of human DNA. Application of this plasmid primer could detect *C. trachomatis* in cervical smears of four symptomatic patients for up to 3 weeks after the start of treatment with doxycycline. In contrast, the MOMP and rRNA gene–directed PCR as well as culture and direct immunofluorescence, gave negative results within 1 week.

Ossewaarde et al, 1992, reported that plasmid primer set detected chlamydial DNA in a higher dilution than the MOMP primer set. The results of the present study was also in total agreement with their observations. Of the 80 specimens subjected for PCR with both MOMP primers and plasmid primers, 11 specimens (13.8%) from 10 patients (12.5%) were positive by PCR with plasmid primer whereas only 3 specimens (3.8%) from 2 (2.5%) were positive by PCR with MOMP primers. The difference in result was found to be statistically significant (McNemar test $P<0.01$). The time taken for the MOMP PCR was more (4 hours and 30 minutes) when compared to plasmid PCR (3 hours). The amount of *Taq* Polymerase added to PCR reaction volume with MOMP PCR was also more (1.5 units) when compared to plasmid PCR reaction. So in terms of time and cost also the Plasmid PCR was found to be rapid and economical respectively.

The advantage in using PCR with MOMP primer is the amplified product can be digested with restriction enzyme or can be DNA sequenced to identify the genotype of *C. trachomatis* provided the amplified product covers the variable domains. But often for routine diagnostic purpose the mere detection of *C. trachomatis* DNA from the clinical specimens and the correlation with the clinical conditions are sufficient to give therapy for the patient. Moreover amplification of a larger portion of MOMP gene to identify the genotype would require more amount of DNA in the clinical specimens. Therefore for detection purpose, PCR with plasmid primers was followed throughout the study.

D. Evaluation of conventional methods against plasmid PCR in detection of *C. trachomatis* DNA in conjunctivitis cases.

A total of 486 specimens collected from 347 patients with clinical diagnosis of primary conjunctivitis were processed for the detection of *C. trachomatis* by PCR, IF and culture. Among these 486 specimens, 27 (5.6%) specimens from 22 (6.3%) patients were positive for *C. trachomatis* by PCR with plasmid primer.. Among these 27 specimens, 3 (0.6%) collected from 2 (0.57%) patients were positive for IF and culture.

The results of this study clearly demonstrated the remarkable and significant reduction in the number of primary conjunctivitis in the adults due to *C. trachomatis* infection reporting to the major Ophthalmic hospitals in Chennai compared to the incidence reported by us 10 years back (1991 – 92). In this earlier study, 127 adult patients were investigated and 44 (34.6 %) were found to have culture proven primary chlamydial conjunctivitis. The present study included twice the number of study population of 347 adult primary conjunctivitis patients, but only one was culture proven. One case of ophthalmia neonatorum also was culture proven with isolation of *C. trachomatis* from both eyes. The shell vial culture technique in McCoy cell line

was verified for its accuracy during the period of study employing controls of cultures of the standard strains of *C. trachomatis*. Obviously the adult chlamydial conjunctivitis is at present an uncommon disease in patient reporting to the major ophthalmic hospitals in Chennai. If the 21-plasmid PCR positive cases were included for comparison purposes, the prevalence is significantly low being 6.0 percent as compared to 34.6 percent of our earlier study. Similar reports on the incidence of adult chlamydial conjunctivitis at different periods of time in any given geographical area are not available on a literature search. Occasional reports indicate occurrence of lower rates of adult and neonatal chlamydial conjunctivitis. In a study from Saudi Arabia reported in 1995, only 3 percent of adult conjunctivitis were positive for chlamydial DNA by PCR (Mellman-Rubin et al, 1995). A decrease in occurrence of chlamydial neonatal conjunctivitis from 4.39 percent in 1995 to 0.78 percent in 1998 was reported from Argentina possibly indicating lowering of the rates of endocervical infections in the mothers (Di Bartolomeo et al, 2001). Mertz et al, (1995) have reported the prevalence of *C trachomatis* infection among black women to have decreased by 19 % in 4 years period.

Majority (24 of 20 cases) of the *C. trachomatis* conjunctivitis in the present study was identified by PCR detecting cryptic plasmid of the bacterium. This indicated that viable and cultivable organisms are not present in the clinical specimens. This was confirmed by passing these 24 McCoy cells inoculated specimens thrice in the cell cultures before considering them as negative for *C. trachomatis*. We modified the amplification protocol of Bailey et al, 1994 to increase the sensitivity of PCR for cryptic plasmid with an initial denaturation at 94^{0}C for 4 minutes and final extension at 72^{0}C for 4 minutes. Cryptic plasmid primers were more sensitive due to the presence of 7-10 copies of plasmid in the organism.

With regard to the techniques used, this study showed that PCR using cryptic plasmid primers has increased clinical sensitivity than conventional smear and culture methods. Though isolation of the organism in cell culture is considered to be the gold standard, its low sensitivity precludes its use in a routine clinical microbiology laboratory. FAT on direct smear is a rapid and specific method but its limitation is the low sensitivity. PCR technique was compared with IF test for chlamydial elementary bodies by Elinfro et al, (1997). They have reported that PCR is likely to play an increasing role in the diagnosis of *C. trachomatis* infection because of its excellent sensitivity and specificity. They have reported that the interpretation of the IF test required meticulous examination of the stained smear, sometimes by two microscopists. In another study conducted by Haller et al, (1996) the positivity rate for chlamydial conjunctivitis was reported as 8.6 percent. They have concluded that PCR proved to be a good alternative to cell culture. Talley et al, (1992), have compared the PCR technique for the detection of *C. trachomatis* from follicular conjunctivitis cases. Among these 4 specimens, two specimens were positive for culture. One specimen was negative by both culture and IF technique. The fourth sample was IF positive but PCR negative. Dean et al, (1989), compared serial tissue culture passage with Polymerase chain reaction amplified DNA probe for conjunctival specimens from trachoma patients from Nepal. In that study of 252 specimens from patients with clinical trachoma only 32 percent of the *C. trachomatis* positive specimens were detected by cell culture compared with 90 percent detected by Polymerase chain reaction. All these findings support our findings that Polymerase chain reaction may have greater sensitivity and compared to IF and McCoy cell culture isolation.

Similar study was conducted by Fan et al, (1993). They have reported that among 49 specimens with conjunctivitis 3 (6.3%) were positive by IF & 23 (47%) by PCR. Bobo et al, (1991) reported that PCR – EIA detected chlamydial eye infection in

49 percent of subjects from all clinical groups compared with 22 percent by IF technique Kowalski et al, 1995 reported the sensitivity of PCR to be 88 percent and specificity to be 100 percent.

The prevalence of *C. trachomatis* eye infection varies in different parts of the world. Woodland et al, (1992) reported the prevalence of *C. trachomatis* infection to be 1.8 percent among individuals diagnosed to have conjunctivitis and Keratoconjunctivitis. Positivity rate of *C. trachomatis* eye infection was reported to be 8.6 percent by Haller et al, 1996;Poets et al, 1986 reported the prevalence of *C. trachomatis* follicular conjunctivitis to be 11 percent. One prevalence rate of *C. trachomatis* eye infection was 4.0 percent. Our current prevalence rate of chlamydial conjunctivitis (6.0%) was near to the prevalence rate reported by Fan et al, (1993) (6.3%).

A clear history of ocular medications in most of the patients included in this study, though clinical histories indicated that they appeared to have had some topical eye drops earlier. It is suggested that this marked reduction in the prevalence of chlamydial conjunctivitis in 10 years in Chennai might be due to frequent self-medication of antibacterial topical drops by the patients for any redness in the eyes because of their easy availability across the counters in pharmacies. This may also explain the low load or absence of viable *C. trachomatis* in the conjunctival specimens positive by PCR.

In conclusion, the incidence of adult chlamydial conjunctivitis among patients reporting to the outpatient departments of major ophthalmic hospitals in Chennai is significantly reduced. PCR using primers for detection of cryptic plasmid of *C. trachomatis* is needed to detect this bacterial infection of conjunctiva.

E. Evaluation of conventional methods for the detection of C. trachomatis and HSV in males with urethritis and females with cervicitis against PCR.

