THE LABORATORY DIAGNOSIS OF LYMPHOGRANULOMA VENEREUM (LGV)

BY

DIMAKATSO MIRRIAM MALEKA

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PRIVATE BAG 7
CONGELLA
4013 DURBAN

DEDICATED WITH LOVE TO MY PARENTS
MALESELA DAVID AND AKAMIYA LORRAINE MALEKA,
WHOSE SUPPORT AND LOVE I TREASURE.

ABSTRACT

This study was carried out to determine the prevalence of lymphogranuloma venereum (LGV) amongst patients with genital ulcer disease (GUD); to evaluate available methods for the diagnosis of LGV; and to serotype chlamydial isolates from genital ulcers. An attempt was also made to correlate urethral infection with isolation from genital ulcers using Ligase chain reaction (LCR) and direct immunofluorescence (DIF) tests.

Genital ulcer specimens were inoculated onto McCoy cells and the culture-positive specimens immunotyped by the microimmunofluorescence (MIF) test. DNA was extracted from McCoy cells of the culture negative and positive specimens, was subjected to specific amplification of the *Chlamydia trachomatis* cryptic plasmid gene by PCR. Specific amplification of the major outer membrane protein (MOMP) gene by PCR, was carried out on all samples in which the 452 bp product of the plasmid gene was detected. First void urine (FVU) specimens and endourethral swab specimens collected for the diagnosis of concurrent urethral infection, were subjected to a plasmid based LCR and examined for the presence of chlamydial antigen by direct immunofluorescence (DIF) test, respectively.

LGV infection was detected by culture in 13 (5.3%) of the 247 patients studied. Using the MIF test, 5 LGV strains (L1=1, L2=4) and 4 oculogenital strains (Ia=2, E=2) were detected. The remaining 4 could not be typed as viability is required for immunotyping and these were not recoverable. PCR for the specific amplification of the plasmid gene detected 22.3% (55/247) of the infections, inclusive of the 13 samples that were positive by culture and an additional 42 that were negative by culture. The specific amplification of the MOMP gene by PCR was less sensitive and detected 26/55 (47.3%) infections detected by the former. In urethral specimens, LCR detected 46/247 (18.6%) of the chlamydial infections while DIF detected 36/230 (15.6%). Of the 54 patients that had urethral infection, 44.4% were found to have chlamydial infection of their ulcers. Of the

193 patients who did not have chlamydial infection in the urethra, 16.1% had *C. trachomatis* isolated from their ulcer specimens. The association of chlamydial infection found in the urethra or in the ulcers was found to be significant ($p=0.001$) with a relative risk of 2.75.

Using the expanded gold standard for the diagnosis of chlamydial infection, this study showed a high prevalence of infection in ulcers (22.3%). The isolation of oculogenital strains from genital ulcer specimens suggests that in addition to the L strains, LGV may be caused by non LGV strains. Since more than one GUD causing pathogens were isolated from several ulcers, contamination by the oculogenital strains remains a possibility. As far as chlamydial diagnosis of genital ulcers is concerned, specific amplification of plasmid DNA by PCR (despite its inability to differentiate between the oculogenital and the LGV serotypes) is the method of choice, above culture alone. However, further investigations on the significance of the oculogenital strains in the pathogenesis of ulcer disease are required.

In this research the statistical planning and analyses, and recommendations arising from these analyses have been done in consultation with the Institute of Biostatistics of the Medical Research Council.

PREFACE

This study represents original work by the author and has not been submitted in any form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

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ABBREVIATIONS

A	angstroms
A260	absorbance at 260 nm
bis	bisacrylamide
bp	base pair
BPB	bromophenol blue
BSA	bovine serum albumin
cm	centimetre
CMGH	chlamydia complete media with glucose and hepes
^0C	degrees celcius
CO_2	carbon dioxide
COMP	Chlamydia Outer Membrane Complex
CrP	cysteine rich protein
CTM	chlamydia transport medium
d	dalton
dATP	deoxyadenine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanidine triphosphate
dH_20	distilled water
DIF	direct immunofluorescence
tdH_2	triple distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymine triphosphate
EB	evans blue
EIA	enzyme immunoassay
FCS	foetal calf serum
FITC	fluorescein isothiocyanate

FTA-Abs	fluorescent treponema antibody absorption
EB	elementary body
EDTA	ethylenediaminetetraacetate
g	gram
G+C	guanine + cytosine
g/l	grams per litre
GIM	granuloma inguinale transport medium
h	hour
HCl	hydrochloric acid
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HSP	heat-shock protein
IFU	inclusion forming units
IgG	immunoglobulin G
kb	kilobase
kDa	kilodalton
KEH	King Edward VIII Hospital
l	litre
LCR	ligase chain reaction
LPS	lipopolysaccharide
μg	microgram
μl	microlitre
μM	micromole
MABP	monoclonal antibody blocking and preservative solution
mg	milligram
MIF	microimmunofluorescence
min	minute
ml	millilitre
mm	millimetre
mM	millimole
M	molar

MAbs	monoclonal antibodies
MEM	eagles minimum essential medium
$MgCl_2$	magnesium chloride
MOMP	major outer membrane protein
MNYC	modified New York City medium
MPC	mucopurulent cervicitis
M_r	molecular weight
M_rM	molecular weight marker
MoPn	mouse pneumonitis
MT	microtitre
NaCl	sodium chloride
NaOH	sodium hydroxide
NGU	non-gonococcal urethritis
NGV	nystatin, gentamicin, vancomycin
AGV	amphotericin B, Gentamicin, Vancomycin
nm	nanometre
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	phosphate buffered saline and tween 20
PCR	polymerase chain reaction
PEG	polyethylene glycol
PGU	post gonococcal urethritis
pH	negative log of hydrogen ion concentration
PID	pelvic inflammatory disease
RB	reticulate body
RE	restriction enzyme
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNAse	ribonuclease
RPR	rapid plasma reagen

rRNA	ribosomal RNA
s	seconds
SDS	sodium dodecyl sulphate
S P	sucrose phosphate
STD(s)	sexually transmitted disease(s)
Taq	*Thermus aquaticus*
TBE	Tris Borate EDTA
TE	Tris-EDTA
TEMED	N,N,N',N' tetramethylenediamine
Tm	melting temperature
TNTC	too numerous to count
TPHA	*Treponema pallidum* haemagglutination
U	unit
UV	ultraviolet
v	volume
V	Volt
VD	variable domain
viz.	namely
VTM	viral transport medium
w/o	without

CHAPTER 1

INTRODUCTION

Chlamydiae are a group of obligate intracellular bacteria that cause a variety of clinical syndromes in humans, other mammals, and birds. Until recently, they were considered to be viruses because of their small size and the difficulty associated with their isolation, particularly since they cannot be grown on synthetic media. *Neisseria gonorrhoeae* was once considered to cause the most prevalent sexually transmitted disease (STD) in both the developed and undeveloping countries. In South Africa, studies conducted from 1979 to 1991, show that 64 96% of urethritis cases were gonococcal in aetiology (Pham-Kanter *et al*, 1996). However, in the last two decades, *Chlamydia trachomatis* has been recognized as a major world health problem not only as the causative agent of trachoma, but also as one of the most common bacterial STDs. In the United States alone, *C. trachomatis* is reported to have an annual incidence of greater than 4 million infections, with the direct and indirect costs exceeding $2.4 billion.

C. trachomatis comprises 18 established serotypes, four of which (L1, L2, L2a, L3) produce lymphogranuloma venereum (LGV), an ulcerative condition associated with lymphadenopathy of the inguinal region. Four serotypes namely (viz.) A, B, Ba, and C, cause trachoma, a chronic inflammatory disease which may lead to conjunctival and corneal scarring. Trachoma is the most common cause of preventable blindness in the world today (Darougar and Jones, 1983; De Sole, 1987; Bailey *et al*, 1989; Piot and Laga, 1989; Loewenthal and Pe'er, 1990). The remaining ten serotypes (D to K) are responsible for causing urethritis, epididymitis, cervicitis, pelvic inflammatory disease (PID), and endometritis complicated by infertility and ectopic pregnancy; they also cause reactive arthritis, ophthalmia neonatorum and infantile pneumonia (Beem and Saxon, 1977; Viswalingam, 1983; Kiviat, *et al*, 1990; Stanley *et al*, 1990; Eley *et al*, 1992; Sands, 1992). The diseases are referred to as oculogenital infections.

Recent studies at various centres in South Africa, have shown that chlamydial infections are on the increase with prevalence rates of 18 34% (Ballard *et al*, 1986; Hoosen *et al*, 1993; O'Farell *et al*, 1994). Co infections of *C. trachomatis* and *Neisseria gonorrhoeae* are also very common. These account for 44-95% of cases where *C. trachomatis* is detected, while its presence in genital ulcer cases ranges between 7% and 12% (Pham-Kanter *et al*, 1996). Syphilis and chancroid have been reported to be the two most common types of sexually transmitted genital ulcer diseases (GUD), with LGV accounting for only 1-7% of GUD in South Africa (Crewe-Brown *et al*, 1982; Coovadia *et al*, 1985; O'Farell *et al*, 1991; O'Farell *et al*, 1994). However, this percentage range is not a true reflection of the frequency of LGV in South Africa, as this ulcerative condition is highly prevalent in some distinct rural and urban regions of the subcontinent such as Kwazulu-Natal, Mpumalanga (previously known as Eastern Transvaal), and Transkei (Pham Kanter *et al*, 1996). Since most centres do not have laboratory facilities which are competent enough to concisely diagnose LGV, underdiagnosis can be expected.

Studies conducted in other parts of Africa like the Gambia, Ethiopia, Kenya, Nigeria, and Zimbabwe, still rank syphilis and chancroid as the most prevalent GUD, and reported LGV to be a minor contributor to GUD with a prevalence of between 0.55% to 8% (Nsanze *et al*, 1981; Mabey *et al*, 1987; Duncan *et al*, 1992; Ghatak, 1992; Tswana *et al*, 1995). However, in one study done in Madagascar, LGV was the second most common GUD isolated with a prevalence of 24%; syphilis was still the most common at 46%, and chancroid third at 16% (Harms *et al*, 1994).

LGV is a chronic disease with a variety of acute and late manifestations and a three stage clinical course (Van Dyck and Piot, 1992). It's primary stage, is manifested as a small, painless genital papule or shallow ulcer of short duration with few symptoms. The most common is the secondary stage characterized by acute inguinal lymphadenitis with bubo formation (inguinal syndrome) and/or acute haemorrhagic proctitis (the anogenitorectal syndrome), together with fever and other symptoms due to the systemic spread of the

infection. Should chlamydia persist, late complications may occur in the anogenital tissue such as ulcers, fistulae and strictures (Schachter and Osoba, 1983; Perine and Osoba, 1984; Piot and Laga, 1989; Van Dyck and Piot, 1992; Chopda *et al*, 1994). In patients who are seropositive for the human immunodeficiency virus (HIV), latent LGV may be reactivated with the development of multiple abscesses in the groins (Latif, 1990; Martin, 1990). At this stage, the response to antichlamydial drugs is poor, and the destruction of inguinal lymphnodes often results in lymphoedema of the genitalia, a chronic condition with persisting suppuration and pyoderma (Greenblatt, 1989).

The increase in chlamydial infections can be attributed to lack of diagnostic facilities and lack of appropriate therapy which leads to continued spread. Undetected infection puts the infected person at risk for serious long term sequelae and sustains transmission within a community. Since symptoms are often mild or absent in the majority of the infected patients, diagnostic testing is the only way to detect infection. Therefore, screening of populations at risk for infection (especially those attending STD clinics), must be a part of STD control and prevention strategies.

The laboratory diagnosis of LGV is usually based on (1) positive chlamydial serology, (2) isolation of *C. trachomatis* from the infected site(s) and/or (3) histological identification of chlamydia in infected tissue (Lefebre *et al*, 1988; Ridgway and Taylor-Robinson, 1991; Taylor Robinson and Thomas, 1991; Centers for Disease Control and Prevention -CDC, 1993). These methods include different staining procedures to demonstrate intracellular inclusion bodies, direct detection of *C. trachomatis* antigens by fluoresceine labelled monoclonal antibodies or by enzyme immunoassay (Tam *et al*, 1984; Stamm and Mardh, 1990; Ridgway and Taylor-Robinson, 1991; Young *et al*, 1991). Although these tests are rapid and simple to perform, none of them show optimal sensitivity and accuracy which is an ongoing quest of chlamydial research. Cell culture is the most sensitive and specific method for diagnosis of *C. trachomatis* and although it is laborious, costly, and requires cell culture facilities unavailable in many laboratories, it still remains the gold standard

(Lefebre *et al*, 1988; Taylor-Robinson, 1991).

With advances in technology with molecular techniques such as polymerase chain reaction (PCR) and ligase chain reaction (LCR), cell culture is no longer being used routinely and large volumes of specimens are now being processed in shorter time and at a low cost (Kellogg, 1989; Claas *et al*, 1990; Ostergaard *et al*, 1990; Frost *et al*, 1993; Chernesky *et al*, 1994; Bassiri *et al*, 1995; Lee *et al*, 1995). Although these assays (PCR, LCR) have higher sensitivities than cell culture or enzyme immnoassays (EIA) due to their ability to detect even the smallest amounts of specific nucleic acid, they also have limitations in that, they cannot differentiate between strains causing LGV and oculogenital infections.

This study proposes to determine the prevalence of LGV amongst patients with genital ulcer disease attending the STD Clinic in Durban; to evaluate available methods for the diagnosis of LGV including an analysis of molecular techniques such as LCR and PCR, and serotype chlamydial isolates from genital ulcers.

CHAPTER 2

REVIEW OF LITERATURE

2.1 HISTORICAL PERSPECTIVE OF CHLAMYDIAE

In the past, the Chlamydia organisms were referred to as: *Bedsonia*, Psittacosis/Lymphogranuloma venereum/Trachoma (PLT) causing agents, *Miyagawanella* and trachoma/inclusion conjunctivitis (TRIC) agents. Due to their unique developmental cycle, they have now been placed in a separate order, *Chlamydiales*, with one family, *Chlamydiaceae*, containing one genus, *Chlamydia* with four species viz. *C. trachomatis*, *C. psittaci*, *C. pneumoniae* and *C. pecorum* (Page, 1968; Fukushi *et al*, 1992).

Trachoma -a blinding eye disease- is one of the oldest recognized human chlamydial diseases having been described in the Ebers papyrus (1500 BC). It has long been known to the ancient Greeks and Romans before its causative agent *C. trachomatis* was discovered. The name is also Greek - $\tau p \alpha x w \mu \alpha$ meaning roughness, and refers to the characteristic conjunctival follicles (Collier and Ridgway, 1989). Common in the Middle East and sub-Saharan Africa, trachoma is believed to have been disseminated to Europe by soldiers returning from military campaigns. It was estimated by the World Health Organization in 1973, that 400 million people had trachoma with blindness occurring in 2 million (Tarizzo, 1973).

Non-gonococcal urethritis (NGU), was described only after Albert Neisser discovered the gonococcus in 1879 (Neisser, 1879; Cited by Goh and Forster, 1993). Initially in the 18th century, *Gonorrhoea virulenta* and *Gonorrhoea simplex* were considered as two entities with the latter being a milder condition, the urethral discharge being mucoid rather than purulent and also having unusual complications. It was when many cases of acute urethritis showed no evidence of gonococci that *urethritis non gonorrhoica* was described.

The aetiology of ophthalmia neonatorum was also poorly understood, though it was known that a connection existed between ophthalmia neonatorum and maternal leucorrhoea. Only when some babies with a milder form of ophthalmia presented with no signs of the usual blinding potential of gonococcal ophthalmia, was *conjuctivite amicrobienne* described by Kroner, in 1884 (Kroner, 1884). The adult syndrome now known as inclusion conjuctivitis, was at that time less well recognized, though it was hypothesised that the eye infection could have been due to contact with non-gonococcal genital discharges.