The high prevalence of urogenital infections due to *C. trachomatis* in the world is well documented (Dean et al, 1998). The results of this study showed that urogenital infection with *C. trachomatis* and HSV in the high-risk group of patients attending the STD outpatient clinic of a major general hospital was not uncommon. The diagnosis was possible mainly by PCR using primers to detect the cryptic plasmid DNA of the bacterium and the virus and in a few by direct smear. Isolation attempts were unsuccessful. PCR detected *C. trachomatis* and HSV in significantly more genital specimens than by direct smear. The non-isolation of *C. trachomatis* could be due to a low number of viable organisms in the specimens. Theoretically, one inclusion would be enough to show a positive result in a PCR-based test, while for a positive culture multiple elementary bodies are needed. In India, reports on the prevalence of *C. trachomatis* genital infection are based on antibody detection by enzyme immunoassay (EIA) using commercial kits. Divekar et al, (2000), reported the prevalence of *C. trachomatis* to be 23.3 per cent among female sex workers (FSW) in Mumbai. Using EIA for the detection of *C. trachomatis* antibodies among women, Tyagi et al, (1998) showed its prevalence in women with tubal infertility to be 74.07 per cent and Shrikande et al, (1995) reported a prevalence rate of 33.0 per cent in patients with pelvic inflammatory diseases in Nagpur. The prevalence among women having vaginal discharge was reported to be 12.2 per cent by Viswanath et al, (2000). The varying prevalence of *C. trachomatis* reported could be an account of the diagnostic tests used. PCR, an advanced nucleic acid amplification test, is rapidly gaining importance in diagnostic microbiology because of its higher sensitivity and absolute specificity. Since *C. trachomatis* contains 7-10 copies of plasmid, in the present study we standardized the PCR to pick up the cryptic plasmid DNA and applied the same on clinical specimens to determine the prevalence of *C. trachomatis* among population at risk of acquiring STDs.

Based on the present study it is concluded that PCR is a sensitive technique over IF and culture for the detection of C. *trachomatis* in genital specimens and this view is supported by reports published by other laboratories throughout the world. The variation in prevalence/ detection rate varies for genital infections due to *C. trachomatis* depending on the method of detection followed (IF, culture and PCR). Dean et al, (2002) compared the performance and cost effectiveness of IF, commercial PCR, ligase chain reaction for the verification of EIA results. Additional 7.0 percent true positive samples were detected by commercial PCR when compared to DFA. The study involved a sample size of 6,571cervical samples. They have reported a greater sensitivity for both commercial and LCR compared with DFA. The specificity was reported to be 100 percent. The overall prevalence was reported to be 3.9 percent. Palmer et al, (1990) compared PCR with IF for the detection of *C. trachomatis* from urethral swabs obtained from 37 men with acute – non-gonococcal urethritis. In comparison with IF technique PCR had a sensitivity of 95 percent. The prevalence of *C. trachomatis* among female sex workers in Senegal, West Africa was reported to be 28.5 percent based as PCR DNA amplification assays (Sturm - Ramieez et al, 2000). Most of them were asymptomatic.

C. trachomatis infection among women was reported to have decreased by 33 percent from 1989 to 1992 by Mertz et al, (1997) on trends in the prevalence of chlamydial infections. The prevalence decreased at least 19 percent among black women 15 to 19 years of age, the group with highest initial prevalence (20.2 %). In another study conducted by Mitike et al, 2002, the prevalence of urogenital *C. trachomatis* infections among males aged fifteen years and above. Among 199 males included, 16.6 percent had laboratory evidence of urogenital chlamydial infections. Postema et al, (1996) studied the epidemiology of genital chlamydial infection in patients with chlamydial conjunctivitis. Among the 65 patients with chlamydial

conjunctivitis 54 percent had a positive chlamydial urethral culture. Seventy percent of them had no genital symptoms. Twenty of the 27 (74 %) examined had a positive chlamydial cervical culture. Sixty percent of these women had no genital symptoms.

Mellman Rubin et al, (1995) reported *C. trachomatis* positivity at a rate of 3 percent among individuals diagnosed to have follicular conjunctivitis. Garland et al, (1995) reported the prevalence of *C. trachomatis* infection to be 2.0 percent among patients with acute conjunctivitis. Cervical and ocular swabs from 100 mothers /new born pairs were assayed for *C. trachomatis* with standard McCoy cell cultures and with biotinylated PCR techniques using primers directed against the MOMP gene and *C. trachomatis* specific cryptic plasmid respectively. Using PCR 20 (20%) mothers and seven (7%) neonates were positive by PCR. Only five of 20 mothers and two of seven neonates positive by PCR were positive by cell cultures.

Loeffelholz et al, 1992, compared biotinylated PCR assay with cell culture in 503 cervical specimens. In their study the biotinylated PCR assay had a sensitivity of 97 percent and a specificity of 85.7 percent, whereas culture technique yielded a sensitivity of 85.7 percent and a specificity of 100 percent. In our study 12.0 percent of male with urethritis and 1.0 percent females with cervicitis were positive for *C. trachomatis* PCR.

Oni et al, (1997) reported isolation of HSV in HEp-2 cell line from 10 patients with non-gonococcal urethritis. The prevalence of genital infection due to HSV in India is mainly determined by antibody detection (Venkataraman et al, 1986). In this study, the HSV prevalence rate as determined by PCR was of 12 per cent in endocervicitis in women and 6.6 per cent urethritis in males with indicating its common occurrence among the STD patients.

Most of our patients gave history of unprotected contacts with multiple partners. One hundred and fifty males with urethritis and 450 women with mucopurulent cervicitis attended the STD clinic during the study period. In this study, 48 per cent of males with urethritis but only 5.6 per cent of females with mucopurulent cervicitis were studied. Though due to small sample size the prevalence rates measured had high variances as indicated by the confidence intervals, a fair assumption could be made that *C. trachomatis* infection was common and HSV infection was not common in urethritis in males. If a similar study is conducted with large sample size, more reliable estimates of prevalence rate can be determined. Though inferences from our study is limited by the small sample size, our study clearly suggested that the PCR technique has increased sensitivity and specificity in the detection of *C.trachomatis* and HSV in the clinical specimens from patients with endocervicitis and urethritis among the high-risk group of subjects attending the STD clinic.

F. Evaluation of Polymerase chain reaction for the detection of *C. trachomatis* in interstitial pneumonia against the conventional methods.

Diagnosis of respiratory tract infections due to *C. trachomatis* in pediatric patients is very important because of its complications and sequelae. Therefore, PCR for the detection of *C. trachomatis* in NPA was evaluated against conventional methods. Seven children were positive for PCR, whereas IF detected *C. trachomatis* in only child. Among the seven children positive for *C. trachomatis* PCR, two of them were positive for Adenovirus DNA. These two among 45 children had co-infection with Adenovirus and *C. trachomatis.* Culture technique was not helpful in the identification of the etiological agent. Savy et al, 1997, reported the sensitivity of IF technique as 37.5 percent for the detection of C. *trachomatis* in NPAs.

Thus PCR technique was helpful in identifying 3 patients with adenoviral respiratory tract infections. There are no reports in India on the prevalence of respiratory tract infections due to *C. trachomatis*. We consider this as the first report on children of *C. trachomatis* by PCR in NPAs.

There are two generally accepted methods for the isolation of *C. trachomatis*. The first method involves yolk sac inoculation of embryonated hen's egg and the second method is the use of tissue cultures. The yolk sac method is time consuming requires one week to grow *C. trachomatis*. The tissue culture method is relatively rapid, the isolation attempt is completed within 3-7 days. Moreover tissue culture methods offer greater advantage in speed and facilitate the handling of large numbers of specimens. Therefore cell culture technique was chosen in our study to isolate *C. trachomatis*.

For the past two decades, the laboratory diagnosis of *C. trachomatis* has been based on the isolation of this organism in cell cultures. The success of laboratories in culturing *C. trachomatis* from clinical specimens depend on the collection of the specimen, the type of material used for the collection of the specimen, transport and storage of specimens. The time duration between collection of the specimen and inoculation also greatly influence the isolation of this organism. Most of the laboratories do not have expertise cell culturing techniques to attempt isolation of *C. trachomatis* from clinical specimens. Collection of specimens for the demonstration of the agent should be carried out before treatment is given. The best recovery rates were reported with cotton swabs resulting in only a decrease in inclusion counts of 0-13.0% (Mahony and Chernesky, 1985). Compared with immediate inoculation, the best recovery occurred after the specimens were held at 4^0C for 24 hours and slow freezing or snap –freezing and storing the specimen at -70^0C. Storage of specimen at 23^0C resulted in a rapid decrease in the isolation rate from 34.4% at 1 day to 0% at 2 days (Mahony and Chernesky, 1985). Blind passage of culture harvest has been

proven to increase the isolation rate of *C. trachomatis* (Jones et al, 1989). We had used sterile cotton swabs for the collection of clinical specimens and the clinical specimens were stored at -70^{0}C and were inoculated with in 24 hours of collection. In spite of all these measures taken to recover *C. trachomatis* from clinical specimens our isolation rate was very poor. This may be attributed to the low number of viable organisms in the specimens. Theoretically, one inclusion would be enough to show positive result in a PCR-based test, while for a positive culture multiple elementary bodies are needed (Mouton et al, 1997). In our country the antibiotics are freely available for even common people to have self-medication and this could have significantly contributed to the low culture results from all clinical specimens for *C. trachomatis.*

G. Evaluation of Polymerase chain reaction (PCR) against the conventional virological methods of antigen detection and virus isolation for the detection of adenovirus conjunctivitis

Conjunctivitis caused by adenovirus is a frequent occurrence. The standard method of detecting adenovirus antigen from clinical specimens is by isolation and by application of immunological tests on smears made from the clinical specimens. Immunofluorescence tests on smears from conjunctiva will aid in the rapid diagnosis but it needs a certain threshold amount of adenovirus antigen to become positive (Kinchington et al, 1994). Cell culture for the isolation of adenovirus and the use of specific antisera to identify the organism is time consuming and may extend to several days. Theoretically cell culture technique requires only a single infection virus in the inoculum and requires careful transportation conditions to maintain infectivity of the virus and the time constraints may delay the etiological diagnosis as the infective virus titer may be reduced during transport and storage of specimens (Madhavan et al, 1993). PCR technique for the detection of Adenovirus DNA from clinical specimen was earlier standardized with nested PCR primers targeted against hexon gene

(Dalapathy et al. 1998). The primers set had a very high analytic sensitivity and high specificity to adenoviral DNA. Therefore a kit was made with these sets of primers by the Alcon laboratories, USA. The analytical sensitivity of the kit was determined to be 0.0030fg. So the sensitivity of the kit was equivalent to the sensitivity of the In-house PCR (0.0032fg).

The hexon protein contains a common group antigen specific for all the human Adenoviruses and is highly stable and conserved. Serological tests for hexon antigens are widely used because of its high stability and is produced in large quantities in infected cells. The 360 KD hexon trimer, the major adenovirion capsomere, possesses type and subgroup determinants. Viable cells of viruses like ortho, paramyxoviruses and adenoviruses can be recovered from the site of disease for a short period only and therefore to obtain a definitive diagnosis by virus culture, nasopharyngeal and conjunctival swabs should be collected as early as possible after the onset of illness. The kit showed a high analytical specificity by amplifying only adenoviral DNA not the other bacterial or viral DNA known to cause conjunctivitis. The specificity was further confirmed by the fact that all the 10 clinical specimens in which virus was isolated were also positive by PCR. In the present study the conjunctival swabs from patients were collected at the earliest possible stage of illness to recover the virus from as many patients as possible. Despite this early sampling the virus was isolated only in 10 specimens, whereas PCR with nested primers was able to pickup adenovirus DNA from 77 specimens. In 8 specimens it remained negative for Adenovirus DNA. The probable presence of PCR inhibitors in these specimens was ruled out as spiking of the known Adenoviral DNA in these specimen were amplified by PCR. The possible reason for PCR negativity in these specimens could be due to any of the following reasons. It could have been a bacterial conjunctivitis or it was conjunctivitis caused by viruses other adenovirus, on the specimen was collected at a very latter stage of illness.

H. Evaluation of PCR against conventional methods of antigen detection and virus isolation for the detection of adenovirus in interstitial pneumonia.

Viruses are the most common causes of respiratory tract infection in children. The case fatality rate during two year study was 0.73 percent by Earballai et al, (2001). Adenovirus infections are exceedingly common in childhood. Most of these infections are asymptomatic, mild and self-limited. But in the immuno-compromised individuals, Adenoviruses can cause severe localized disease. Ribavirin an antiviral agent licensed in aerosol form for the treatment of respiratory syncytial virus (RSV) infection, has documented activity against adenovirus in vitro (Shetty et al, 2000;Chacon et al, 1998; Gavin et al, 2002). Therefore children with severe respiratory tract infections due to Adenoviruses may be treated thereby reducing the mortality rate can be brought down. Hence timely and accurate diagnosis is necessary. The occurrence of Adenoviral respiratory tract infection was determined by 8.8 percent based on PCR. Maitreye et al, 2000 has studied the etiological agent (viral) of lower respiratory tract infections in northern India. Rapid centrifugation enhanced cultures followed by indirect immunofluorescence was used to determine the etiological agent. Adenoviruses accounted for 1.5 percent of lower respiratory tract infection. They have also reported that respiratory viruses accounted for 44.5 percent cases of acute lower respiratory tract infections in India. John et al, (1991) studied the etiology of acute respiratory infections in children in tropical southern India. Viruses accounted for 49 percent cases of acute lower respiratory tract infection and adenoviruses were the third most common viral agent accounted for LRTI. Studies done throughout world shows that the rate of Adenovirus infection ranged from 2.8 to 27.3 percent among acute respiration tract infections. (Avilla et al, 1990; Earballai et al, 2001; Avila et al, 1989, Yun et al, 1995, Al-hajjan et al, 1998, Straliotto et al, 2002, Videla et al, 1998).

In this study 8.8 percent of children with lower respiratory tract infections, the etiological agent was found to be adenovirus. The value of PCR against conventional method in detection of Adenovirus in NPA was evaluated. Among the 45 specimens processed 4 (8.8%) were positive by PCR whereas 1 among these 4 was IF technique. None of the specimen was positive by culture. Addition 3 children were diagnosed to have Adenoviral infection by PCR. Though the number of specimens included in the study is less. To have more information it can be concluded that PCR is a more sensitive test than IF and culture techniques.

The average period of time needed for IF technique was 2 ½ hours and for culture technique it was seven days and for PCR, only eight hours. PCR inhibitors were not encountered in NPA as the spiking of known DNA work out well. Application of PCR technique on NPA will help in the rapid, accurate differential diagnosis of Adenoviral infections.

I. DNA sequencing of amplified products to genotype the *C. trachomatis*

The plasmid amplified products of the 3 laboratory isolates, 3 genital and 5 ocular specimens showed a single nucleotide substitution at the 1939th position. This substitution shows that our strains are trachoma biovars. Otherwise no significant information was inferred that would help to genotype the organism. So plasmid sequencing was not helpful in genotyping *C. trachomatis.*

J. MOMP DNA sequencing to genotype *C. trachomatis*

In order to study the disease epidemiology of *C. trachomatis* infections, laboratory techniques for differentiating the serovars and variants have been developed. The diversity in MOMP is thought to be necessary for the bacteria to survive in its environment. Hence MOMP typing is given importance to study the epidemiology of the organism. MOMP serotyping is the traditional method followed

to identify the serovars of *C. trachomatis* isolates. Differences in reactivities with monoclonal and polyclonal antibodies have led to the identification of a large number of serovariants (Morre et al, 1998). The classification in MOMP serotyping is based on immunoepitope analysis of the MOMP with polyclonal and monoclonal antibodies (Kuo et al, 1983; Ossewaarde et al, 1994). The need for multiple passages in cell culture and a large panel of monoclonal antibodies are major drawbacks of MOMP serotyping.

Typing by RFLP analysis of *ompl* gene is a simple, rapid, and powerful tool in epidemiological studies (Frost et al, 1991;Lan et al, 1993; Lan et al,1994; Rodriguez et al,1991). Nucleotide sequencing of the MOMP1 gene provides a definite typing method. Hence this envisaged MOMP sequencing method to genotype C. *trachomatis.*

C. trachomatis isolated from both the eyes of a 10 day old children with clinical diagnosis of ophthalmia neonatorum was sequenced with the amplified product of the MOMP1 gene. The sequence analysis of the variable domains showed that it was *C. trachomatis* genotype B. Though serotypes B and Ba cause trachoma, they have been recovered from the genital tract (Dowson, 1996; Morre, 1998). We postulate that the source of infection in this infant was the infected genital tract of the mother. But the endocervical swab from the mother was not cultured since the mother refused to give the swab. So the source of infection was not proved.