The invasive biovar of *C. trachomatis*, lymphogranuloma venereum (LGV), which causes the systemic sexually transmitted disease, was described for the first time, as a suppurative inguinal bubo by John Hunter, in 1786. He thought that it could not be syphilitic, but might be "scrofulous". Similar cases were apparently, also reported in 1833 by the Irish surgeon William Wallace who recorded fistula formation and by the French venereologist Phillipe Ricord in 1867 who described it as "bubo d'emblee". However, it was not until 1913 that Durand, Nicolas and Favre established the disease as a clinical and pathological entity (Favre and Hellerstrom, 1954). They called it *lymphogranuloma inguinale*, together with various names (cited: Perine and Osoba, 1984) until eventually, settling for *lymphogranuloma venereum* (LGV).

Combinations of vulval elephantiasis with or without ulceration (esthiomene) and rectal strictures known as the "genito-anal rectal syndrome", which is a late complication of LGV, had been known since the middle of the 19th century, despite the uncertainty surrounding its cause. This disease did not become established as a chlamydial disease until the 1940s, although its diagnosis had been facilitated by the delayed hypersensitivity test introduced by Wilhelm Frei in 1925; he later managed to show that the "genito ano-rectal syndrome" was linked to LGV. The first LGV case report on prepubertal children was reported in 1925 by Weiss and Cain; it involved a 14 year old girl presenting with suppurative inguinal bubo (Weiss and Cain, 1925). However, it was not until 1957 that T'ang *et al* in Peking, succeeded in growing *C. trachomatis* into the yolk of embryonated

hen's eggs (T'ang *et al*, 1957) and this provided additional proof that LGV and other chlamydial diseases were all caused by a similar agent.

The causative agent, *Chlamydia trachomatis* was first observed in stained conjunctival scrapings by Ludwig Halberstaedter (a dermatologist) and Stanislaus von Prowazek (a microbiologist) in 1907 (Halberstaedter and Prowazek, 1909; Becker, 1978; Jones 1983). The diagnostic intracytoplasmic inclusions were recognized first experimentally, in specimens from subhuman primates infected with human trachomatous material. In 1910, Lindner *et al*, described epithelial inclusions in the cervix of the mother of a neonate with atypical inclusion conjunctivitis or "inclusion blennorrhoea" (cited: Jones, 1983). However, the first isolation of *C. trachomatis* from a neonate with non gonococcal ophthalmia and from the cervix of the mother of another such infant, were reported by Jones, Collier and Smith (1959). Lindner, as well as Heyman and Stargardt (Jones, 1983; Cassell *et al*, 1982), proceeded to demonstrate epithelial inclusions in the cervix of a woman whose partner had an inclusion positive NGU. Halberstaedter and von Prowazek called the newly discovered organisms Chlamydozoa (Greek $X \cap AMU\Sigma$, a mantle), because of the blue staining matrix in which the elementary bodies are embedded. These discoveries laid the clinical and epidemiological groundwork for understanding oculogenital chlamydial infection.

Chlamydia researchers found themselves at a dead end due to lack of a sensitive and specific laboratory diagnostic technique especially when the yolk sac method turned out to be good but unsuitable for screening large numbers of specimens. The method is time consuming and labour-intensive, and the necessity to grow chlamydia within cells as for viruses, has precluded many bacteriological and genetical approaches. A breakthrough only came in 1965, when Gordon and Quan introduced cell culture techniques for *C. trachomatis* isolation (Gordon and Quan, 1965; Gordon *et al*, 1969). It was also discovered that, by inhibiting the replication of cells, favours *C. trachomatis* in its competition for ATP with host cells. Methods improved and years later, in 1970, Wang

and Grayston introduced the microimmunofluorescent (Micro IF) test for the detection of antibody and for immunotyping (Wang and Grayston, 1970). These developments led to the elucidation of the wide spectrum of this organism especially in the confirmation of the etiologic role of *C. trachomatis* in NGU.

Psittacosis (Parrot fever), caused by *C. psittaci*, was first described by Ritter (1879) in Switzerland when several cases of unusual pneumonia associated with exposure to tropical birds began to surface (Schaffner, 1990). In 1894, Morange in Paris, investigated an outbreak and concluded that parrots were the source of infection. He named the illness after the Greek word for that bird, *psittakos*. According to Meyer (cited: Meyer, 1942), a pandemic resulted between 1929 and 1930, after large scale importation of infected South American birds to Europe and the United States. In 1930, Bedson managed to isolate the filterable agent from human and avian tissues while Rivers and colleagues, studied the infection in laboratory animals which led to the conclusion that the probable route of human infection is by the upper respiratory tract due to the inhalation of dried excreta of birds shedding the agent.

The designation of the TWAR strain, currently known as *C. pneumoniae*, was derived from laboratory codes of the first two isolates viz. TW 183, isolated in 1965 (during Trachoma studies in Iran and Taiwan) from conjunctival swabs, and AR 39, which was isolated in 1983, in Seattle, United States, from the pharynx of a college student with an upper respiratory infection (Grayston *et al*, 1986; Grayston *et al*, 1989a; Grayston *et al*, 1990). Another atypical strain (strain IOL 207) was isolated in the 1970s, at the Institute of Ophthalmology (London), from an Iranian child with Trachoma (Forsey *et al*, 1986). TWAR was initially considered as a subspecies of *C. psittaci* but subsequent analyses have demonstrated that this organism is distinct from both *C. psittaci* and *C. trachomatis*. TWAR is now recognized as the third species of *Chlamydia* (Grayston *et al*, 1989a). Like *C. trachomatis*, its strains appear to be circulating among humans without an avian reservoir.

2.2 GENERAL CHARACTERISTICS OF THE GENUS *CHLAMYDIA*

The Chlamydiae are small, coccoid, non-motile, gram-negative bacteria which are obligate, intracellular parasites. The special combination of viral and bacterial properties that characterize *Chlamydia* has led to both taxonomic and terminologic confusion. Long classified as large viruses due to their size (200-400 nm in diameter) and their intracellular parasitism (Schachter and Dawson, 1979; Ward, 1983), *Chlamydia* organisms are now considered as a special type of bacteria. They differ from viruses by possessing both DNA and RNA, have cell walls analogous to gram-negative bacteria, are susceptible to antibiotics, possess a number of enzymes, divide by binary fission inside host cells, and contain ribosomes. They cannot synthesize ATP and are therefore considered as energy parasites (Moulder, 1984; Cook and Honeybourne, 1994). Neither muramic acid nor a peptidoglycan layer are present in *Chlamydia* cell walls (Caldwell *et al*, 1981). The structural rigidity of the cells is caused by a n-acetyl glucosamine polypeptide layer, for which construction penicillin-binding proteins are used (Barbour *et al*, 1982). These are similar in location (inner membrane), size, and affinity for the antibiotic to those of bacteria with peptidoglycan in the cell wall (Matsumuto and Manire, 1970).

C. trachomatis consists of three biovars: the trachoma (caused by serotypes A, B, Ba and C), LGV (caused by L1, L2 and L3 serovars), and murine (mouse pneumonitis) biovar. The trachoma biovar also includes serotypes D through K which are responsible for human oculo-genital diseases (Table I). *C. psittaci* which is primarily an animal pathogen, causes atypical pneumonia (Borg) and endocarditis in man. It consists of +/ 120 strains which include 6C (parakeet), psittacosis meningopneumonitis (Cal 10), bovine (EBA-59-795) and ovine chlamydial abortion, feline and ovine pneumonitis, bovine encephalomyelitis, epizootic chlamydiosis, pigeon and turkey ornithosis, and sheep polyarthritis (Becker, 1978; Cassell *et al*, 1982; Barnes, 1989). The newly recognized species *C. pneumoniae* (TWAR strain), causes human respiratory disease (Grayston *et al*, 1993) and has drawn much attention due to difficulties involved in its diagnosis. *C. pecorum* on the other hand, is reported to cause diseases such as encephalomyelitis, polyarthritis, pneumoniae, and

enteritis in cattle and sheep. *C. pecorum* type strain is strain Bo/E58 (=ATCC VR628), which was isolated in 1953 from the brain of a calf that had sporadic bovine encephalomyelitis. The isolated organism was subsequently identified as a member of the genus *Chlamydia* (Wenner *et al*, 1953). Other reports available on this species, involve information on monoclonal antibodies used for micro-immunofluorescence typing for its serovars, together with those of other *Chlamydia* species (Fukushi *et al*, 1992; Kuroda-Kitagawa *et al*, 1993).

C. trachomatis strains are sensitive to sulphonamides, produce a single round inclusion that displaces the nucleus of host cell, and also stains with iodine due to the presence of glycogen. *C. psittaci* and strains are resistant to sulphonamides, produce glycogen-negative inclusions that rupture early distributing themselves around the host cell nucleus without displacing it (Collier, 1989). *C. pneumoniae* has less than 10% DNA homology with the other species, produce glycogen-negative inclusions with typically pear-shaped elementary bodies. *C. pneumoniae* strains are also immunologically distinct, lacking cross reactivity when exposed to monoclonal antibodies to strains of *Chlamydia* (Campbell *et al*, 1987). Table I shows some characteristics of *Chlamydia* species.

2.3 CELL MORPHOLOGY AND DEVELOPMENTAL CYCLE

The interaction of Chlamydiae with the host is complex, parasitic and most often, results in cell destruction. Chlamydiae are highly pleomorphic and share a general sequence of events in their reproduction. They exist in two distinct forms: the infectious, non dividing elementary body (EB) which is a "spore like" structure adapted for extracellular survival and transit between host cells, and the non infectious reticulate body (RB) or the "initial body" that divides intracellularly by binary fission (Pearlman and McNeeley, 1992; Mathews *et al*, 1993). The EBs are spherical cocci of about 300-400 nm in diameter; they contain electron dense nuclear material with numerous ribosomes surrounded by a rigid trilaminar wall. The RBs are larger forms of 800 1000 nm in diameter, have less dense, fibrillar nuclear material, more ribosomes and non rigid trilaminar walls (Becker, 1978; Moulder, 1991).

The developmental cycle begins with the infection of the susceptible host cell by the EB, through sexual contact or direct exchange of body fluid (Dunlop *et al*, 1972; Schachter *et al*, 1975; Grayston *et al*, 1985). Evidence suggests the involvement of the chlamydial adhesins in promoting chlamydial attachment and of the host receptor, in promoting chlamydial adhesion. After attachment at a specific site, the EB enters the host cell inside a phagosome. *Chlamydia* do not enter the cytoplasm of the host cell, but remain and multiply within the phagocytic vacuole which is somehow protected from fusion with lysosomes (Bowie and Holmes, 1990; Zhang and Stephens, 1992), and which eventually expands to fill much of the cytoplasmic volume of the cell. The rate of attachment and phagocytosis depends on both the infecting strain and the mode of presentation to the host cell (Ward and Clarke, 1990).

The reproductive cycle usually takes 30-48 h, but can take as long as 96 h in a less hospitable atmosphere. During the first 6 8 h after infection, the EB reorganizes into RB. Then approximately 8-10 h after infection, RB starts to divide by binary fission within the

membrane-bound vacuole. From 10 20 h post infection, RBs can be found at various stages of division viz. EBs, intermediate forms and RBs. At 18-24 h after infection, while the RBs continue to divide and to reorganize, the size of the individual bodies diminishes and the RBs reorganize themselves into large numbers of EBs by a poorly understood reorganization or condensation process. Towards the end of the cycle, an inclusion may contain hundreds of EBs filling almost the entire cytoplasm of the host cell. Thus, approximately 48-72 h post infection, depending on the strain, the mature infectious EBs are released from the host cell by a phenomenon similar to exocytosis (Todd and Caldwell, 1985) to initiate another cycle of infection. Due to high multiplicity and large size of the inclusions, the host cell may burst and die (Moulder, 1991). The yield of EBs per host cell varies from 10 to 1000, depending again on both the chlamydial strain and the host cell and among other factors, the culture conditions. Figure 1, illustrates the development cycle of the Chlamydiae.

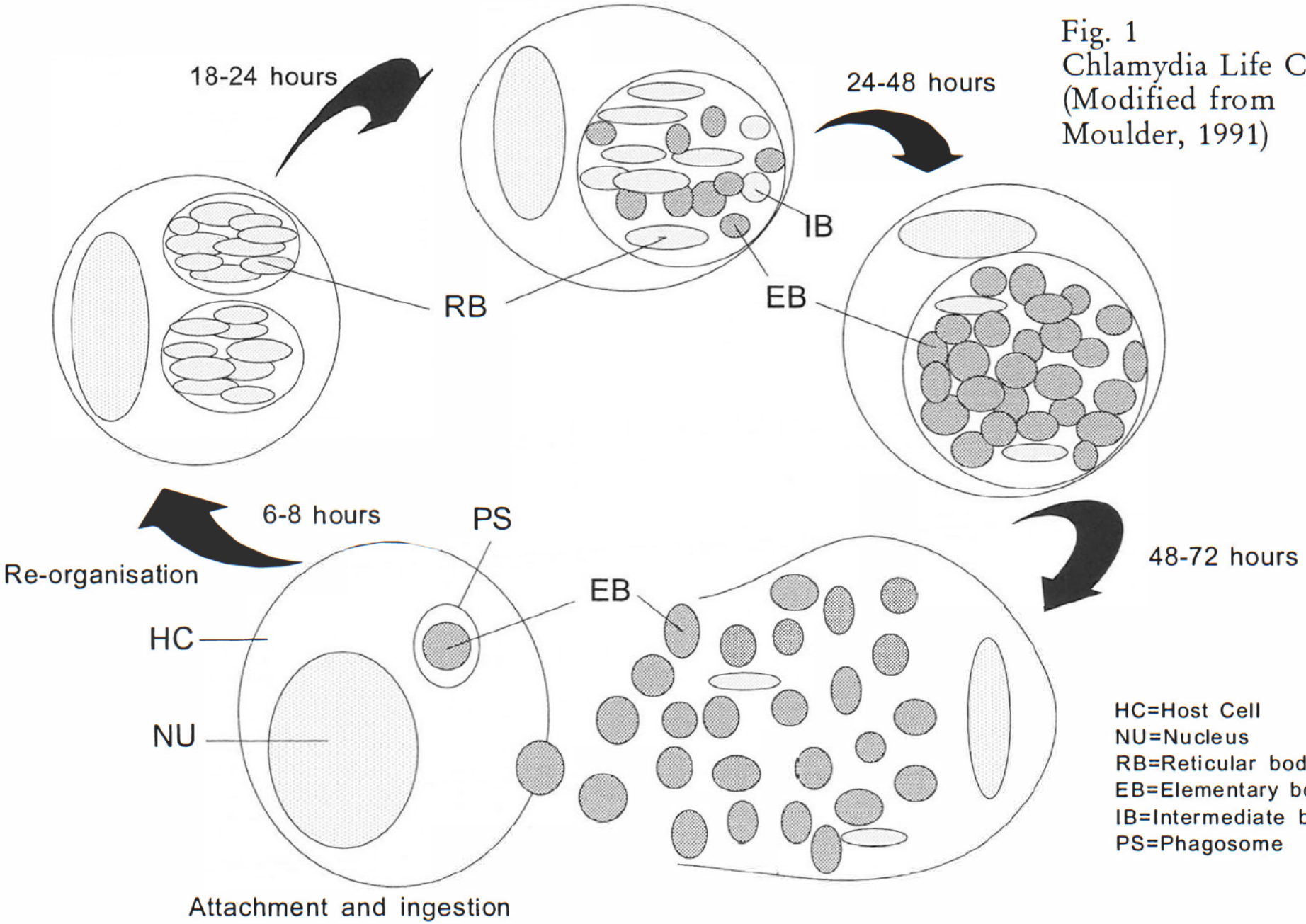


Fig. 1
Chlamydia Life Cycle
(Modified from
Moulder, 1991)

2.4 MOLECULAR BIOLOGY OF CHLAMYDIAE

2.4.1 Genetics

The Chlamydiae have relatively small genomes compared to other prokaryotes. The genome of *C. trachomatis* is composed of circular double-stranded deoxyribonucleic acid (DNA) with a molecular weight (M_r) of approx. 6.6 X 10^8 daltons (11 X 10^5 nucleotide pairs) (Becker, 1978; Moulder, 1984a). The Guanine-plus-cytosine content (mol% G+C) of the DNA is 42.5% (Tm). This mol % G+C composition is determined by thermal denaturation of the DNA of a number of chlamydial strains and has been found to range from 41 to 44 mol%, with *C. psittaci* strains at the lower end of a continuous spectrum of values while *C. trachomatis* biovar trachoma strains are at the higher end (Moulder *et al*, 1984b). Tm is the melting temperature of DNA at which absorbance A_{260} is half complete. This Tm, increases with every increase in percentage Guanine+cytosine (% G+C) due to the relative number of hydrogen bonds in G+C as compared to Thymine and Adenine (A+T) pairs (Freifelder, 1986).