In the literature, there are many reports on sequence typing of *C. trachomatis* isolates with the genital isolates. Three hundred and thirty isolates collected over a period of 2 years from patients attending STD clinic were genotyped using sequencing of MOMP 1 gene by Jonsdottir et al, (2003). The predominant genotypes found in descending order of prevalence were D, E, J, F, K, G, H and I. They have reported that no significant changes in frequency of genotypes were noted. A

relatively stable situation of genotypes was noted by them and suggested an ecological advantage of serotype E. Sylvan et al, 2002, reported genotype E as the most prevalent organism causing genital infection among men and genotype D among women. In another study conducted by Bandea et al, (2001) to assess the prevalence of *C. trachomatis* infections in the cities of Bangkok and Chiang Rai, Thailand, genotype F was predominantly isolated from urine samples collected from asymptomatic pregnant women. In yet another study conducted by Jurstrand et al, (2001) in Sweden, serotype E followed by F was identified as the predominant genotype causing urogenital *C. trachomatis* infections. They had also reported that MOMP 1 gene was highly conserved among genotype E and F as there were no nucleotide difference observed among them. Whereas one to four nucleotide substitutions were observed among the less predominant genotypes G and H.

The predominant *C. trachomatis* strains responsible for sexually transmitted disease world wide are serotypes E, F and D serovars of *C. trachomatis.* (Dean and Millman ,1997). But to the best of our knowledge no reports on MOMP sequencing of ocular isolates from cases of conjunctivitis of *C. trachomatis* is reported from India. This is the first time *C. trachomatis* type B has been isolated from a case of ophthalmia neonatorum is reported. Though MOMP gene 1 sequencing is laborious and expensive it is proved beyond doubt that it is the definite method to study the epidemiology of *C. trachomatis* infection as the organisms are typed accurately. In a study conducted by Pedersen et al, (2000), discrepancies between the genotyping results obtained by PCR-RFLP and by sequencing method were reported. *C. trachomatis* genotypes F isolates from 14 samples were mistaken for genotype C and in one case genotype G was mistaken for C and in another genotype J was mistaken for genotype H . Therefore though MOMP 1 gene sequencing is an expensive method it is the accurate method for genotyping *C. trachomatis.* Therefore serotype B can be associated with ophthalmia neonatorum.

K. Determination of MIC and MBC of antibiotics against *C. trachomatis*.

Earlier MIC of drugs were measured by exposure of the standard number of *C. trachomatis* particles to varying concentrations of the drugs and determined its growth microscopically after iodine or Giemsa staining of the cell cultures (Blackman et al, 1977). Being non-specific, these staining methods yielded highly inaccurate results. This was improved upon by using the specific staining method of micro-immunofluorescence (MIF) (Borsum, 1990) but this also had its own limitations of observer variability because of the presence of stained *C. trachomatis* particles of the inoculum. Reverse transcriptase Polymerase chain reaction (RT-PCR) to determine the MIC of drugs against Chlamydia spp. have been described (Khan et al, 1996; Cross et al, 1999) and was reported as accurate, reproducible and the MIC values were higher than that determined by the IF method. In the present study, we standardized RT-PCR based drug sensitivity test for *C. trachomatis* and applied the technique to determine the MIC of ciprofloxacin, erythromycin, roxithromycin, and sparfloxacin against the standard strains of trachoma serovars A, B, Ba and C, and 3 clinical isolates of *C. trachomatis* were determined. Antimicrobial susceptibility testing of *C. trachomatis* is not a routine procedure, but a difficult and drug resistance among *C. trachomatis* is rarely reported. Indian reports on MIC of antibiotics against *C. trachoamtis* are not found except one. Madhavan et al, (1996) determined the MIC of Ciprofloxacin and tetracycline against 27 ocular isolates of *C. trachomatis* and have the MIC of ciprofloxacin were 2.9µg/ml and for tetracycline 5.7 µg/ml.

Determining the MIC of drugs against *C. trachomatis* is a difficult, expensive and cumbersome work, as it requires tissue culture facility.

The developmental cycle of *C. trachomatis* makes it more difficult and the culture harvests were needed to be passed two to three times in cell cultures without the drugs for confirmation of non - viability of *C. trachomatis*. This resulted in

prolonged period of time for testing. Minimum inhibitory concentrations of drugs determined by MIF method may not be accurate because of observer variability and presence of non-viable organisms may also be read as positive for growth of the organisms. RT-PCR picks up only live organism and passing the culture on to cell culture for confirmation of the results is not necessary. Cross et al in their experiments pointed that passing the material from IF negative but RT-PCR positive wells at antibiotic concentration close to the MIC, proved to be successful in several cases, but major limitation is that inclusions which are very small or aberrant bearing no morphological resemblance to classical inclusions may not be seen with certain antimicrobials . Though drug resistance is rarely reported among *C. trachomatis* RT-PCR based sequencing method using specific genes will be of great help to understand the exact molecular basis. In our study we found that RT-PCR was a method for accurate measurement of MIC of drugs against *C. trachomatis*. Once standardized RT-PCR technique can be a useful technique to monitor drug therapy particularly in patients with recalcitrant chlamydial infections.

L. Influence clinical specimen on the MIC of ciprofloxacin, roxithromycin and erythromycin against *C. trachomatis*

Five conjunctival swabs collected from individuals with out any clinical symptoms of conjunctivitis were spiked with *C. trachomatis* serotype Ba. The spiking was done with very low concentration of the organism such a way that these specimens were negative by IF staining but positive for PCR. In two conjunctival swabs spiked with *C. trachomatis* Ba MIC against Ciprofloxacin and erythromycin was determined. The MIC value determined by RT-PCR and MIF varied from that obtained without spiking. The MIC of Ciprofloxacin and erythromycin in the presence of clinical specimen was found to be lower this could have been due to the presence of augmented effective of antibacterial substances present in the clinical specimen. The MIC of ciprofloxacin by MIF was determined as 0.25µg/ml, 0.5 µg/ml. The MBC

value determined by RT-PCR (1.25 µg/ml) was higher than that of MBC value obtained by IF technique (0.20 µg/ml). The MIC value for erythromycin determined by IF was 2.5 µg/ml. The MBC value determined by RT-PCR technique was higher (3.5 µg/ml). Whereas the MIC value for roxithromycin was higher when compared to what value obtained with spiking the specimen. The MIC value for roxithromycin was 0.05 µg/ml by IF and by RT-PCR. The spiking experiment shows that the clinical specimen with fewer copy number of organism can be directly exposed to varied concentrations of antibiotics to determine the MBC.

Neutralization Test

The neutralization test with the ten-(10) adenovirus isolates was done with Adenovirus four type specific serum only as the isolates were typed as Adenovirus 4 by multiplex PCR. Therefore serotyping by neutralization test was easier. Neutralization test is a laborious time consuming test. The test also requires virus isolations and the titer of the virus should be more to perform the test. It also involves the use of panel of expensive type specific antisera, which are not available freely in India.

For typing Adenovirus isolates first a provisional subgroup classification is obtained by determining the agglutination characteristics of the isolate with monkey, human group O and rat RBC. The isolates are then typed against antisera of that subgroup. Then they are typed using type specific antisera, which should include a panel of antiserum, unless clinical or epidemiological data for the isolates suggest a smaller set of sera like in our study, where the isolates were identified as type 4 by multiplex PCR. Neutralization test also takes a minimum of 3 days for reporting.

M. Multiplex PCR to determine the subgenus typing of adenovirus isolates

Human adenovirus are implicated in numerous diseases and are widespread throughout the world. Adenovirus are divided into six different subgenera A to F based on a neutralization haemagglutination and DNA genome homologies the length of fibers and oncogenicity of the virus. It has been reported that by hexon-X ray crystallography analysis and sequencing, hypervariable regions (HVRs) that participate in type-specific neutralization have been found to exist on the surface of Ads (Takeuchi et al, 1999). The existence of serotype-specific hyper variable regions are thought to be important regions for serotype identification. The serotype specific sequence are restricted to seven independent HVRs and neutralization epitopes are located in one to several sites in these regions. Comparison of hexon HVRs revealed homology within the same sub genus. This means that the HVR s reflect the subgenus, which is a group that has similar DNA properties. It is suggested that the hexon that contains the HVRs may also be associated with disease specificity. Based on the subgenus-specific sequence identified in the hexon region, primer sets were designed which would amplify specifically these DNA sequences. These primer sets were also specific for the conserved hexon gene region. Among the six subgenus A Adenoviruses are isolated most commonly isolated from infants diagnosed to have gastroenteritis.