Data derived from DNA homology studies have shown that of the presently recognized four species, only the most recently acknowledged member, *C. pneumoniae*, is a single species having 60-70% DNA/DNA homology (Storey *et al*, 1993). Only 10-30% DNA relatedness has been found between *C.trachomatis* and *C. psittaci* by the thermostability test, which is a thermal denaturation measurement (Kingsbury and Weiss, 1968; Moulder, 1991). Storey *et al* (1993), described the characterization and taxonomic status of N16, a *C. pneumoniae* strain isolated from the respiratory tract of a horse. This strain of equine origin, shows 94.5% and 94.4% DNA homology with TWAR isolates IOL-207 and AR-39 respectively, and 70.9% to 71.1% homology with *C. pecorum*. Thus, DNA homology and monoclonal antibody data from this study, suggest that horse Chlamydiae, as exemplified by N16, form a new second biovar of *C. pneumoniae*.

However, the intraspecies homology among strains of *Chlamydia* is near 100%, even though the mouse pneumonitis biovar (MoPn) of *C. trachomatis* displays 30 70% homology to other human strains of that species (Weiss *et al*, 1970; Moulder *et al*, 1984a; Moulder *et al*, 1984b). There is complete DNA homology between the trachoma and LGV biovars in all strains tested.

Electron micrographs of chlamydiae inside host cells show that RBs have many more ribosomes than EBs do. The EB has equal DNA and RNA content whereas RB contains three to four times more RNA than DNA. The *Chlamydiae* also contain 23S, 16S, and 5S ribosomal RNA (rRNA) as 50S and 30S subunits. The 16S rRNA sequences of *C. trachomatis* LGV Biovar, *C. pneumoniae* 6BC strain and *C. psittaci* strains differ by 5% (thus exhibiting greater than 94% homology) and have been found to be extremely different from the sequences (16S rRNA) of the other known eubacteria (Cox *et al*, 1988; Gaydos *et al*, 1993). *C. trachomatis* also has two rRNA operons that differ from other prokaryotes (Engel and Ganem, 1987).

Plasmids of 4.4 X 10^6 daltons (7.5 kilobases kb) has been described (Peterson and de la Maza, 1983). These plasmids were first identified in *Chlamydia* in 1980 by Lovett *et al*, and it was originally believed that all chlamydial isolates possessed a plasmid but, according to Campbell *et al* (1987) and McClenagan *et al* (1988), certain types of *C. psittaci* and *C. pneumoniae*, lack this plasmid. *C. pneumoniae* of equine origin does contain a typical 7.5 kb plasmid, although it is absent in the human isolates (Storey *et al*, 1993). *Chlamydia* plasmids are "cryptic", meaning that they convey no discernible phenotypes. On the other hand, Moulder *et al* (1984a,b), have reported on a 4 X 10^6 dalton extra-chromosomal DNA which is common to both *C. trachomatis* and *C. psittaci* thus, still confirming the lack of plasmids in *C. pneumoniae* strains. The mouse pneumonitis (MoPn) biovar is known to carry a plasmid (Palmer and Falkow, 1986) which apparently does not

cross hybridize with the plasmid from human strains. Restriction endonuclease analysis has shown that plasmids from different trachoma biovars are very similar and closely related to those of the LGV biovar. However, the *C. psittaci* plasmid has been found to be different from those of *C. trachomatis* (Stephens, 1990). Several studies including the one reported by Palmer and Falkow (1986), have found that, there are ten times (10X) more copies of plasmid per *Chlamydia* chromosome equivalent. According to Comanducci *et al*, (1988, 1990), this plasmid could be required for growth within mammalian cells and might therefore play a role in *C. trachomatis* pathogenicity.

Genes encoding heat shock proteins (HSPs) have been characterized. These include among others, genes encoding the genus-specific 57 kDa antigen, and the Dna-K homologue which has been cloned from all three species (Danilitton *et al*, 1990; Kornak *et al*, 1991). Gene cloning and sequence analysis of the gene encoding the 57-kDa protein revealed that this protein was a member of the family of widely conserved 60-kDa HSPs (hsp60), and also to be closely related to the groEL protein of *Escherichia coli* (Morrison *et al*, 1989a). This sarkosyl soluble hsp60 (groEL) has been associated with a delayed-type hypersensitivity response in chronic infections (Morrison *et al*, 1989b). Antibodies against the 75-kDa HSP (Dna-K) have been reported to neutralize infectivity even though the antigen does not elicit any delayed hypersensitivity activity (Maclean *et al*, 1988; Taylor *et al*, 1990). The groEL homologue of *C. pneumoniae* shows a 95 and 97% amino acid sequence similarity to *C. trachomatis* and *C. psittaci* homologs, respectively. Its Dna-K homologue on the other hand, shares only 87% amino acid similarity with the *C. trachomatis* protein.

2.4.2 Antigenic Composition

The main constituents of the chlamydial outer membrane complex (COMP) are three cysteine rich proteins (CrPs) of M_r 40, 60 and 12 kilodaltons (kDa) together with lipopolisaccharide (LPS). The 40-kDa component is the major outer membrane protein (MOMP) comprising 60% of the membrane protein and has a highly immunogenic species specific epitope (Caldwell *et al*, 1981). This protein (MOMP), has been a major focus of attempts to develop a chlamydial vaccine and more is known about its genetic structure and immunochemistry than of any other chlamydial antigen. The MOMP encoding gene, termed *omp1* or *ompA* for all the *C. trachomatis* serovars have been completely determined (Dean *et al*, 1991; Hayes *et al*, 1990; Lampe *et al*, 1993; Stephens *et al*, 1986; Stephens *et al*, 1987; Yuan *et al*, 1989). MOMP sequences are also currently available for five strains of *C. psittaci* and for two strains of *C. pneumoniae* (Carter *et al*, 1991). Campbell *et al* (1990), described a 39.5 kDa MOMP protein in all Chlamydiae, but others have found this protein to be cross reactive among chlamydial species (Campbell *et al*, 1990; Freidank, 1993; Iijima *et al*, 1994).

MOMP is believed to provide structural integrity to the chlamydial cell wall by extensive disulphide bond cross-linking (Newhall and Jones, 1983; Carter *et al*, 1991). This protein also has porin functions i.e has pore forming capabilities that permit exchange of nutrients for the reticulate body form (Bavoil *et al*, 1984). Monoclonal antibodies (MAbs) have been described which define neutralizing epitopes, suggesting that proteins encoded by *omp1* may serve as an adhesin (Su and Caldwell, 1991). Analysis of several MOMP genes have shown that the MOMP gene encodes for nine different amino acid sequences called regions. These consist of five highly conserved regions that do not vary from serovar to serovar, and four variable domains (VDI through VDIV) that contain serovar specific epitopes (Stephens *et al*, 1987; Lampe *et al*, 1993). The four VDs are presumably arrayed on the monomer face. Gene sequence studies indicate that this diversity

is due to clustered nucleotide substitutions for closely related serovars and insertions and deletions for distantly related serovars (Stephens *et al*, 1987).

The other major components of the COMP are the 60 kDa and 12 kDa CrPs with determinants that are biovar and species specific (Zhang *et al*, 1987). The 60 kDa protein appears as a doublet in the LGV biovar, whereas only a single band on polyacrylamide gel has been detected in the trachoma biovar (Batteiger *et al*, 1985). Unlike MOMP, which is present in RB, these two proteins are reported to be developmentally regulated and to play a role in the condensation process which accompanies the transition from RB to EB (Sardinia *et al*, 1988). Evidence by Newhall (1987), suggests the involvement of MOMP MOMP, MOMP 60 kDa and 12-12 kDa links in conferring structural rigidity of the EB. These CrPs viz. MOMP, 60 kDa and 12 kDa, are also similar in M_r and structure in all *Chlamydia* species (Perez *et al*, 1993). A 98-kDa CrP has also been described; however, it could be detected only in the outer membrane complex of *C. pneumoniae*. This finding led to the idea that its presence might have a role in providing more rigidity to the membrane structure in order to sustain the pear shaped morphology that is unique to *C. pneumoniae* strains.

All chlamydiae possess LPS, another major constituent of the COMP. It is a heat stable, lipoprotein carbohydrate complex with an immunodominant 2 keto 3 deoxyoctanoic acid as the reactive moeity. It contains genus specific epitopes (absent in other Gram negative bacterium) used for both sero diagnosis and antibody based chlamydia detection tests viz. Complement fixation and immunofluorescence. Although LPS antigens are surface exposed, they are incapable of stimulating the production of neutralizing antibodies as do MOMP antigens. Chemical cross linking studies show that MOMP lies within 10 Angstroms (A) of LPS molecules (Birkelund *et al*, 1988). Monoclonal antibodies and monospecific polyvalent antisera to this LPS, have been used in enzyme

Table I Differentiation and characteristics of the species and biovars of the genus *Chlamydia*
(Modified from Bergey's manual of systematic bacteriology, Vol.1 1984)

Characteristics	species and biovar				
	C. trachomatis			*C. psittaci*	*C. pneumoniae*
	Trachoma	LGV	Mouse		
MOMP 40 000 MW	+	+	N/A	+	+
Folate biosynthesis	+	+	+	-	-
Net synthesis of ATP	-	-	-	-	-
Mol % G+C of DNA	44.0	41.9	42.9	41.3	40.0
DNA, molecular weight (10^6)	N/A	660	N/A	500	N/A
% DNA homology to Trachoma biovar	100	100	30-60	10	10
% DNA homology to TWAR	10	10	N/A	10	100
Presence of 4 X 10^6 dalton plasmid	+	+	N/A	+[a]	+[b]
Genus specific antigen (demonstrated by CF)	+	+	+	+	+
Type specific antigens (demonstrated by Micro IF)	A K[c]	L1 L3	N/A	N/A	N/A
Number of serovars	14	4	N/A	N/A	1
Natural Infections (host)	Humans	Humans	Mice	Birds, Lower mammals	Humans
Preferred site of infection	Squamo columnar epithelial cells (oculo genital and respiratory)	Lymph nodes	Lungs	Multiple	Squamo columnar (respiratory)
Inclusion morphology	Oval, vacuolar	Oval, vacuolar	Oval, vacuolar	Variable shape, dense	Oval, dense
Elementary body morphology	Round	Round	Round	Round	Pearshaped
Glycogen in inclusions	+	+	+	-	-
Mouse lethality intracerebrally	-	+	-	+	-
Follicular conjunctivitis in lower primates	+	-	-	-	-
Cell Culture Enhancement By: Centrifugation of inoculum onto monolayer	+	-	-	-	+
Effect of treatment of host cells with DEAE dextran	+	weak	-	weak	weak
Plaques in mouse fibroblasts (L cells)	-	+	+	+	N/A

N/A data not available
Some strains do not contain plasmids:[a]Campbell et al, 1987; [b]Storey et al, 1993.
[c]Sexually transmitted *C. trachomatis* serovars D K are within this group

immunoassay (EIA) systems for the detection of chlamydial antigen in clinical specimens (Pugh *et al*, 1985; Howard *et al*, 1986).

Since the introduction of the micro immunofluorescence (MIF) technique for the detection of antibody and for immunotyping by Wang and Grayston in 1970 (Wang and Grayston, 1970), 18 known serovars of *C. trachomatis* have been identified including three recently found serovars, Da, Ia, and L_2a (Wang and Grayston, 1991). These were defined by using polyvalent antisera (Wang and Grayston, 1971) and monoclonal antibodies (Wang *et al*, 1985). *C. trachomatis* can be divided further into subspecies (serogroups) and serovars (serotypes). Serovars are each designated by a letter (A to K; and L1 to L3), and are distinguished by both the antigenic variation in the MOMP and the clinical disease they cause. Serovars are also grouped into three separate biovars: Trachoma Biovar, which include types A, B, Ba, and C frequently isolated from patients with ocular disease in areas of endemic trachoma (Fig. 2). Types D, E, F, G, H, I, J, and K, are primarily isolated from genital infections or neonatal infections acquired from the mother's cervix during birth, but they also cause ocular infections in adults. Most non LGV genital infections ($\sim$70%) are caused by serovars D, E, F, and G (Kuo *et al*, 1983). Types L1, L2, and L3 are isolated almost exclusively from LGV patients (Schachter and Meyer, 1969). The remaining biovar contains a single serovar, viz. the MoPn, which is involved in murine infections.

The 18 serotypes, are further grouped into three subspecies (serogroups). Each serogroup corresponds to one of the three *C. trachomatis* complexes as defined by Yuan *et al* (1989) from the homologies of the MOMP amino-acid sequences (Table II). Within each group, the serovars can be arranged in a hierarchy of antigenic complexity (Wang and Grayston, 1971). MOMP M_rs also differ among serovars, with MOMPs of B Complex serovars having lower M_rs than those of the C Complex (Newhall *et al*, 1982). The first type, B-Complex includes the L1, L2,

L2a, E, D, Da, B, and Ba serovars. The second type, the F Complex includes serovars F and G; while the last type, the C-Complex, includes serovars A, C, J, K, H, I, Ia, and L3.