Adenoviruses belonging to subgenus C causes respiratory disease in small children, which also show prolonged excretion after infection. Serotypes belonging to subgenus e can also be associated with sudden infant and death syndrome (Pring-Akerblom et al, 1999). Subgenus B viruses are associated with acute respiratory infection, follicular conjunctivitis and haemoerhagic cystitis. Subgenus D viruses are associated with epidemic keratoconjunctivitis. Ad4 the only member belonging to subgenus E can also cause conjunctivitis and respiratory disease.

Since in the present only respiratory and conjunctival specimens were processed for the detection of Adenovirus and subsequent typing, primer sets specific for the subgenus B, C & E, which would identify ocular and respiratory adenoviruses, were chosen. The primers were absolutely specific for the adenovirus and the specificity was further confirmed by the fact that all the 10 clinical isolates were also positive by the primer set specific for subgenus E. The analytic sensitivity of these set primers was low, as the detection limit of the Adenoviral DNA was only 20 nanogram of DNA. Therefore we were not able to apply the multiplex PCR for subgenus typing on clinical specimens from conjunctivae and NPA. Since Adenovirus H is the only serotype classified under subgenus E, we were able to identify the serotype of 10 Adenovirus isolates recovered from patients diagnosed to epidemic viral conjunctivitis.

The clinical specimens collected from these 10 patients from whom the virus was isolated were earlier positive for nested PCR primer specific for the hexon gene. Attempts were made to increase the sensitivity of the primer by decreasing the annealing temperature from 40 ^{0}C to 38 ^{0}C. As there were non-specific amplication seen, the annealing temperature was maintained at 40^{0}C only. The amplified products of all the sets of primers were distinguished easily by 2% agarose gel electrophoresis as the minimum difference in size ranged from 66 between the amplified products size of subgenus E & B. So the cumbersome PAGE technique was not needed to differentiate the subgenus specific amplified products. So we were able to identify the serotype within 2 hours from the time of completion of DNA extraction from the culture harvest. Pring Akeslom et al, (1999) had employed this multiplex PCR with 6 sets of primers specific for the subgenus typing on 100 clinical specimens, 65 stool specimens, 23 ocular swabs and 12 throat swabs. Subgenus A, C & F adenoviruses were found in stool samples subgenus D & E were detected from ocular specimens. Subgenus B adenoviruses were detected in all three different specimens. Adhikary et

al 2001 had developed a typing method of only oculopathogenic strains of subgenus D. The primers set was specific for fiber genes, which amplified all the 9 oculopathogenic prototypes. Followed by PCR amplification. *Dde* I *Hint* I and Rsai restriction enzymes were used to differentiate the serotypes. They have reported an increased PCR sensitivity C fibre based primer than culture.

N. PCR-RFLP to genotype adenovirus isolates from conjunctivitis

Adenovirus isolates were earlier typed by neutralization test (Fitch et al, 1989). Then genomic DNA isolation followed by restriction digestion with restriction enzyme was done to identify the serotype of Adenovirus involved in particular epidemic outbreak (Kemp et al, 1983, Wadell et al, 1981). Large epidemiological studies have been done with Adenoviruses to identify the change in etiology of conjunctivitis and to demonstrate the strain variation within a serotype. (Kemp et al, 1986). Kemp et al 1986 had collected Adenovirus type eight-(8) strain over a 19-year period from eye specimens from patients with Keratoconjunctivitis. Genomic DNA isolated from these strain were divided by restriction enzyme analysis with endonucleases *Sal* I, *Hind* III, *Sac* I, *Kpa* I, Sma I into 3 subgroups. The prototype 8, 8C, 8D was found throughout the United States from 1966 through 1985 and also in Taiwan and Greece in the early 1980s. Genotype 8C was isolated from Maryland to Missouri to Alabama from 1971 through 1974. Genotype 8D was found only in an epidemic of eye disease among Vietnamese refugees. They had also reported that genotype 8C and D were distinct from genotypes 8A & B described from Japan in 1975 through 1981.

Kemp et al, 1983 have shown that restriction enzyme analysis is a reliable means of differentiating between serologically identical strains with this method the Ad19 prototype isolated in different periods.

Similarly Ad37 isolates been isolated over in two different periods have shown to be different by restriction enzyme analysis. Fuji et al 1983 demonstrated that two genotypic variants designated Ad 8A and 8B circulated in the population of Sapparo, Japan between 1975 and 1981. With the advent of PCR in 1983, PCR based RFLP techniques were used to determine the genotypes (Chang et al, 2001, Tanaka et al, 2000). Since serotypic variations in Adenovirus are determined by the variation in the hexon protein. Primers are designated to amplify the hexon gene. This was then followed by restriction digestion with restriction enzymes. Compared to the whole genome restriction digestion PCR – RFLP technique is more rapid and less cumbersome (Saiton- Inagawa et al, 1996). Saithoh – Ingawa et al, 1996, had performed PCR, RFLP technique to differentiate 14 Adenovirus prototypes by combination of restriction enzymes *ECOT* 141, *Hae* III & *Hinf* I. PCR – RFLP was performed on 69 samples collected from patients diagnosed to have acute conjunctivitis, epidemic keratoconjunctivitis, pharyngoconjunctivitis. They were identified as Ad 37, Ad 3, Ad 11, Ad 8 and Ad 4 by PCR- RFLP technique. PCR-RFLP technique on fiber- gene on amplified product to differentiate subgenus D was reported by Adhikary et al, (2001).

Extensive molecular works have not been reported in India to identify the serotype of etiological agents of epidemic conjunctivitis. The detection of the etiological agent responsible for an epidemic have been based on identification tests done only on few isolates selected at random by several other workers (Gogate & Padeiri 1981; Pal et al, 1983; Broor et al, 1992). Earlier reports of epidemics of conjunctivitis in India have been either due to EV 70 (Kono et al, 1981; Bhide et al, 1994) or coxsackie virus A 24 variant strains (Gogate et al, 1978; Gogate and Padbidri, 1981). Dalapathy et al 1998 reported that adenovirus serotype 7a was the likely causative agent of epidemic conjunctivitis in 1998.

In the present study Adenovirus Genotype 4 was identified as the causative agent of epidemic conjunctivitis that occurred during August – Dec 2002. Saition-Inagawa et al, (2001) reported discrepancies in results between neutralization and PCR – RFLP analysis. The Adenovirus isolate was identified as 34 by neutralization test and as Ad14 by PCR- RFLP analysis. Late by genomic analysis, the isolate was typed as Ad34 a. Similar observation was reported by Takeuchi et al, 1999. But in our study the PCR- RFLP pattern was identical with the prototype RFLP pattern 4. Moreover with multiplex PCR, the isolates were typed as subgenus E. Hence we conclude that PCR- RFLP is an accurate, rapid method to identify Adenovirus genotypes.

PCR- RFLP to genotype adenoviruses detected in interstitial pneumonia patients

PCR- RFLP typed the Adenovirus detected in 3 patients with clinical diagnosis of interstitial pneumonia as Adenovirus 4. As the sensitivity of the primers for subgenus typing had a low analytical sensitivity. The clinical specimens did not get amplified with the primers. The RFLP pattern was identical with RFLP pattern of the standard strain 4. A few reports only were available in the literature for comparing our results. Viquesnel et al, (1997) reported Adenovirus type 7 pleuropneumonia in an immunocompetent adult. The virus was reported to have caused a severe infection and the patient required a mechanical ventilation with tracheostomy. The mortality rate of pneumonia due to Adenovirus vary with the population and the etiologic serotype reaching as high as 60 percent in immuno compromised patients and 15 to 20 percent of the children with Adenovirus type 7 pneumonia (Zahradnik, 1987). Adenovirus 7 h were reported to have constituted 2.4% of 1233 cases of acute respiratory infections in fine hospitals Murtagh et al, 1993. Pneumonia and bronchiolitis were the principle diagnosis sixteen children developed a severe pulmonary disease which required intensive care. Their observations suggest a possible high pathogenicity of adenovirus 7 h .

Lower respiratory tract infections including bronchitis and pneumonia is a common complication of Adenovirus infection, particularly with serotypes. Severe Adenovirus pneumonia are occasionally fatal, occur in young infants and children and only rarely in adults and are mostly associated with types 3, 4, 7 and 21 (Hierholzer, 1989). Epidemics of acute respiratory disease occur predominantly in military recruits and the Ad4 and 7 are most frequently involved in the United States: type 3, 14 and 21 are more frequently involved in Asian European countries. Pneumonia is also a common complication of acute respiratory disease (ARD) in military recruits and the pulmonary involvement can be prolonged and extensive. Fatal cases associated with Ad 4 and Ad 7 have been documented in this population (Hierholzer, 1989). To the best of the knowledge, no serotype / genotyping of Adenoviruses done from NPAs. Therefore the results of PCR-RFLP results on the amplified products is not compared.

Comparison of multiplex PCR, PCR – RFLP and neutralization test.

On comparison of PCR – RFLP, multiplex PCR and neutralization test, PCR – RFLP test is considered as the method of choice for genotyping the Adenovirus isolates. Combination of restriction enzymes can be used to identify the isolates with PCR-amplified products. The RFLP pattern can be matched with the RFLP pattern of the standard strains to accurately identify the isolate. Though DNA sequencing is considered as the ultimate to identify the genotype, only few laboratories in India have sequencing facility. Therefore it is not possible for all the laboratories to perform DNA sequencing. Next to PCR – RFLP technique, multiplex PCR with allele specific primers will be useful to identify the genotype of isolate. In case any discrepancies are encountered more than one typing method should be made use of to verify the result. In the absence of allele specific primers, subgenus typing with primers specific for the subgenus determinates on the hexon gene can be done since often in clinical practice subgenus typing would suffice.

Summary and Conclusion

SUMMARY AND CONCLUSIONS

- Six continuous cell lines were tested for their relative susceptibility to grow *C. .trachomatis* serotype A, B, Ba & C. Among the six cells lines (McCoy, HeLa, HEP - 2 , A549, Vero, BHK–21), McCoy and HeLa were more sensitive to grow *C.trachomatis.* Next to these two cell lines Vero was sensitive. BHK– 21 and HEP – 2 were not very useful to grow *C. trachomatis* A549 cell line did not allow the growth of *C.trachomatis.*

- Three in-house DNA extraction methods were evaluated and Guanidine thiocyanate method was observed as the most efficient method. Therefore, this method was applied on all the clinical specimens.

- Two sets of primers, one set specific for part of plasmid DNA sequence and the other set specific for MOMP gene sequences was evaluated for their specificity and sensitivity for the detection of *C. trachomatis* in patients with diagnosis of primary conjunctivitis. Both the primer sets were absolutely specific for *C. trachomatis* DNA, but the plasmid primers were more sensitive than the MOMP primers in detecting *C. trachomatis* DNA in 11(13.8%) specimens 9 (additional specimens). Whereas MOMP primers were able to amplify DNA in only 3 (3.8%) specimens. Hence the plasmid primers were used for the detection of *C. trachomatis* from clinical specimens for diagnostic purposes.

- PCR for the detection of *C.trachomatis* in conjunctivitis was evaluated against conventional methods, IF and culture. PCR had increased clinical sensitivity when compared to IF and culture. Among the 486 specimens processed PCR detected *C.trachomatis* in 27 (5.6%) specimens whereas IF and culture detected *C.trachomatis* in only 3(0.6%) specimens. PCR was concluded as a

sensitive, specific technique for the detection of *C.trachomatis* in conjunctivitis. The prevalence of *C. trachomatis* conjunctivitis has reduced drastically compared to the prevalence of 34.6% in 1990.

- PCR for the detection of *C.trachomatis* in NPAs collected from patients with clinical diagnosis of interstitial pneumonia was evaluated against conventional methods. Among the 45 specimens processed for PCR, IF culture, PCR was positive in 7(15.6%) specimens whereas IF detected *C. trachomatis* in only one (2.2%) specimen. PCR had increased the clinical sensitivity by 13.4%. Two patients had coinfection with *C .trachomatis* and Adenovirus. PCR helped in the rapid differential diagnosis in interstitial pneumonia.

- PCR was evaluated for the detection of *C.trachomatis* in 25 female patients with cervicitis and 72 male patients with urethritis against conventional method. Among the 25 women, one (4.0%) was positive for *C. trachomatis* and 3 (12.0%) were positive for HSV by PCR. FAT and culture were negative. Nine (12.0%) of the urethral swabs positive for *C. trachomatis* and 5 (6.6%) were positive for HSV by PCR. Among the 9 positive by PCR for *C. trachomatis*, 3 (4.0%) were positive by FAT. Cultures for both organisms were negative. Endocervicitis and male urethritis due to *C trachomatis* and HSV are not uncommon among high-risk individuals.

- The etiological agents of epidemic conjunctivitis was investigated by PCR, IF culture on a total 85 conjunctival specimens collected from 62 patients. The etiological agent was identified as Adenovirus. PCR technique detected Adenovirus DNA in 77 (90.0%) specimens, whereas IF could detect only in 63(74.1%) specimens. Adenovirus was isolated only from 10 specimens. Thus PCR had increased the clinical sensitivity by 14.0 per cent.

- PCR was evaluated against IF and culture for the detection of adenovirus in 45 NPA diagnosed to have interstitial pneumonia. PCR detected Adenovirus DNA in 4 (8.8%) clinical specimens whereas by IF only one (2.2%) specimen was positive. Thus PCR helped in the differential diagnosis in 4 specimens. Among these 4 specimens, 2 specimens had *C. trachomatis* infection. PCR had increased the clinical sensitivity by 6.6%.

- The 3 *C. trachomatis* isolated recovered from both the eyes of a child diagnosed to have ophthalmia neonatorum and from a patient with inclusion of conjunctivitis and 8 PCR plasmid amplified products (3 genital and 5 ocular specimens) were sequenced. There were no significant nucleotide variations observed, which could be associated with a particular genotype, hence the plasmid DNA sequencing was not helpful in identifying the genotype of *C. trachomatis*. On comparing with the plasmid referral sequence a single nucleotide substitution at the 1939th position was observed. This showed that our strains belong to trachoma biovars. Plasmid DNA sequencing does not help to genotype *C. trachomatis*.

- DNA extracted from the 2 isolates from a case of ophthalmia neonatorum and the four standard strains were amplified with primers specific for the MOMP gene of *C . trachomatis* Upon DNA sequencing, the two isolates were identified as *C .trachomatis* B. Sequences of the four variable domains were identical with the variable domain sequence of *C. trachomatis* reference strain B from genital tract. The isolation of *C. trachomatis* B is rarely reported. *C. trachomatis* serotype B is commonly associated with trachoma. But the isolation of genotype B from cases of ophthalmia neonatorum is not reported in the literature. Our results show that genotype B cause conjunctivitis in new

born and the organism could have been acquired from the genital tract of mother. As the mother of this child refused to give cervical swab, this hypothesis was not proved.

- RT – PCR to determine the MIC and MBC of all the three isolates and the standard strains was standardized. The MBC range of the 4 antibiotics tested with in the acceptable limit as determined by IF and RT – PCR. *C .trachomatis* Ba showed a profound resistance to erythromycin up to a concentration of 160 µg / ml as determined by RT – PCR whereas by IF the MIC and MBC was determined to be 80 µg / ml.

- The MIC range of the 4 drugs determined by MIF staining was four fold lower than RT – PCR except for Ciprofloxacin against *C. trachomatis* Ba.

- In reading the MIF results, difficulties were encountered it needed two experts to read the result. Sparfloxacin was showing auto fluorescence and this interfered with reading the results.

- The influence of conjunctival specimens on antibiotics, used for *C. trachomatis* infections was determined by spiking known quantity of IFU of *C. trachomatis* in the clinical specimen. The spiked clinical specimens were exposed to varied concentrations of antibiotics. The MIC and MBC values were within normal range. The MBC values of antibiotics were accurately determined by RT-PCR technique.