Table II Complexes and serotypes of *C. trachomatis*

Complex	Serovars
B complex	L2, L2a, L1, E, D, Da, B, Ba
F complex	F and G
C complex	A, C, J, K, H, I, Ia, L3

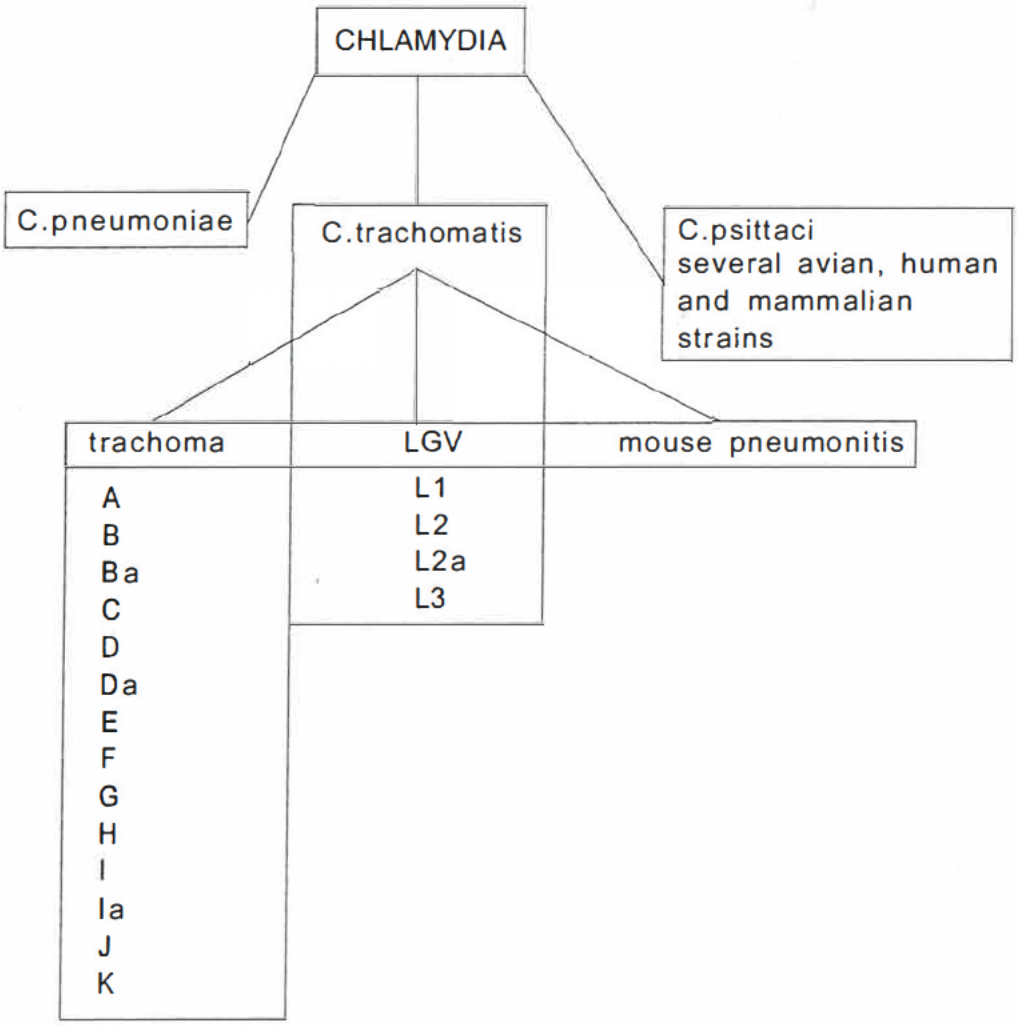


Fig. 2 Schematic representation showing the taxonomic divisions of the genus *Chlamydia* and the biovars of *C. trachomatis*

2.5 DISEASE SYNDROMES ASSOCIATED WITH *CHLAMYDIA*

Chlamydial infections are a host of clinical syndromes caused by infection with any of the various chlamydial serotypes. These infections are difficult to diagnose, with most being asymptomatic. Untreated asymptomatic infection may lead to serious sequelae, but if detected early, these sequelae can be eliminated by treatment with adequate doses of antichlamydial drugs. Chlamydial infections are prevalent among adolescents and young adults regardless of their demographic background. The association of the organisms with different diseases are listed in Table III.

Table III Clinical spectrum of *Chlamydia*

SPECIES	SEROTYPE	DISEASE
C. psittaci	Many unidentified serotypes	Psittacosis, ornithosis, Abortion, Endocarditis,
C. pneumoniae (TWAR)	Not known	Community-acquired pneumonia, bronchitis, pharyngitis, sinusitis, ?coronary artery disease
C. trachomatis	A, B, Ba, C	Hyperendemic trachoma
C. trachomatis	L1, L2, L2a, L3	Lymphogranuloma venereum (LGV)
C. trachomatis	D,E,F,G,H,I,Ia,J,K	
Male		Nongonococcal urethritis, Postgonococcal urethritis, Conjunctivitis, Proctitis, Reiter's syndrome, epididymitis, Sexually acquired arthritis (SARA)
Female		Mucopurulent cervicitis, Proctitis, Urethritis, Conjunctivitis, Salpingitis, Perihepatitis, Ectopic pregnancy, Endometritis, Infertility
Neonates		Inclusion conjunctivitis, Pneumonia, Otitis media, Rhinitis, Proctitis, Pharyngitis, Premature birth, ? Stillbirth

2.5.1 *Chlamydia psittaci* Infections

The distribution of *C. psittaci* infections is worldwide and virtually any species of bird can serve as host for the organism. Over 120 different species have been found to harbour the organism. *C. psittaci* is a common avian pathogen that causes sporadic cases of human ornithosis and psittacosis, feline pneumonitis and bovine abortion (Schachter and Dawson, 1979; Cassell *et al*, 1982; Yung and Grayston, 1988). Human psittacosis is a zoonosis, meaning that, it is a disease in nature communicable to man.

The organism is shed in the faeces of the birds, contaminating the environment and getting spread by aerosol. In humans, the lung is usually the organ involved most prominently. The organism enters the body via the respiratory tract and is transported to the reticuloendothelial cells of the liver and spleen which, due to infection, becomes enlarged thus, causing focal necrosis. *C. psittaci* infections (85%) have been associated with exposure to infected avian species (Schachter and Dawson, 1979; Yung and Grayston, 1988; Verweij *et al*, 1995). Person-to-person transmission has also been suggested; Bourke *et al* reported on a case involving one family member who had been exposed to pet birds and transmission of the organism to other unexposed family members (Bourke *et al*, 1989).

Diagnosis is dependent on complement fixation (4-fold antibody rise) tests and chest x-ray results. The clinical course of psittacotic infection can vary widely, with the incubation period ranging from 5-15 days or even longer (Schachter and Dawson, 1979; Cassell *et al*, 1991; Cunha, 1991). Infection is abrupt in onset, starting with chills and fever (38-40.5^{0}C) over a 2-4 day period. Other symptoms include a severe headache, malaise, anorexia, painful myalgia (particularly of the back and neck), arthralgia, and Horder's spots (a pale macular rash). A persistent non-productive cough is prominent with complications such as consolidation and

crepitations in the lung, and evidence of hepatitis (Yung and Grayston, 1988; Bourke *et al*, 1989; Cunha, 1991). Severe pneumonia, respiratory and multi-organ failure may occur in severe cases. Overall mortality is estimated to be about 0.7% and a poor prognosis has often been associated with the elderly and those with leukopenia, renal impairment and multilobular involvement (Cassell *et al*, 1982; Verweij *et al*, 1995). *C. psittaci* has also been described as a pathogen in infectious endocarditis, as well as myocarditis and pericarditis; a possible involvement in myocardial infarction has also been suggested (Odeh and Oliven, 1992).

C. psittaci has also been implicated in human abortions. In the United Kingdom (UK) and France, pregnant women (mostly farmers' wives) have been reported to have aborted after exposure to *Chlamydia* infected sheep during the lambing season (Roberts *et al*, 1982; Johnson *et al*, 1985). These women also exhibited antibody responses and chlamydiae were isolated from their placental samples. This aetiological association was confirmed by DNA restriction analysis, which showed that the strains isolated from human foetuses and placentae, were of ovine origin (Herring *et al*, 1987).

2.5.2 *Chlamydia pneumoniae* Infections

Being a newly described species, *C. pneumoniae* has received considerable attention in the past decade. Much of the knowledge on this organism has been derived from serologic studies using the *C. pneumoniae*-specific micro-immunofluorescence (MIF) test. *C. pneumoniae* seems to be a primary human pathogen, and attempts to identify zoonotic reservoirs have been unsuccessful. It appears to have a worldwide distribution, with the antibody prevalence being higher in tropical countries than colder climates (Forsey *et al*, 1986; Wang and Grayston, 1990). The mode of transmission remains uncertain, though it is believed to be from

person to person via respiratory secretions; reports on the acquisition of infection by droplet aerosol during a laboratory accident supports the above suggestion (Kleemola *et al*, 1988; Mordhorst *et al*, 1992; Theunissen *et al*, 1993). The incubation period is several weeks, which is longer than that for other respiratory pathogens (Kishimoto *et al*, 1994; Mordhorst *et al*, 1994).

Infection is most common among school-age children and very rare in children less than 5 years of age (Saikku *et al*, 1988; Wang and Grayston, 1990; Haidl *et al*, 1994; Jantos *et al*, 1995), though data varies with geographic location. Seroconversion occurs from age 5 through 14 years, being accelerated through adolescence, slowing down in the older age group (Wang and Grayston, 1990). Seroprevalence is almost equal in both sexes under 15 years of age; however, due to other factors (smoking is also implicated), antibody titers are more markedly elevated in adult males than in adult females (Marrie *et al*, 1987; Wang and Grayston, 1990; Mendall *et al*, 1995).

The spectrum of diseases associated with *C. pneumoniae* is expanding, with most infections being mild or asymptomatic. *C. pneumoniae* is reported to cause an average of 10% of community acquired pneumonia cases and 5% of bronchitis and sinusitis cases (Marrie *et al*, 1987; Grayston *et al*, 1989c). Studies have shown that only 10% of these infections result in clinically apparent pneumonia (Saikku, 1992a; Grayston, 1992). *C. pneumoniae* causes acute respiratory disease associated with a non-productive or mildly productive cough, pyrexia, headache, sinusitis and pharyngitis (Grayston *et al*, 1986; Marrie *et al*, 1987; Grayston *et al*, 1989b; Grayston, 1992; Grayston *et al*, 1993; Pruckl *et al*, 1995). Initial reports emphasized atypical pneumonia clinically resembling that associated with *Mycoplasma pneumoniae* (Grayston *et al*, 1986); however, pneumonia caused by *C. pneumoniae* cannot be clinically differentiated from other pneumonias.

The role of *C. pneumoniae* as an opportunistic pathogen among immunocompromised persons has not been well defined. The organism has been successfully isolated from the lungs of human immunodeficiency virus (HIV) infected patients (Augenbraun *et al*, 1991) and is believed to be responsible for about 20% of respiratory infections as identified by polymerase chain reaction-enzyme immunoassay (PCR EIA) and serology (Gaydos *et al*, 1993). Seroepidemiologic studies have shown evidence of chronic infection due to *C. pneumoniae* in the elderly, smokers and in patients with chronic obstructive pulmonary disease. Complications associated with the disease include myocarditis associated with sudden death (Wessleri *et al*, 1992), Guillain Barre Syndrome in a child (Haidl *et al*, 1992) and with atherosclerosis (Thom *et al*, 1991; Saikku *et al*, 1992b; Kuo *et al*, 1993; Poulakkainen *et al*, 1993; Mendall *et al*, 1995). *C. pneumoniae* has been reported in cases with reactive arthritis and Reiter's Syndrome (Braun *et al*, 1994).

Evidence of the presence of *C. pneumoniae* in atheromatous plaques has been obtained by electron microscopic studies, immunocytochemical staining and PCR testing of coronary atheroma (Kuo *et al*, 1993). Then Campbell *et al*, proceeded to demonstrate that 33% of patients with primary lesions and 86% of patients with re-stenosis had chlamydial DNA associated with plaque formation (Campbell *et al*, 1995). However, Weiss *et al*, were unsuccessful; they failed to detect the organism in atheromas by culture, electron microscopy or PCR (Weiss *et al*, 1994). Even though these studies show the association of *C. pneumoniae* with atheromatous plaques, the role of *C. pneumoniae* infection in the pathogenesis of atherosclerosis is still unknown.

2.5.3 *Chlamydia trachomatis* Infections

Chlamydia trachomatis is amongst the most prevalent bacterial pathogens. Its preferred site of infection is primarily, epithelial surfaces in the eye and genital tract. It is also a major cause of reproductive tract morbidity throughout the world, particularly in women. *C. trachomatis* also causes ocular infections, the most common being trachoma. Trachoma is the commonest infectious cause of blindness affecting about 500 million people, of whom 7 million become blind (Dawson *et al*, 1981).

Chlamydial infections are difficult to diagnose and require very expensive screening programmes to detect and eradicate. Urinary tract infections caused by *C. trachomatis* are much more commonly symptomatic in men than in women. Both gonorrheal urethritis and *C. trachomatis* urethritis may cause "sterile" pyuria, and both infections may be asymptomatic. Infections in males include among others non gonococcal urethritis, post-gonococcal urethritis and epididymitis (Storey *et al*, 1987).

Chlamydial genital infections in women are important because of their frequency and their consequences. They are mostly asymptomatic and may go undetected for a long time. The recovery of *C. trachomatis* from the cervix of sexually active women ranges from 5 35%, depending on the characteristics of the population. Risk factors for genital chlamydial infections include young age ($\leq$24 years), single marital status, concomitant STDs, multiple sexual partners, history of new partner,and use of nonbarrier or oral contraceptives. (Handsfield *et al*, 1986; Wölner-Hanssen *et al*, 1990). *C. trachomatis* causes a spectrum of genital infections in women; clinical manifestations of chlamydial lower genital tract include postcoital bleeding or induced mucosal bleeding, mucopurulent cervical discharge, acute urethral syndrome, acute Bartholinitis, and proctitis.

Transmission of chlamydial infections from mother to baby occurs when a baby passes through the birth canal of a mother with cervical chlamydial infection. These babies are at a higher risk of being infected or colonized with the organism, than babies born through caesarian section. Clinical manifestations include inclusion conjunctivitis seen in 30-40% of babies, and pneumonia, occurring in 10 20% of these babies. Apart from the eyes and respiratory tract, infection at various other sites have been documented viz. the middle ear, nasopharynx, pharynx, rectum and vagina. These infections remain asymptomatic until 6-7 months post-partum due to the suppressive effect of maternal antibody (Hobson *et al*, 1983).

2.5.3.1 Trachoma

Trachoma is the commonest cause of ocular morbidity and preventable blindness in the world (Darougar and Jones, 1983). It is endemic in tropical and subtropical countries, with North Africa, Middle East and northern parts of the Indian subcontinent, affected the most. Studies conducted in Tunisia (Hanna *et al*, 1976), Ethiopia (De Sole, 1987; De Sole and Martel, 1988), Gambia (Bailey *et al*, 1989), and in Kenya (Loewenthal and Pe'er, 1990), show that trachoma is still a major cause of blindness in most parts of Africa, with the prevalence reaching 45.1%. It is also prevalent in the northern regions of Transvaal (now called Northern Province) in South Africa (Ballard *et al*, 1983; Bucher and Ijsselmuiden, 1988; Scott, 1993), but there are no reports of this disease in Kwazulu Natal (KZN) province. Trachoma is caused by serovars A, B, Ba and C of *C. trachomatis*, and can be spread from eye to eye, affecting both children and adults. It may begin as a mucopurulent conjunctivitis and is often complicated by secondary bacterial infection. The incubation period could vary from 2 14 days but, it is difficult to establish the date of contact. Trachoma is defined as a chronic keratoconjunctivitis characterized by follicles, palpebral conjunctival scarring and pannus keratitis with scarring of the cornea; other symptoms include mucopurulent discharge, swelling of eyelids, redness, irritation and itching. The blinding consequences of trachoma

are caused by conjunctival scarring that in turn leads to trichiasis (inturned eyelashes), then corneal scarring. The degree of scarring increases with the duration and severity of the conjunctival inflammatory disease (Dawson *et al*, 1989; Dawson *et al*, 1990).

2.5.3.2 Inclusion Conjunctivitis

Inclusion conjunctivitis infections affect both adults and newborn babies. These infections are caused by *C. trachomatis* oculogenital serovars (D through K), and are acquired by direct contact between eyes of normal individuals and material (including genital secretions) from infected individuals. The incubation period of adult conjunctivitis is generally 1-2 weeks. The disease is manifested acutely with foreign body sensation, photophobia, mucopurulent discharge, and follicular conjunctivitis often with keratitis. The follicles, unlike those of Trachoma, are more pronounced in the lower than the upper lid. Pannus (a membrane like structure produced by superficial vascularization of the cornea with infiltration of granulation tissue) is unusual but well documented (Viswallingam *et al*, 1983). In the absence of reinfection, the lesions tend to heal over a period of several months to 2 years.