- Multiplex PCR for subgenus typing of Adenovirus was standardized with 3 sets of primers which would detect adenoviruses that causes respiratory tract and conjunctival specimens.

- Multiples PCR was employed to type 10 Adenoviruses that were isolated from conjunctival swabs of patients during the epidemic outbreak of August – December 2002. The isolates were found to belong to subgenus E. As Adenovirus 4 is the only serotype grouped under subgenus E, the isolates were typed as Adenovirus 4. Compared to neutralization test multiplex PCR technique was more accurate and rapid to serotype adenoviruses.

- These 10 isolates were amplified with primer sets specific for the hexon gene, and the amplified products were digested with *Hae* III enzyme. The RFLP patterns of the isolates were identical with the RFLP pattern of Adenovirus prototype 4. Therefore the etiological agent of epidemic conjunctivitis was identified as adenovirus type 4.

- The isolates were confirmed as Adenovirus 4 by PCR – RFLP technique. Therefore the causative agent of epidemic outbreak of conjunctivitis was identified as type 4.

- The PCR amplified products of Adenovirus from Nasopharyngeal aspirates were digested with restriction enzyme *Hae III* to identify the serotype. The restriction pattern of the PCR - RFLP were identical with that of Adenovirus 4.

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Paper Publications & Presentations

PAPERS PRESENTED

1. "PCR for the detection of *C. trachomatis* from primary conjunctivitis and genital specimens", J. Malathi, HN. Madhavan., K. Lily Therese. Presented at XXVth IAMM conference held at Delhi from 23-25th November 2001.

2. ''PCR for the detection of *C. trachomatis* in conjunctivitis. J. Malathi, H.N.Madhavan, K. Lily Therese, HN. Madhavan. Presented at the 9th IERG meeting held at L.V.Prasad Eye Institute, Hydrabad.

3. "Standardization of reverse transcriptase PCR (RT-PCR) the detection of minimum for inhibitory concentration of antibiotics against (MIC) *chlamydia trachomatis* " J. Malathi, HN. Madhavan, K. Lily Therese Presented at XXVI th IAMM conference held at Bangalore, 21-25th November 2001.

4. "Evaluation of polymerase chain reaction with primers coding for MOMP and plasmid genes for direct and genotyping of *chlamydia trachomatis* in primary conjunctivitis" J.Malathi, H.N.Madhavan, K.Lily Therese. Presented at the SERI ARVO meeting held at Singapore Eye Research Institute, Singapore between 7th- 9th February 2003.

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Prevalence of *Chlamydia trachomatis* & herpes simplex virus in males with urethritis & females with cervicitis attending STD clinic

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Background & objectives: **Cervicitis and urethritis due to *Chlamydia trachomatis* are common sexually transmitted diseases. However, there is a paucity of information on urethritis and mucopurulent cervicitis due to herpes simplex virus (HSV) from India. We used polymerase chain reaction (PCR) to find out the prevalence of *C. trachomatis* and HSV associated urethritis in males and mucopurulent cervicitis in females attending a sexually transmitted diseases (STD) clinic.**

Methods: **Twenty five endocervical swabs from 25 women with mucopurulent cervicitis and 75 urethral swabs from 72 males with urethritis were processed for the detection of *C. trachomatis* and HSV by antigen detection by fluorescent antibody test (FAT), culture and PCR.**

Results: **Among the 25 women, one (4.0%) was positive for *C. trachomatis* and 3 (12.0%) were positive for HSV by PCR. FAT and culture were negative. Nine (12.0%) of the 75 urethral swabs were positive for *C. trachomatis* and 5 (6.6%) were positive for HSV by PCR. Among the 9 positive by PCR for *C. trachomatis*, 3 (4.0%) were positive by FAT. Cultures for both organisms were negative.**

Interpretation & conclusion: **Endocervicitis and male urethritis due to *C. trachomatis* and HSV are not uncommon among high-risk individuals. The diagnosis could be established mainly by PCR.**

Key words *Chlamydia trachomatis* endocervicitis - *C. trachomatis* urethritis - HSV endocervicitis infection - HSV urethritis - polymerase chain reaction

Chlaymydia trachomatis is an obligate intracellular Gram negative bacillus and the oculogenital serovars D through K are considered to be the most common sexually transmitted bacterial pathogens[1,2]. In women, urogenital chlamydial infections results in a broad spectrum of clinical manifestations including urethritis, cervicitis and pelvic inflammatory diseases leading to serious complications such as ectopic pregnancy and tubal infertility[3,4]. In men, urogenital *C. trachomatis* infection causes urethritis and rarely ascending infections like epididymitis[5,6]. Herpes simplex virus (HSV) has been isolated from all visceral and mucocutaneous sites[7]. Involvement of the cervix and urethra with HSV results in endocervical and urethral discharge respectively[8,9]. The diagnosis of these infections is based on demonstration of their antigens by immunological methods such as fluorescent antibody test (FAT) or enzyme immunoassay (EIA) and isolation in cell cultures.

The sensitivity of these methods has been shown to be low[10,11]. DNA amplification methods such as polymerase chain reaction (PCR) to detect *C. trachomatis* and HSV in urogenital infections are currently available and reported to be far more sensitive than direct antigen detection methods and cell culture techniques[12,13]. Since there is a paucity of information on the prevalence of these organisms causing genital infections in India, a study was undertaken to determine the same among the high risk population attending the sexually transmitted diseases (STD) outpatient clinic at General Hospital, Chennai.

Material & Methods

Patients & collection of enodcervical and urethral specimens: The present study was carried out in July and August 2001. Endocervical swabs were collected in duplicate from 25 women with mucopurulent cervicitis and urethral swabs were collected in duplicate from 72 males with urethritis who had not been on antibiotic treatment, consecutively three days a week during the study period.

One female and 3 male patients were seropositive for human immunodeficiency virus (HIV) infection. Specimens were collected using sterile cotton-tipped swabs. Two swabs were collected from each patient. The first swab was placed in 0.2M sucrose phosphate buffer (2 SP) at *p*H 7.2 and transported to the laboratory for PCR assay for *C. trachomatis* plasmid DNA and HSV DNA and for cultivation of *C. trachomatis* in McCoy cell culture and HSV in Vero cell cultures. Using the second swab, smears were made on clean microscopic glass slide and fixed in methanol. Fluorescent antibody test (FAT) for the detection of *C. trachomatis* and HSV was done on the smears. The transport medium containing the first swab was vortexed in a cyclomixer and the swab was discarded after squeezing it on the side of the tube to remove the cells sticking to it. This was further processed for isolation of *C. trachomatis* and HSV and PCR assay for detection of DNA of *C. trachomatis* and HSV (performed within 3 h of collection) and culture. Five hundred µl of the specimen was placed in a sterile Eppendorf storage vial that was stored in -20°C for PCR assay. The remaining specimen was decontaminated with gentamicin (50 µg/ml) (Hi-Media, India) and amphotericin B (10µg/ml) (Hi-Media, India) and stored at -20°C until processed for cultures.

Fluorescent antibody test (FAT) for direct detection of C. trachomatis: Smears were stained by FAT using fluorescein isothiocynate (FITC) labeled monoclonal antibody for detection of major outer membrane protein (MOMP) of *C. trachomatis* (Orion Diagnostics, Finland) as per the instructions of the manufacturer. The smears were observed under Epi-fluorescence microscope with a blue filter (Optiphot, Nikon, Japan).

C. trachomatis isolation: Cultivation of *C. trachomatis* was done as described by us earlier[14] for ocular specimens except that the maintenance medium contained 10 per cent foetal calf serum, 5mg/ml glucose, 3.0 per cent L-glutamine, 1µg/ml of cycloheximide (all the reagents supplied by Hi-Media, India). The cultures incubated at 37°C for 48 h and at the end of this period the cover slips were gently washed and fixed in methanol for 10 min. The infected monolayer on the cover slip was stained by FAT for MOMP antigen of *C. trachomatis* as described earlier for the direct smear and observed under Epifluorescence microscope with a blue filter (Optiphot, Nikon, Japan).

Standardization of PCR for the detection of C. trachomatis in endocervical & urethral swabs: PCR was standardized with DNA extracted from *C. trachomatis* Ba [ATCC-VR 341 procured from the Centres for Disease Control (CDC), Atlanta, USA] grown on McCoy cell cultures. DNA extraction with the standard strains and the clinical specimens was done by the guanidine thiocyanate (GTC) method[15].