Chlamydial eye infection in neonates, is more common than gonococcal ophthalmia in the United States. Neonatal inclusion conjunctivitis is usually subclinical with the symptoms appearing within 1 week post partum. The disease is characterized by an acute copious mucopurulent discharge, inflammation or oedema of the eyelids, and conjunctival erythema. If left untreated, the disease tends to resolve over 2-3 months but, may later result in conjunctival scarring. Complications are seen mostly in untreated infants.

2.5.3.3 Neonatal Pneumonia

The possible involvement of *C. trachomatis* in causing afebrile pneumonitis was first suggested by Schachter *et al.* in 1975, then Beem and Saxon (1977) proceeded to characterize the syndrome even further (Schachter *et al*, 1975; Beem and Saxon, 1977). The onset is gradual, ranging from 2-14 weeks after delivery. Antecedent or concomitant conjunctivitis is frequently noted. Initial manifestation is rhinitis, followed by a staccato cough and tachypnea. Other symptoms include malaise, cyanosis, and poor weight gain. Evidence that *C. trachomatis* is a cause of pneumonia in the newborn include among others, the recovery of the organism from neonatal lung specimens. Chest x-rays reveal hyperexpansion with symmetrical diffuse interstitial and patchy alveolar infiltrates. Laboratory findings include eosinophilia and elevated serum immunoglobulin levels. Serous otitis media is reported as a complication of pneumonia but may also occur independently. The diagnosis is confirmed by culture, antigen detection of a pharyngeal aspirate, or the appearance of IgM antibodies to *C. trachomatis*. Left untreated, the disease runs a benign course, leading to apparent recovery.

2.5.3.4 Genital Infections in the Male

2.5.3.4.1 Non-gonococcal Urethritis

Non-gonococcal urethritis (NGU) is the most common STD in men in industrialised countries, with up to 50-60% of the cases caused by *C. trachomatis* (Holmes *et al*, 1975; Felman and Nikitas, 1981). In South Africa, *N. gonorrhoeae* is still the leading cause of urethritis in adult males with a prevalence of 70-80%. In our own study on adult male urethritis in Durban, *C. trachomatis* was responsible for 28% of the NGU cases (Small and Maleka, 1994). There is no clinical difference between chlamydial and non-chlamydial NGU. The incubation period is between 1 and 3 weeks; by which time patients complain of dysuria and urethral irritation,

and on investigation, a mucoid or mucopurulent urethral discharge is often found. Chlamydial infections may be symptomless even though mild erythema or any signs of urethritis may be present. A presumptive diagnosis of NGU is defined as >4 polymorphonuclear cells per field in a Gram-stained smear using magnification X1000, and >15 polymorphonuclear cells per field in the resuspended sediment of a centrifuged 10 ml first catch urine specimen examined at magnification X400 (Swartz and Kraus, 1979; Shafer *et al*, 1986). Cell culture and antigen testing can also be used as diagnostic measures. Chlamydia urethritis tend to be less symptomatic compared to gonococcal urethritis. Organisms also implicated in causing between 10 25% of NGU cases include among others, *Ureaplasma urealyticum, Candida albicans, Trichomonas vaginalis, Mycoplasma genitalium* and Herpes simplex virus (Bowie and Holmes, 1977; Bowie *et al*, 1977; Oriel, 1981; McCormack and Taylor Robinson, 1984; Shanson, 1988).

2.5.3.4.2 Post-gonococcal Urethritis

Post-gonococcal urethritis (PGU) is defined as a persistent NGU following treatment for gonococcal urethritis in men. It is due to co infection with *Neisseria gonorrhoeae* and one of the organisms known to cause NGU. PGU results after treatment with a single dose of penicillin, cephalosporin or a quinolone which is directed against eradication of gonorrhoea and not of chlamydia. Co infection with *N. gonorrhoeae* and *C. trachomatis* is seen more frequently in heterosexual men (15 30%) than homosexual men (5%). Approximately 80% of men infected by *N. gonorrhoeae* and *C. trachomatis* develop PGU after single dose therapy (Oriel *et al*, 1975; Oriel, 1983). *C. trachomatis* is responsible for 30 50% of PGU cases.

2.5.3.4.3 Epididymitis

The aetiology of acute epididymitis varies with age and sexual activity of the patient. In young men under the age of 35 years, infection is often due to *C. trachomatis* and *N. gonorrhoea*, with the former being responsible for 30 60% of the cases. Where gonorrhoeae is uncommon, *C. trachomatis* is usually the major cause (Berger *et al*, 1978; Doble *et al*, 1989a; Eley *et al*, 1992). In South Africa, Fehler *et al*, (1989) reported a 17% prevalence of *C. trachomatis* in sexually acquired acute epididymitis, and 18% of the patients who were negative by culture, had elevated antichlamydial antibody titres of $\geq$1:64. In men over 35 years of age, epididymitis is often associated with instrumentation, and caused by *Enterobacteriaceae* and *Pseudomonas aeruginosa*. Symptoms of epididymitis include unilateral scrotal pain, scrotal swelling, tenderness and fever. When urethral discharge is associated with epididymitis in older men, gonococcal or chlamydial infection is likely to be the cause. Whereas the presence of midstream pyuria and bacteriuria in older men without urethral discharge, is suggestive of *Enterobacteriaceae* and other bacterial infection.

2.5.3.4.4 Prostatitis

This condition occurs in both acute and chronic forms. Symptoms of prostatitis include perineal or suprapubic discomfort, dysuria, frequency, fever and urethral discharge. *N. gonorrhoeae* and *Enterobacteriaceae* have been found to cause acute prostatitis (Oriel and Ridgway, 1983). However, the role of *C. trachomatis* in this disease is dubious. Although *C. trachomatis* has not been recovered from prostatic fluid of men with chlamydial urethritis and has reportedly been isolated from prostatic tissue (Poletti *et al*, 1985; Doble *et al*, 1989b), there is still no convincing evidence to associate *C. trachomatis* with either acute or chronic prostatitis.

2.5.3.4.5 Proctitis and Proctocolitis

Among homosexual men engaging in anoreceptive intercourse, non-LGV serovars of *C. trachomatis* have been associated with proctitis which is characterized by inflammation limited to the rectum, while LGV serovars seemed to be associated with either proctitis or proctocolitis (Quinn *et al*, 1981; Quinn *et al*, 1983; Munday and Taylor-Robinson, 1983). With non LGV serovars, symptoms include mild-to-moderate rectal discharge, mild anorectal pain, tenesmus, and constipation. Rectal mucosa is usually erythematous and friable; a rectal Gram stain reveals numerous polymorphonuclear leukocytes and biopsy specimens show a polymorphonuclear leukocyte infiltrate in the lamina propria. In contrast, symptoms due to LGV serovars are severe as that seen in Crohn's disease; the rectal mucosa usually shows haemorrhages and ulcers, fever and inguinal lymphadenopathy may occur as well (Quinn *et al*, 1981; Quinn *et al*, 1993).

2.5.3.4.6 Reiter's Syndrome

Reiter's syndrome is an asymmetric, reactive polyarthritis or tenosynovitis associated with uveitis and urethritis (Stamm and Holmes, 1984; Sands, 1992); however, Reiter related disease manifestations are also, frequently seen. In the absence of uveitis, Rheumatoid-factor negative arthritis associated with genital infection, is referred to as sexually acquired reactive arthritis (SARA) (Keat, 1983). Approximately 1-3% of NGU cases are complicated by this condition. The syndrome has been found to occur 20 times more frequently in men than in women and like all Rheumatoid-factor negative arthritis, also significantly more often in HLA B27 antigen-positive individuals (Inman *et al*, 1987; Keat *et al*, 1987). There is usually multiple joint involvement in descending order of frequency knees, ankles, wrist and metatarsophalangeal joints. Mucocutaneous manifestations include balanitis, vulvitis, and conjunctivitis. Other ocular manifestations include keratitis, iritis, and retinitis. In patients with Reiter's syndrome, synovial fluid

culture for *C. trachomatis* is negative, although fluorescent antibody testing of synovial fluid has demonstrated the presence of *C. trachomatis* elementary bodies in the synovial fluid (Keat *et al*, 1987).

2.5.3.5 Genital Infections in Females

2.5.3.5.1 Mucopurulent Cervicitis

Chlamydial infection of the cervix is the most common genital infection in women in the industrialised world and almost 50% of these infections are asymptomatic. *C. trachomatis* is the major cause of mucopurulent cervicitis (MPC) and pelvic inflammatory disease (PID). *C. trachomatis* accounts for 60% of the cases and co infection with *N. gonorrhoeae* is common (Brunham *et al*, 1984). As with post gonococcal urethritis in men, women with gonorrhoea and concurrent *C. trachomatis* infection receiving antimicrobials only active against *N. gonorrhoeae*, often have persistent post gonococcal chlamydial cervicitis.

MPC is the counterpart in women of urethritis in men. It often goes unrecognized because of the absence of abundant discharge, itching or foul smell. Observation of >10 polymorphonuclear leucocytes per high power field on a Gram's stain is diagnostic of mucopurulent cervicitis. Apart from the mucopurulent discharge, presumptive diagnosis of MPC include a positive swab test (by culture or antigen detection), increased erythema, oedema, and induced mucosal bleeding in the area of ectopy and the transformation zone (Paavonen *et al*, 1986; Sands, 1992). Histopathological findings include the presence of dense stromal inflammation (plasma cell infiltrations), intraepithelial and intraluminal inflammation, and well-formed lymphoid follicles comprising transformed lymphocytes (germinal centres) (Kiviat *et al*, 1990).

2.5.3.5.2 Pelvic Inflammatory Disease

Pelvic inflammatory disease (PID) refers to infection of the uterus, fallopian tubes, and adjacent structures. PID is almost always an ascending infection in which pathogenic micro organisms spread from the vagina and cervix to the upper genital tract (McCormack, 1994), causing severe pelvic organ damage. The true incidence and prevalence of PID is unknown, although *C. trachomatis* is estimated to be responsible for 20 30% of PID cases. In our study at King Edward VIII hospital, Durban, *C. trachomatis* was detected in 24% of the patients presenting with PID (Chetty and Maleka, 1995). The clinical spectrum of chlamydial PID ranges from subclinical endometritis to frank salpingitis, pelvic peritonitis, infertility, and ectopic pregnancy (Mardh *et al*, 1989). PID in sexually active women is usually caused by *N. gonorrhoeae* and *C. trachomatis* alone, in conjunction with endogenous flora of the lower abdominal tract. According to Sweet *et al* (1986), clinical symptoms vary with respect to *C. trachomatis* serovar group. They found that women with group F G infections tend to have milder symptoms when compared with infections caused by B and C group infections (Sweet *et al*, 1986). Any PID and subclinical PID in particular, is very difficult to diagnose and the traditional clinical and laboratory criteria for the diagnosis of PID are considered to be insensitive and non-specific. Thus, laparoscopy (gold standard), endometrial biopsy, or vaginal ultrasound examination have been incorporated in the diagnosis of PID in order to improve accuracy (Paavonen *et al*, 1987; Eddowes *et al*, 1990; Sellors *et al*, 1991).

Clinically, the symptoms and signs of chlamydial endometritis or salpingitis are usually mild (Stanley *et al*, 1990). Histological evidence of endometritis is seen in approximately 50% of women with mucopurulent cervicitis. Endometritis usually arises after instrumentation of the uterus and has been reported to occur in up to 20% of asymptomatic chlamydia infected women undergoing suction abortion (Blanco *et al*, 1985). Symptoms typical of endometrial chlamydial infection include

heavy, prolonged or irregular menses. Women without symptoms of abdominal pain, who have chlamydial cervicitis often have a plasma cell endometritis with or without lymphoid follicles (Paavonen *et al*, 1985; Kiviat *et al*, 1990). Salpingitis on the other hand, usually presents with lower abdominal pain occasionally described as atypical or mild and often coinciding with the onset of menses, as in gonococcal PID (Svensson *et al*, 1980). Common physical findings include a mucopurulent cervical exudate, uterine and adnexal tenderness, and pain on movement of the cervix. At laparoscopy, women with chlamydial salpingitis have been found to have disease as severe as women with gonococcal salpingitis. Laparoscopic findings often reveal severe tubal inflammatory changes out of proportion to clinical findings.

PID is the single most preventable cause of infertility and adverse pregnancy outcome. The majority of patients with tubal factor infertility or tubal pregnancy have serum antibodies to *C. trachomatis* indicating past chlamydial infection (Mardh *et al*, 1989; Paavonen, 1990). Women with salpingitis are at considerable risk of developing infertility due to peritubal adhesions or tubal occlusion. Each repeat episode of PID doubles the risk of tubal damage (Brunham *et al*, 1985; Patton *et al*, 1990). The prevalence of antibody to *C. trachomatis* is higher among women with infertility and tubal occlusion than in comparison groups. Subclinical and asymptomatic episodes of PID has also been associated with increased risk for ectopic pregnancy, which contributes to 50% of infertility cases (Cates and Wasserheit, 1991). A 2 to 3-fold increased risk for ectopic pregnancy in women with an IgG titre $\geq$1:64 has been reported in a case control study. Ruptured tuboovarian abscesses have been noted in advanced PID patients as well as periappendicitis with serosal inflammation of the appendix (Paavonen, 1990). Tissue damage due to delayed type hypersensitivity is caused by chlamydial stress proteins belonging to 60 and 70 kDa heat shock protein families characteristic of several micro organisms known to cause chronic infections (Morrison *et al*, 1989b; Danilitton *et al*, 1990).

Data on the importance of *C. trachomatis* as a cause of spontaneous abortions, stillbirths, chorioamnionitis, premature rupture of membranes, premature delivery, and neonatal deaths are conflicting. Martin *et al* (1982), were the first to report an increased risk for stillbirth and neonatal death, which were occurring 10 times more frequently in women infected with chlamydia. They found the gestation period to be significantly shorter in women with antenatal chlamydial infections (Martin *et al*, 1982). *C. trachomatis* IgM sero-positivity has been linked with poor obstetrical outcome. This was confirmed by the findings of Gravett *et al* (1986) and Sweet *et al* (1987) in the case of pre-term labour and premature rupture of membranes.

2.5.3.5.3 Acute Perihepatitis (Fitz-Hugh Curtis Syndrome)

Acute perihepatitis was described for the first time in 1919 (cited: Bolton and Darougar, 1983), however, its association with *C. trachomatis* as the aetiological agent, has only recently been described (Sands, 1992). It is estimated that between 3-5% of women with acute PID develop perihepatitis with symptoms of pleuritic right upper quadrant abdominal pain, and tenderness often without pelvic pain. However, adnexal tenderness consistent with salpingitis has been recorded as well. The characteristic "violin-string" adhesions seen between the liver and the anterior abdominal wall which are associated with *C. trachomatis* infection, were first thought to be caused by *N. gonorrhoeae*; however, 80% of patients with proven Fitz-Hugh-Curtis Syndrome were found to be positive for *C. trachomatis* by culture or serology, while only 10% were positive for *N. gonorrhoeae* (Holmes *et al*, 1990).

2.5.3.6 Lymphogranuloma Venereum

Lymphogranuloma venereum (LGV) is a systemic disease caused by one of the three serovars (L1, L2, and L3) within the species *C. trachomatis* (Schachter and Osoba, 1983; Perine and Osoba, 1984; Osoba, 1987). This venereal disease occurs mainly in tropical and subtropical areas of southeast Asia, India, east and west Africa, the Caribbean, and certain parts of the United States and South America. The few sporadic cases reported in Europe are mainly imported. Cases of LGV in these areas, have been largely reported in adults, however, there have been no studies of antenatal and paediatric cases in these endemic areas over the past three decades despite the introduction of more specific tests for LGV. LGV is usually described in three stages with the primary stage involving a superficial lesion, usually on the external genitalia, which develops 1 to 2 weeks after exposure (Van Dyck and Piot, 1992). The lesions are painless and may be vesicular or ulcerative.

The secondary stage, occurring a week or more after the primary stage, is characterized by inguinal lymphadenopathy. This is the typical stage with which men often present at clinics. Symptoms include a tender cord like swelling along the shaft of the penis that is caused by lymphangitis, buboes (enlarged inguinal lymph nodes) that are classically multilocular, and fever and chills. Women do not often present with this form of the disease. Suppuration may occur in the lymph nodes, resulting in a multilocular abscess (Latif, 1990; Martin, 1990).

If there is a delay in treatment, the disease usually progresses to the tertiary stage, known as the anogenital syndrome. Due to the asymptomatic nature of chlamydial infections and the lack of obvious early manifestations of LGV, women often seek medical help when the disease is at its tertiary stage. This stage is characterized by the inflammation and scarring of the genitalia and anorectal canal; fistulae,

strictures, and genital elephantiasis may also occur. In females, this form of elephantiasis of the vulva is known as an "esthiomene". Often, genital elephantiasis caused by chronic fibrosis, and destruction of inguinal lymph nodes and rectal strictures, caused by the destruction and fibrosis of perirectal and pelvic lymph nodes, may require surgical treatment (Schachter and Osoba, 1983; Perine and Osoba, 1984; Piot and Laga, 1989; Van Dyck and Piot, 1992; Chopda *et al*, 1994).

Infection has been noted to occur on various sites other than the genitalia, depending on where the organism was implanted. Parinaud's oculoglandular syndrome may develop if the eye is the site of infection, and when implantation is within the oral cavity, cervical lymphadenopathy occurs. LGV proctocolitis is also common among homosexual men. Other systemic complications include hepatitis, pneumonia, arthritis, and meningoencephalitis.

2.6 LABORATORY METHODS FOR THE DIAGNOSIS OF CHLAMYDIAL INFECTIONS

The choice of diagnostic methods for the detection of chlamydial infections depends on factors such as the number of samples to be processed, cost of the test, the sites of infection, and the purpose of the test. For laboratory diagnosis, isolation in cell culture systems has traditionally been the most accurate method. For this reason, it has been considered as the "gold standard" against which other methods were measured (Lefebre *et al*, 1988; Ridgway and Taylor-Robinson, 1991; Taylor-Robinson and Thomas, 1991). In the last few years, a number of non-culture methods have been introduced and the use of non-invasive techniques has increased. However, the success of the laboratory culture of chlamydia or any other test to be performed, is directly related to the collection of the appropriate (good quality) specimen by the physician and then adequate preservation of chlamydiae in the specimens before and after arrival in the laboratory. Effective swabbing of the urethra in men (by inserting the swab 2cm or more into the urethra, then rotating

it) and the cervix in women (by removing excess ectocervical mucopus prior to collection), has been the traditional approach for specimen collection (Centers for Disease Control and Prevention CDC, 1993). Chlamydiae are obligate intracellular parasites that infect columnar epithelium, therefore, the specimen must contain columnar epithelial cells.

As chlamydiae are extremely labile organisms, even with the use of a special transport medium and maintenance of the specimen at $4^{0}C$, the viability of any chlamydiae present is quickly lost if the specimen is kept for more than 24 hours. If rapid transport to the laboratory is not possible, storage at $70^{0}C$ is preferred when cell culture is to be attempted. The nature of the swabs may also influence the recovery of chlamydiae, particularly in cell culture systems. Dacron swabs on metal or plastic shafts should be used because of the toxic effect wood and cotton have on the growth of this fastidious organism (CDC, 1993). Other disadvantages of cell culture technique are that it is expensive, labour-intensive and takes several days to produce a result (Young, 1990; Taylor Robinson, 1992). These limitations have led to the development of non-culture techniques, thereby limiting the use of cell culture to specialised and research laboratories.

Non culture diagnostic techniques include: direct visualisation of the organisms using fluorescein labelled monoclonal antibodies (direct immunofluorescence DIF),detecting the presence of antigens in specimen by enzyme immunoassay (EIA), and nucleic acid hybridization (DNA probe) test. The main advantages of these methods is that viability of organisms is not important and the specimen can be kept for several days after collection, at either room or refrigerator temperature. Recent developments include the polymerase chain reaction (PCR), which is highly sensitive and allows the detection of even the smallest amounts of specific nucleic acids using repeated amplification steps of a specific target sequence.

2.6.1 Tissue Culture Isolation

Although the sensitivity of various tissue culture systems is accepted to be less than 100%, they remain the standard for identifying Chlamydiae. Since the initial isolation and growth of *Chlamydia* in embryonated hen's eggs in 1955, a number of cell lines have been developed for culture. McCoy (mouse fibroblasts), HeLa 229 (Helen Lang's carcinoma of cervix), HL (human lung), BHK 21 (baby hamster kidney) and BGMK (Buffalo Green Monkey Kidney) are among the cell lines that have been used (Kuo *et al*, 1972; Ripa and Mardh, 1977; Hobson *et al*, 1982; Cles and Stamm, 1990). These cell lines have proved to be at least 10-fold more sensitive than the yolk sac method. Cles and Stamm (1990) reported to have found HL cells to be more sensitive than other cell lines in the isolation of *C. pneumoniae*. However, McCoy cells which are mouse fibroblasts (L cells), are widely used as the cell line of choice for the isolation of *C. trachomatis*.

A number of monolayer cell replication inhibitors have been used in the pretreatment of different cell lines to increase the sensitivity of *C. trachomatis* isolation. Such inhibitors favour the organism in its competition for ATP with the host cells. These include: irradiation, cyclohexamide, IUdR (5 iodo-2 deoxyuridine), cytochalasin B, and mitomycin C (Kuo *et al*, 1972; Ripa and Mardh, 1977; Sompolinsky and Richmond, 1974; Evans and Taylor-Robinson, 1979; Woodland *et al*, 1987). Centrifugation at forces between 2000 3500 g enhance infection of monolayers by the organisms. This is thought to result from an improved contact of the microbes with cells. Iodine, Giemsa, methylene blue, acridine orange, DNA binding dyes, and immunofluorescence among others, have been utilized to identify inclusion bodies. Many workers prefer using specific fluorescent monoclonal antibodies which are very sensitive thus offering more rapid detection of chlamydial inclusions (Thomas *et al*, 1977).

2.6.2 Enzyme Immunoassays

Enzyme immunoassays (EIAs), were introduced in the late 1980s, although only those with IDEIA were available. Unlike cell culture, EIAs are easy to perform, lacks subjectivity, and allow large numbers of samples to be tested within a few hours at a low cost. The drawback about these tests is the grey zone that exists between definite positive readings of optical density (OD) and definite negative readings. All currently available EIA tests utilise either polyclonal or monoclonal antibodies directed predominantly against chlamydial LPS (Ridgway and Taylor-Robinson, 1991), thus making them all genus specific. The widely used EIAs are MicroTrak (Syva), Chlamydiazyme (Abbott Diagnostics), IDEIA (Novo Nordisk), Clearview Chlamydia (Unipath), and Kodak Surecell (Kodak) (Caul and Paul, 1985; Pugh *et al*, 1985; Barnes, 1989; Arumainayagan *et al*, 1990; Hammerschlag *et al*, 1990; Young *et al*, 1991; Kellogg *et al*, 1992; Shafer *et al*, 1993).

These tests detect Chlamydia antigen using an antibody labelled with an enzyme and colour substrate. The results are then measured spectrophotometrically. In view of the fact that the antibody reacts to any type of Chlamydiae, a blocking agent is added to decrease the false-positive rate. Unfortunately, the sensitivity of these assays has varied from study to study, and in general are less than that of cell culture and false positive reactions are not uncommon. The use of EIAs in testing rectal specimens is highly discouraged due to the number of false positives that occur (Rothburn *et al*, 1986). Different studies have reported sensitivities and specificities ranging from 70-96% and 97-99% respectively (Thomas *et al*, 1989).

2.6.3 Direct Fluorescent Antibody (DFA) Test

Whereas culture requires the existence of viable organisms, immunofluorescence techniques do not. These non-culture diagnostic methods using monoclonal antibodies directly on patient material have been found to be rapid, simple and sensitive and comparing well with culture (Tam *et al*, 1984). There are several commercially available kits; some use species-specific monoclonal antibodies that bind to MOMP, while others use genus-specific antibodies that bind to LPS (Alexander *et al*, 1985; Cles *et al*, 1988; Stamm and Mardh, 1990). The former detect only *C. trachomatis*, while the latter detect all three species and are unable to distinguish between them.

The widely used commercial direct immunofluorescence (DIF) test is the Syva MicroTrak (Syva) which takes about 30 min to perform. This test, which uses monoclonal antibodies directed against MOMP, the specimen is smeared onto a glass slide treated with a fluorescent monoclonal antibody that binds to the elementary body (EB) of *C. trachomatis*. The technique is also useful for the evaluation of specimen adequacy, as the presence or absence of columnar epithelial cells can easily be noted. The major disadvantage of DIF is the necessity for a skilled microscopist because of difficulty in differentiating between true elementary bodies (EBs) and artifacts or background immunofluorescence. Several studies have recommended that a minimum of 10 EBs should be visualised to consider a specimen positive. This criteria has resulted in improved specificity (87-99% for men; 82-100% for women) but decrease in sensitivity (70-100% for men; 68-100% for women) (Taylor-Robinson and Thomas, 1991). Despite that, DIF test has been reported to be more sensitive and specific when compared with EIAs.

2.6.4 DNA Probes and Polymerase Chain Reaction

When DNA probes were first introduced, radioisotopic (^{125}I) DNA probes complementary to the ribosomal RNA (rRNA) of the target organism, were available commercially. However, they did not prove to be user-friendly and have now been replaced by a non-isotopic label. Palva *et al* (1984), were the first group to publish information about DNA probes for detecting *C. trachomatis*. The DNA probe test, checks for chlamydial rRNA by linking luminescent DNA with rRNA. This test, although specific, is not sensitive (Gratton *et al*, 1990; Ossewaarde *et al*, 1992).

Due to the apparent lack of sensitivity of many nucleic acid probes, the polymerase chain reaction (PCR) was developed for the diagnosis of chlamydial infection. This technique was developed in 1987 by Kary Mullis in an effort to imitate the replication of genetic material which occurs in nature when cells divide PCR is an *in vitro* method based on the amplification of a specific nucleic acid fragment of defined length and sequence. Amplification of greater than 10^6 fold can be obtained from very small amounts of template. In practical terms, this is achieved by means of two flanking oligonucleotides sequences called primers and repeated cycles of amplification with the enzyme DNA polymerase and deoxynucleotide triphosphates (dNTPs - dATP, dCTP, dGTP and dTTP) (Saiki, 1990). The use of a thermostable enzyme isolated from the bacterium *Thermus aquaticus*, called *Taq* DNA polymerase, has made it possible to automate this procedure.

PCR has significantly transformed the usefulness of nucleic acid probe tests and is now being used in many laboratories worldwide. It has so far, been applied in a number of areas such as prenatal diagnostics, forensic medicine, detection of foreign organisms and many others. The first report on the use of PCR for the

detection of *C. trachomatis* in clinical specimens came in 1989 (Griffais and Thibon, 1989). Since then, several studies have been conducted using plasmid, MOMP or rRNA gene targets (Claas *et al*, 1990; Frost *et al*, 1991). PCR has proved to be extremely sensitive and specific (Mahony *et al*, 1992; Jaschek *et al*, 1993; Frost *et al*, 1993) but prone to false positive results if laboratory contamination is not meticulously avoided. Other disadvantages are that, it relies highly upon the knowledge of the sequence in order to design the primers, and also that only a small part of a gene can be conveniently amplified and examined at any one time. In addition to that, there are many complex interactions which occur during the PCR, therefore, making it unlikely that there will be a set of conditions optimal for all reactions.

Commercial DNA amplification system for *C. trachomatis* such as Amplicor (Roche Products Ltd.), have been available since late 80's. The recent system, Ligase Chain Reaction (LCR) is similar to PCR and can detect *C. trachomatis* from both swab and first void urine specimens. The LCx (Abbott Diagnostics) *C. trachomatis* assay uses the nucleic acid amplification method LCR to directly detect the presence of *C. trachomatis* cryptic plasmid DNA in clinical specimens. This plasmid is found in the conserved regions of all serovars of *C. trachomatis* at approximately 10 copies per elementary body (EB) or reticulate body (RB) (Palmer and Falkow, 1986), but is not found in other species (Joseph *et al*, 1986). The LCx assay employs four oligonucleotide probes designed in pairs, to recognize and hybridize to a specific target sequence located within the *C. trachomatis* DNA. The two pairs of oligonucleotide probes are labelled with immunoreactive chemical groups called haptens, and only the LCR amplified products with both haptens covalently attached, generate a fluorescence signal which is detected by the LCx analyzer.

Studies performed using this plasmid-based assay (LCR) have reported sensitivities ranging from 87% to 98% and specificities of 99.8% to 100% (Chernesky *et al*, 1994a; Chernesky *et al*, 1994b; Schachter *et al*, 1994; Bassiri *et al*, 1995; Lee *et al*, 1995). LCR thus provides a rapid, highly sensitive and specific noninvasive screening method for detecting genital chlamydial infections in both men and women.

2.6.5 Serology

Chlamydial infections, usually those resulting in deep-seated infections, induce in individuals the formation of antibodies that can be detected serologically. Several serological techniques such as agglutination, immunodiffusion, haemagglutination, have been used in the diagnosis of chlamydial infections by detecting antibodies to the three species of the genus (Taylor-Robinson and Thomas, 1991). In common with the use of serology for the diagnosis of many infections, a change in antibody titre is the most reliable criterion, particularly the demonstration of a fourfold or greater rise in titre (Hanuka *et al*, 1987). The oldest and most common method has been the complement fixation test which detects the genus specific anti-LPS to measure antibody. This test is insensitive in that it only detects the deep-seated chlamydial infection, such as psittacosis (ornithosis), LGV, infant pneumoniae and PID but not localized ocular or genital infections; it is also incapable of differentiating between causative organisms. This lack of sensitivity and specificity has led this test to be superseded by other tests such as the micro immunofluorescence (Micro-IF) (Ridgway and Taylor-Robinson, 1991; Taylor-Robinson, 1992).

The Micro-IF was devised by Wang and Grayston in 1970 and is the only sensitive and specific serological assay for any of the Chlamydiae (Treharne *et al*, 1983; Barnes, 1989). This test with TWAR antigen, is specific for *C. pneumoniae*. By

using this technique, 15 chlamydial serovars were defined during the first decade of chlamydial serotyping. This test can also distinguish between antibodies in the IgG and IgM fractions, which makes it helpful in distinguishing recent from past infection, and reinfection from primary infection (Taylor Robinson and Thomas, 1991). However, this test is technically demanding, and the antigens are not yet commercially or rather readily available.

Characterization of *C. trachomatis* isolates has always been carried out by serotyping since the 1960s. It was initially done with a bioassay based on serovar specific prevention of toxic death after intravenous chlamydial challenge in mice, and later replaced with an indirect microimmunofluorescence (micro IF) method which employs polyvalent mouse antiserum. The test has been simplified somewhat by the use of formalinised *C. trachomatis* organisms (Wang *et al*, 1979) or the use of reticulate bodies as antigens (Yong *et al*, 1979), but the methodology is generally considered too complex for routine clinical laboratories.

CHAPTER 3

PATIENTS AND METHODS

3.1 STUDY PERIOD

The clinical aspects of this study were carried out between 3 July and 22 August 1995. All clinical assessments and specimen collection were performed during this period.