(*i*)*Primers*: The primers used were custom synthesized by Invitrogen Life Technologies, Grand Island, New York, USA. The primer sequences were CTP1: 5'TAGTAACTGCCACTTCATCA3' and CTP2:5'TCCCCTTGTAATTCGTTGC3' which generated a 201-bp of cryptic plasmid of *C. trachomatis* on PCR amplification[16,17].

(*ii*) *Specificity of PCR*: The specificity of PCR for *C. trachomatis* was tested by performing the same on DNA extracted from adenovirus serotype2 (ATCC 846-VR), *Staphylococcus aureus* (ATCC 25293), *Staphylococcus epidermidis* (ATCC 12228), *Klebsiella pneumoniae* (ATCC 138880) and laboratory strains of *Propionibacterium acnes*, β-haemolytic streptococci, *Corynebacterium xerosis*, *Candida albicans* and DNA extracted from human blood.

(*iii*) *Estimation of detection limit (sensitivity) of primers*: The sensitivity of PCR was done by performance of the same on serial 10-fold dilutions of DNA extracted from the cell culture harvest of *C. trachomatis* serotype Ba (ATCC VR-341). Each dilution was kept for amplification and the amount of DNA present in each dilution was estimated spectrophotometrically. The corresponding number of elementary bodies (EBs) that would be present was enumerated by counting the same in the pinhead smear made with 1µl of cell culture harvest. Reagent controls were included whenever amplification was carried out to rule out the presence of amplicon contamination.

While performing the PCR assay measures were taken to prevent the risk of amplicon contamination by including reagent controls, sample extraction controls and by use of different laboratory rooms for DNA extraction, PCR amplification and the product analyses. PCR was performed in a laminar flow bench with dedicated pipettes and single use double sterilized tips, microfuge tubes. Milli-Q filtered water (Millipore Corporation, Bedford, MA, USA) was used for the preparation of DNA extraction reagents and PCR cocktail.

(*iv*) *Amplification profile*: The DNA was amplified in a thermal cycler (Perkin-Elmer thermalcycler Model No. 2400, USA) for 40 cycles with 50 picomoles of plasmid primers, 200 µM dNTPs, 10X PCR buffer, 1.5 units of *Taq* polymerase. The amplification profile is denaturation at 95°C for 1 min, annealing at 53°C for 1.5 min, and extension at 72°C for 1.5 min. The amplified products were run on a 2 per cent agarose gel incorporating 0.5 µg/ml ethidium bromide and visualized under UV (302nm) transilluminator (Pharmacia, USA).

Investigations for the detection of HSV in endocervical and urethral swabs: The conventional methods of antigen detection (FAT) and culture for HSV were done[18] as described by us earlier[19].

PCR for the detection of HSV in endocervical and urethral swabs: PCR using primers for the detection of DNA polymerase gene of HSV was done by the method described by us earlier[20].

Statistical method: The results were statistically analysed using McNemar test[21].

Results

The primers were absolutely specific for *C. trachomatis* plasmid DNA only and there was no amplification of DNA of any of the microorganisms and human DNA tested. The detection limit of the plasmid DNA was 0.2ng equivalent to 10 EBs of *C. trachomatis*. The comparative results of microbiological investigations and PCR for detection of *C. trachomatis* are shown in the Table. Of the 25 endocervical swabs (from 25 women) tested 1 (4.0%) was positive for *C. trachomatis* by PCR. None of the specimens were positive by smear and culture for *C. trachomatis*. Among the 75 urethral swabs from 72 male patients processed, PCR detected, *C. trachomatis* plasmid DNA in 9 (12.0%) specimens. Among these, 3 were positive by direct smear for *C. trachomatis* showing more than 10 EBs in direct smear. One of these patients was serologically positive for HIV infection. *C. trachomatis* was not isolated in any of these patients. PCR detected *C. trachomatis* in 7 (7.0%) additional specimens as compared to FAT which detected in 3 (3.0%) of the 100 specimens

Thus the detection of *C. trachomatis* by PCR was found to be significantly more compared to FAT (McNemar test, $P<0.05$) and culture (McNemar test, $P<0.01$).

HSV DNA was detected in 3 endocervical specimens (12.0%) by PCR (Table). Five (6.6%) urethral swabs were positive for HSV PCR. Smear

Table. Comparative results of microbiological investigations and polymerase chain reaction (PCR) for detection of DNA of *Chlamydia trachomatis* and herpes simplex virus (HSV)

No. of patients n=97	No. of specimens n=100	Specimens positive for *C. trachomatis* no. (%) [95%CI]			HSV No. (%) [95%CI]		
		FAT	Culture	PCR	FAT	Culture	PCR
25 females with mucopurulent cervicitis	25	None	None	1(4.0%) [0.0-11.7%]	None	None	3(12.0%) [0.0-24.7%]
72 males with urethritis	75	3 (4.0%)* [0.0-8.4%]	None	9 (12.0%) [4.6-19.3%]	None	None	5(6.6%) [1.0-12.4%]

CI- 95% confidence interval; FAT, fluorescent antibody test;* 3 specimens positive by FAT were also positive by PCR for *C. trachomatis*

and culture were negative for all the specimens. Thus PCR detected HSV in 8 (8.0%) specimens compared to none by smear and culture (McNemar test $P<0.01$).

Discussion

Our results showed that urogenital infection with *C. trachomatis* and HSV in the high-risk group of patients attending the STD outpatient clinic of a major general hospital was not uncommon. The diagnosis was possible mainly of PCR using primers to detect the cryptic plasmid DNA of the bacterium and the virus and in a few by direct smear. Isolation attempts were unsuccessful. PCR detected *C. trachomatis* and HSV in significantly more genital specimens than by direct smear. We believe that non-isolation of *C. trachomatis* could be due to a low number of viable organisms in the specimens. Theoretically, one inclusion would be enough to show a positive result in a PCR-based test, while for a positive culture multiple elementary bodies are needed[6]. In India, reports on the prevalence of *C. trachomatis* genital infection are only a few and many of these reports are based on antibody detection by enzyme immunoassay (EIA) using commercial kits. Divekar *et al*[22] reported the prevalence of *C. trachomatis* to be 23.2 per cent among female sex workers (FSW) in Mumbai. Using EIA for the detection of *C. trachomatis* antibodies among women, Tyagi *et al*[23] showed its prevalence in women with tubal infertility to be 74.07 per cen and Shrikande *et al*[24] reported a prevalence rate o 33.0 per cent in patients with pelvic inflammator diseases in Nagpur. The prevalence among wome having vaginal discharge was reported to b 12.2 per cent by Viswanath *et al*[25]. The varyin prevalence of *C. trachomatis* reported could be a account of the diagnostic tests used. PCR, a advanced nucleic acid amplification test, is rapid gaining importance in diagnostic microbiolog because of its higher sensitivity and absolu specificity. Since *C. trachomatis* contair 7-10 copies of plasmid[26, 27], in the present study w standardized the PCR to pick up the cryptic plasm DNA and applied the same on clinical specimens determine the prevalence of *C. trachomatis* amo population at risk of acquiring STDs.

Oni *et al*[28] reported isolation of HSV in Hep cell line from 10 patients with non-gonococc urethritis. The prevalence of genital infection due HSV in India is mainly determined by antibo detection[29]. In our study, the HSV prevalence r of 12 per cent in women with endocervici and 6.6 per cent in males with urethritis indica its common occurrence among the STD patients.

Most of our patients gave history of unprotec contacts with multiple partners. One hundred fifty males with urethritis and 450 women w

mucopurulent cervicitis attended the STD clinic during the study period. In this study, 48 per cent of males with urethritis but only 5.6 per cent of females with mucopurulent cervicitis were studied. Though due to small sample size the prevalence rates measured had high variances as indicated by the confidence intervals, a fair assumption could be made that *C. trachomatis* infection was common and HSV infection was not common in urethritis in males. If a similar study is conducted with large sample size, more reliable estimates of prevalence rate can be determined. Though inferences from our study is limited by the small sample size, our study clearly suggests that the PCR technique has increased sensitivity and specificity in the detection of *C. trachomatis* and HSV in the clinical specimens from patients with endocervicitis and urethritis among the high-risk group of subjects attending the STD clinic.

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