3.2 PATIENTS

A total of 247 adult male patients attending the City Health STD Clinic, Durban, were investigated. Patients presenting with any form of genital ulcer(s) during the study period were recruited. The exclusion criteria for participation in the study were the use of antimicrobial agents in the preceding two weeks, the inability to provide a urine specimen, and refusal to be included in the study. Patients were informed of the purpose of the study and the need for specimen collection. Only patients giving verbal consent were included in the study. This study was approved by the Ethics Committee of the Faculty of Medicine, University of Natal.

3.3 CLINICAL EVALUATION

All patients were evaluated by means of a standardized interview form (Appendix 6) that included information on patients' age, marital status, past and current sexual history, and genital signs and symptoms. A detailed genital examination was performed by the study clinician.

3.4 COLLECTION AND TRANSPORT OF SPECIMENS

Genital ulcer specimens were collected after cleaning the base and edges of the ulcer(s) with a cotton tipped swab and a subsequent swab was used to prepare smears on clean glass slides for Gram staining (Dangor *et al*, 1990).

A plastic loop was then used to scrape cellular material off the edges of the ulcer(s) and immediately placed in 0.5 ml of phosphate buffered saline (PBS).

Endourethral specimens were obtained by inserting narrow shafted calcium alginate tipped swabs 2 to 3 cm into the urethra. Swab specimens were used for the preparation of smears on clean slides for *C. trachomatis* antigen detection by the Direct Immunofluorescence (DIF) test (MicroTrak, Syva), for Gram staining, and direct inoculation onto Modified New York City medium (MNYC) and into 2 ml Chlamydia transport medium (CTM) for the isolation of *N. gonorrhoeae* and *C. trachomatis* respectively (Hoosen *et al*, 1993).

All inoculated agar plates were immediately placed into candle extinction jars and transported to the laboratory within 2 hours, where the plates were streaked out and then incubated at 37^0C, in 5% CO_2 for 48 hours (h).

Three tubes (5 ml each) of venous blood were collected aseptically from each patient, from a peripheral vein. These were kept at room temperature until transported to the laboratory.

Each patient was requested to collect about 20 ml of the first portion of voided urine sample in a sterile plastic, screw cap tube. The urine specimens were kept at 4^0C until transported to the laboratory.

3.5 PROCESSING OF SPECIMENS

The PBS ulcer samples (0.5 ml) were vortexed and 100 μl aliquots were added into different culture transport media i.e chlamydia transport media (CTM), viral transport media (VTM), granuloma inguinale transport media (GIM) and Tryptic Soy broth (containing 1% Isovitalex), for the isolation of *C. trachomatis*, herpes simplex virus, *Calymmatobacterium granulomatis* and *Haemophilus ducreyi* respectively (Ripa and Mardh, 1977; Dylewski *et al*, 1986; Hoosen *et al*, 1987; Taylor Robinson and Thomas, 1991; Kharsany *et al*, 1997). All culture specimens were transported to the laboratory and processed within 24 h.

With regard to *Chlamydia* culture, of the 247 clinical specimens collected, the first 200 specimens (P 1 45, and U 1 155) were processed within 24 h of collection, while the last 47 specimens (U 156 202) were stored at 70^0C for a week prior to processing. The reason for the delay in processing of the last 47 specimens was due to excessive workload which had accumulated from the first 200 specimens.

Smears for DIF were air dried, fixed with acetone, wrapped in foil and then stored at 20^0C until processed (Taylor Robinson and Thomas, 1991).

Serum was separated from clotted blood specimens and aliquoted into three cryovials. One vial was sent to the Serology laboratory, Department of Medical Microbiology, King Edward VIII Hospital (KEH), Durban, for syphilis serological tests i.e screening with the

Rapid Plasma Reagen (RPR) test (Romanowski, 1991; Sischy, 1991). All positive sera were titred and confirmed by the *Treponema pallidum* haem agglutination (TPHA) test and the Fluorescent Treponema Antibody Absorption (FTA Abs) test (Hunter, 1990; Sischy, 1991). The second vial was sent to the Serology laboratory, Department of Virology, KEH, for human immunodefiency virus (HIV) serology which comprised screening with an ELISA (Abbott Diagnostics) and confirmation by indirect immunofluorescence.

Aliquots of 10 ml of unspun urine specimens were pipetted into polyethylene cryotubes, labelled and then frozen at 70^0C for the performance of the Ligase Chain reaction (LCR) testing (LCx, Abbott Diagnostics).

3.6 PROCESSING OF SPECIMEN FOR *CHLAMYDIA TRACHOMATIS*

3.6.1 Direct Antigen Detection of *C. trachomatis*

Endourethral smears were examined using a *C. trachomatis* direct fluorescent antibody reagent test (MicroTrak, Syva). The reagent contains fluorescein isothiocyanate conjugated (FITC), purified murine monoclonal antibodies specific for *C. trachomatis*, Evans Blue counterstain and suppressors of nonspecific staining in a protein stabilized buffer solution. The use of highly specific monoclonal antibodies allows the visualization of individual chlamydial elementary bodies in direct patient specimens. Tests were performed according to the manufacturer's instructions. The procedure used was as follows:

The direct specimen reagent together with the acetone fixed slides (removed from 20^0C) were left at room temperature for 30 minutes before use. Smeared slides were stained using 30 μl of diluted monoclonal antibody which was spread over the entire area of the well. The slides were incubated at room temperature for 15 min

in a moist chamber. After incubation, slides were gently rinsed in distilled water for 10 seconds to remove unbound antibody and then air dried. A drop of mounting fluid was added to the centre of each well and a coverslip mounted. Slides were examined with a fluorescent microscope (Olympus) at a wavelength of 540 nm. A positive diagnosis was made when two or more elementary bodies were observed. Elementary bodies (EBs) were identified as characteristic individual pin-points of medium to bright apple green fluorescence. These were then visualised using 100X oil objective for verification. To quality control the performance of the staining procedure and the microscope, both *C. trachomatis* positive and negative control slides provided by the manufacturer were stained and read in parallel with each series of test slides.

3.6.2 Culture and Isolation of *Chlamydia trachomatis*

3.6.2.1 Reconstitution of Cultured Cells

An ampoule/vial (to be reconstituted) containing 10^6/ml McCoy cells was removed from the liquid nitrogen freezer and immediately plunged into a bottle containing sterile triple distilled water (tdH_20) at a temperature of 37^0C. The thawing process has to be rapid because of the trauma of the process of freezing and thawing to the cells. Immediately upon thawing, the vial was wiped with 70% alcohol and opened aseptically in the laminar flow safety cabinet and the contents diluted 1:10 in growth medium [Eagle's minimum essential medium (MEM) with L glutamine (2 mM)] in a mini flask. Heat inactivated Foetal Calf Serum (10% FCS) (i.e 1 ml in 10 ml of MEM), was added and the flask placed at 37^0C in a 5% CO_2 incubator. After 24 h, the cells were trypsinized if they were confluent and if not, the medium was changed and the cultures re incubated at 37^0C in 5% $C0_2$ until confluency.

3.6.2.2 Heat Inactivation of Serum

A 500 ml bottle of foetal calf serum was removed from 20^0C and placed at room temperature for 3 4 h before thawing completely in a 37^0C waterbath. The bottle was removed from the waterbath, agitated to mix the contents and then, placed into a 56^0C preheated waterbath with the water level above the serum level. Another bottle with an equivalent water volume with a thermometer inserted inside, was placed in the same waterbath as a control. The temperature of the waterbath was monitored to ensure that it stayed at 56^0C while the serum was occasionally mixed by swirling. When the temperature in the control bottle had reached 56^0C, a timer was set for 30 min. At 15 min intervals, the temperature was checked and the bottle swirled. After 30 min incubation, the bottle was removed from the waterbath and the serum aliquoted into 20-ml bottles and stored at 20^0C until required.

3.6.2.3 Trypsinization/Passaging Procedure

When the cells had reached the required percentage of confluency, the spent medium was removed and the cell monolayers washed thrice with 5 ml of PBS. The washing fluid (PBS) was decanted and replaced by 2 ml of warmed trypsin/EDTA solution (equal parts of 0.25% trypsin and 0.02% EDTA). The flask was swirled gently in order to spread the solution evenly over the cell monolayer. The trypsin was allowed to work for 20 seconds, then decanted leaving a small amount (approximately 100 μl) to cover the surface of the flask. Table IV depicts the relationship between flask size and volume of trypsin/EDTA used.

Table IV Flask size - Volume of trypsin/EDTA added

a. 75 cm^2 flask	- 2.5 to 5.0 ml
b. 105 cm^2 flask	5.0 to 10.0 ml
c. 850 cm^2 roller bottle	10.0 to 20.0 ml

It was found that, the longer the time frame between confluency and subculturing, trypsinization took longer. Thereafter, the flask was incubated at 37^0C until cells detached as a film from the flask surface (ca. 2 min). Cells were immediately resuspended in 3 ml of FCS to stop the action of the trypsin and thereafter topped with 7 ml of MEM. The cell suspension was titurated thrice i.e pipetting back and forth, using a syringe and an 18G needle and once through a 25G needle in order to break cell clumps and obtain single, dispersed cells.

The cell suspension was transferred to a new flask and topped with 20 ml of growth medium containing 10% FCS and antibiotics (Appendix 1.2). The flask was incubated at 37^0C for 4 days before the next passage. After two days of incubation, the medium was changed with MEM containing 5% FCS and antibiotics. On the fourth day, the flask was removed from the incubator and cells trypsinized in the same manner as above. After trypsinization, the McCoy cells were seeded at a 1:40, 1:20 and 1:5 ratio and incubated; occasionally, the cells were counted by the Trypan Blue exclusion method (see below) using a haemocytometer and an inverted light microscope.

3.6.2.4 Determination of Cell Numbers

For an accurate determination of cell numbers in suspension cultures, a haemocytometer was used. This consists of two chambers, each of which is divided into nine large (1 mm) squares. The four large squares at the corners are each ruled into sixteen smaller squares and the large square in the middle is ruled into twenty five smaller squares which in turn are ruled into sixteen even smaller squares. The depth of the chamber is 0.1 mm therefore, to convert to ml, one has to multiply by 10^4 (1/10000).

Prior to counting, the haemocytometer and the cover glass were properly cleansed with distilled water (H_20), followed with absolute ethanol and then wiped dry. The cover glass was placed over the haemocytometer chamber. Cell suspension (0.5 ml) was transferred into a 1.8 ml vial and mixed with 0.5 ml of diluted Trypan Blue dye. Trypan Blue is used to differentiate between viable and dead cells, since it is excluded by the membrane of viable cells whereas the nuclei of dead cells take up the stain. Using a clean glass capillary tube (Becton Dickinson), both chambers of the haemocytometer were filled by capillary action. Cells in the 5 large squares (each corner square and the middle square) of both chambers including cells lying on the top and the left hand margins of each square were included in the count for that square. The cells similarly located on the bottom and the right hand margins were omitted and vice versa. When over 10% of the cells represented clumps, then the entire sequence was repeated.

The total number of cells in the original culture was calculated as follows:
cells/ml = average count per square X 10^4
Total cells = cells per ml times any dilution factor e.g 2, if 0.5 ml of cells plus Trypan blue (0.5 ml) is used X total volume of cell preparation from which sample was taken.

E.g n = total no. of cells counted in 5 large squares
y = 2 (dilution of solution in trypan blue)
6-1 = 5 (volume of cell preparation dilution i.e 0.5 ml X 2)
n/5 = no. of cells in one large square

Equation: $\frac{10^4\ ny}{5} = \frac{10^4 \times n \times 2}{5} \times 5$

Count was repeated to check reproducibility. The desired cell concentration was then adjusted by diluting the cells in growth medium with a factor of X/5 where X = the number of cells calculated in the squares.

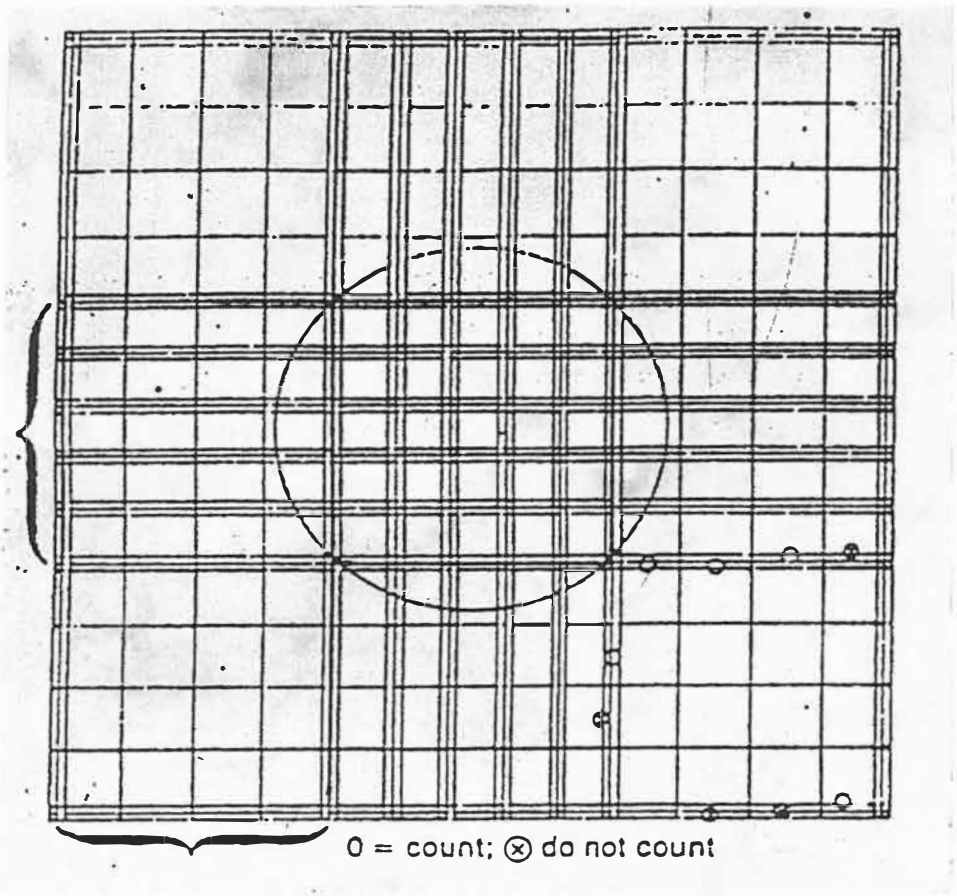


Fig. 3 Diagram of a haemocytometer. The ruled area of the improved Neubauer counting chamber. O = count; X = do not count

3.6.2.5 Cryopreservation of McCoy Cells

After trypsinization and cell count, the cell mixture was resuspended in growth medium at a concentration of 2.5 to 4.0 X 10^6 cells/ml. McCoy cells were stored at 10^6 in 1 ml cryo vials. For example: if in the remaining 19.5 ml of MEM/cell mixture, there were approximately 15.210.000 cells, the number of cells was divided by 10^6 to get the number of cells per vial to be expected. In this case, the amount was 15.21, therefore, 15 vials were used. The cell suspensions during the procedure were kept cool at all times in an ice box.

The 19.5 ml of the tissue culture fluid in the tissue culture flask was then poured into 15 ml centrifuge tubes and the tubes centrifuged at 2100 rpm for seven min. After centrifugation, the supernatant was discarded leaving the cell pellet at the bottom. This was topped up with 15 ml or more of freezing medium (MEM) containing 10% FCS and 10% glycerol.

The suspension was titurated using a syringe and an 18G needle and thereafter, 1 ml aliquots of the suspension were transferred into sterile cryotubes labelled with the type of cell line, passage number and the date. The vials were placed at 4^0C for 1 h in a polystyrene box, after which, the vials were stored at 70^0C overnight giving a cooling rate of 1^0C per min. The following day, the vials were removed from the 70^0C freezer and stored in liquid nitrogen container. At approximately 3 monthly intervals, one vial was taken out and reconstituted to check whether the cells were still viable.

3.6.2.6 Seeding of McCoy Cells

Having trypsinized the cells, counting and adjusting the cell concentration in growth medium, 1 2 ml of McCoy cell suspensions were planted onto 13 mm glass coverslips contained in 15 mm-diameter (1-dram) disposable flat bottomed track vials. Approximately 1 X 10^5 to 2 X 10^5 cell concentration was always selected to give a light, confluent monolayer after 24 to 48 h of incubation at 37^0C in a CO_2 incubator. If the monolayers were to be used the next day, a dilution of 2 X 10^5 cells/ml was used.

It is possible to dispense cells at a lower concentration, incubating for a longer time until they are confluent. In this case the required cell count must be adjusted accordingly.

Prior to returning the track vials to the incubator, they were gently swirled to remove any bubbles beneath the glass coverslips. For optimal results, the cells were used within 24 to 72 h after reaching confluency.

3.6.2.7 First Passage Isolation

Prior to inoculation of McCoy cell lines, the following were carried out:

Each track vial was examined using an inverted microscope (objective X5, ocular X8) to check whether the cells had formed a confluent monolayer. Only those with 70 80% cell confluency were selected; those with cracked tubes, broken coverslips, "patchy" distribution of cells or overcrowded cells were discarded. Each track vial cap was then marked with the corresponding specimen number.

For the clinical specimens: sterile glass beads were added aseptically in each specimen tube. The specimens were vortexed with glass beads for 2 min.
This procedure is safer and more convenient than sonication, although the latter has been reported to increase sensitivity.

The isolation technique for *C. trachomatis* using McCoy cells was performed as described by Ripa *et al* (1977), with slight modification. To inoculate the monolayers, growth medium was aspirated from each track vial and monolayer rinsed once with PBS. This was replaced with 0.5 ml of cycloheximide overlay medium (Bio Whittaker) and 1 ml of the inoculum (1:2) [two vials per specimen]. Cycloheximide is a glutarimide antibiotic which inhibits the DNA and protein synthesis of eukaryotic cells, but does not affect prokaryotic cells such as *Chlamydia*.

The specimen was then centrifuged onto the cell monolayer at 3000 rpm at 35^{0}C for 1 h. The infected monolayers were incubated at 35^{0}C in the atmosphere of 5% CO_2 for 2 h before the medium was aspirated and replaced with 1 ml of cycloheximide overlay medium (Bio-Whittaker).

The cells were re incubated for a further 48 to 72 h at 35^{0}C in 5% CO_2, after which one of the cultures (one of the two vials for each specimen) was fixed in 95% ethanol and stained with 30 μl of FITC monoclonal antibody, *Chlamydia trachomatis* confirmation test (MicroTrak, Syva) and examined for inclusion forming units using a fluorescence microscope (Olympus).

This test was performed according to the manufacturer's instructions. A known positive control and a negative control (uninoculated McCoy cell monolayer) were processed and stained in parallel with every batch of patient specimens.

3.6.2.8 Fixing, Staining and Evaluation of McCoy Cells

After 48 h incubation of the cultures, one vial from each set of specimen vials was selected and placed on a tray. The medium was aspirated carefully from the vials without disturbing the cell monolayers on the coverslips. The monolayers were immediately rinsed twice with 1 ml of PBS by slowly adding PBS on the side of the track vial, then gently agitating the vial for at least 1 min and finally aspirating PBS with a pasteur pipette.

To fix the cultures, 1 ml of 95% ethanol was added to the vials and left to stand for 10 min. The ethanol was aspirated from the vials and the coverslips removed using forceps. A drop of DPX mountant was placed onto clean, labelled

microscope slides upon which, the coverslips were placed with cell side facing upwards. These were left to dry overnight, at room temperature in the dark.

Slides were stained the next day or wrapped in foil and stored at 20^0C until required. Slides used immediately or if stored at 20^0C were brought to room temperature, and the detection of *Chlamydia trachomatis* was confirmed using fluorescein isothiocyanate (FITC) conjugated monoclonal antibody. FITC conjugated monoclonal antibody (30 μl) was added to each monolayer, covering the entire coverslip. The slides were incubated at 37^0C for 30 min in a moist chamber. The antibodies were not allowed to dry onto cells since drying may cause non specific binding.

Following incubation, excess reagent was aspirated and the slides rinsed in deionized water for 10 sec. Slides were left to air dry. A drop of mounting medium was placed onto each appropriately labelled microscope slide and a square coverslip placed on top of the drop.

Slides were examined for the presence of characteristic apple green round inclusion forming units (IFU) using a fluorescence microscope (Olympus). Inclusions were confirmed at 100X magnification. For a positive result, 2 or more inclusions were required. Slides were read within 24 h of staining or stored at 4^0C in the dark to be read within 48 h or at -20^0C until read.

3.6.2.9 Second Passage Isolation

When a blind passage of negative material was required, then, 72 to 96 h post-inoculation i.e after first passage, the one remaining vial of each specimen was

passaged. Sterile glass beads were added to each of the vials, the cell monolayer disrupted and the cells detached by shaking with glass beads on a vortex mixer for 20 to 30 sec.

Cycloheximide overlay base medium (0.5 ml) (Bio Whittaker) was added to two fresh vials (at least 70% confluent) and the monolayers were inoculated as described above (section 3.6.2.7).

After 48 h incubation one vial of each specimen was fixed, stained and read as described above (section 3.6.2.8). When no inclusions were found on reading the first and second passage coverslips, then the specimen was considered negative for *C. trachomatis*.

3.6.2.10 Storage of Chlamydia Cultures

C. trachomatis positive and negative cultures in the remaining vials, were processed as in second passage (3.6.2.9). If more than two vials were processed from each specimen, then suspensions from the same inoculum were combined. An equal volume of 0.4 sucrose phosphate buffer (0.4 S P) was added to each suspension. Then 0.1 ml of FCS for each millilitre (ml) of the mixture was added. 1 ml of the culture mixture was dispensed into 1.8 ml cryo vials and labelled with the specimen number, the number of times that it had been passaged and the date. The same data was also recorded in the laboratory culture book. The isolates were then stored at 70^0C.

3.6.2.11 Repassage of Chlamydial Isolates

Stored isolates (70^0C), which were known to be infected with chlamydia, were used as the inoculum for the newly prepared monolayers. Following the same isolation method as detailed above, more than two track vials for each isolate were inoculated depending on the number of chlamydial inclusions that were expected to be present in the infected monolayer. After centrifugation, change of medium and incubation, one vial was fixed and stained, one stored and the other(s) repassaged into more track vials as appropriate. This procedure was continued until an adequate number of isolates was stored.

3.6.3 Ligase Chain Reaction (LCR)

The plasmid based LCR assay for the detection of *C. trachomatis* was performed according to the manufacturer's instructions (LCx, Abbott Laboratories). Three different areas were used in order to prevent amplicon contamination. These consisted of Area 1 (sample preparation area i.e the hood) where specimen processing and addition to LCR reaction mixture took place, Area 2 thermocycling and pulse centrifugation area, and Area 3 where loading of reaction cells and the amplification vials into the carousel and detection of amplified product took place.

3.6.3.1 Specimen Processing

On the day of LCR testing, the 247 urine specimens were completely thawed and briefly vortexed to resuspend any settled material.

Using a pipettor with aerosol barrier pipette tips (Art, S.A. Scientific), 1 ml of urine was dispensed (in the hood) into appropriately labelled 1.7 ml Urine Specimen Microfuge Tube from the Urine Specimen Preparation Kit (No. 3B21).

The samples were centrifuged at 13,000 X g for 10 min (Eppendorf Centrifuge, Abbott). After removal of the supernatant, the pellet was resuspended in 1 ml Urine Resuspension Buffer which is provided with the LCx kit and the tubes were sealed with cap locks and vortexed until the pellet was resuspended. The samples were then placed in an LCx dry bath at 97^0C for 15 min to lyse the cells. After being cooled to room temperature (~ 15 min), the urine samples were held at 4^0C until further processing.

As the pellet was often translucent, fine tipped pipettes were used to remove the supernatant so as not to disturb or dislodge the pellet. The time period between centrifugation and removal of supernatant was also kept to less than 15 min.

3.6.3.2 DNA Amplification

For testing, 100 μl of the Activation Reagent was added to each bottle of negative controls and calibrators. After vortexing, 100 μl of each processed urine sample and of each control was pipetted into appropriately labelled, individual unit dose tubes containing 100 μl of the LCR reaction mixture.

Controls consisted of two negative controls and two calibrators of purified DNA with each batch of samples processed. The mixture contained four oligonucleotide probes specific for *C. trachomatis* cryptic plasmid, NAD, magnesium, thermostable DNA polymerase and a thermostable DNA ligase which acts to fill in and join adjacent oligonucleotide primers that line up on complementary target DNA, allowing the reaction to commence.

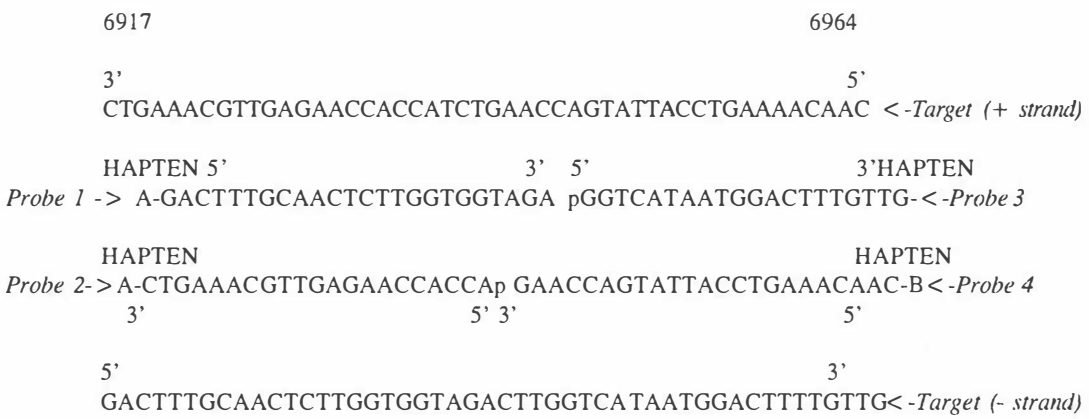


Fig. 4 DNA sequences for plasmid target and probe set 6917 6964 used in the LCx *C. trachomatis* assay.

Samples were then transferred to Area 2 where they were placed immediately into the LCx thermal cycler (Model 80056). Only 48 samples at one time were amplified for 40 cycles over a 2 hour period. The amplification protocol was as follows:

Assay Step Cycle File:

Segment 1 93^0C for 1 sec
Segment 2 59^0C for 1 sec
Segment 3 62^0C for 1 min 10 sec
Cycle count 40 cycles
The Assay Step Cycle file is "linked to" the Soak File at 25^0C, indefinitely.

Two negative controls as well as two calibrators were assayed with each batch of samples. After thermocycling, the controls, calibrators and the samples were pulse-centrifuged for 10 15 seconds and then transferred to Area 3 where LCx reaction cells were placed into a Micro particle Enzyme Immuno Assay (MEIA) carousel.

3.6.3.3 LCx Detection and Inactivation of Amplification Product

The LCx Chlamydia amplification vials were placed into the LCx reaction cells in the following order: negative controls in positions 1 and 2, calibrators in positions 3 and 4 and specimens in the remaining positions. The LCx Chlamydia Detection Reagent Pack was removed from 4^0C storage, gently inverted 5 times making sure that no bubbles were formed, then the reagent pack bottles were opened in increasing numerical order: 1 4. Thereafter, the run was initiated.

Amplification products were qualitatively detected in an LCx analyzer (Abbott

Laboratories), which is a microparticle sandwich immunoassay based on covalent labelling of the four probes with two different haptens. The amplified product was captured onto the microparticles by immobilized antibodies directed against one of the haptens (carbazole and adamantane) whereas the other end of the product which contained the second hapten, was recognized by a second antibody conjugated to a reporter enzyme (alkaline phosphatase).

Only the LCR amplified products with both haptens covalently attached generated a detectable signal in the analyzer. The LCR results were expressed as counts per second, with a positive result defined as a value equal to or greater than the product of the mean of the two calibrator values multiplied by 0.45.

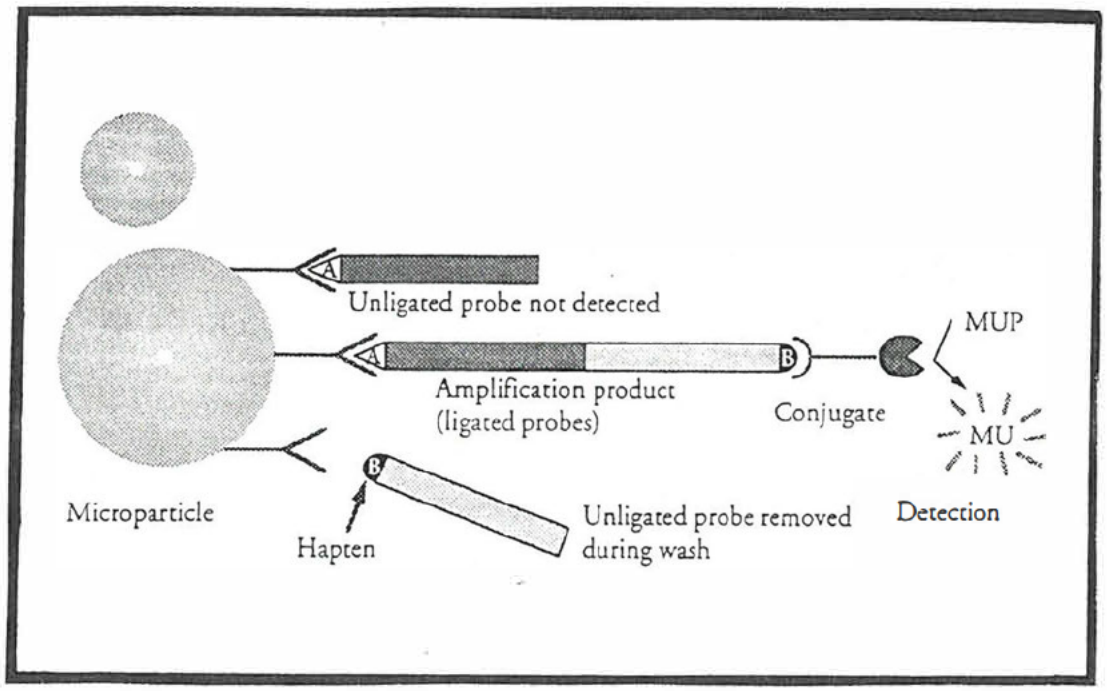


Fig. 5 Schematic diagram of the capture hapten binding to a microparticle and the detection signal of *C. trachomatis* LCR-amplified products.

3.6.4 Processing of Specimens For Polymerase Chain Reaction (PCR)

3.6.4.1 *C. trachomatis* Culture Preparation for PCR

Reference strains and clinical strains of *C. trachomatis* were recovered on cycloheximide-treated McCoy cell monolayers (in duplicate) by incubating for 72 h at 35^0C in 5% CO_2. Inclusions were detected using the direct fluorescent culture confirmation test (MicroTrak, Syva). Serial passages were carried out until the monolayers had 15 to 30 inclusions per high-power microscopic field and 30-50% of the cell monolayers were infected. Sterile glass beads were added to each track vial and the cells disrupted by vortexing for 1 min.

3.6.4.2 Chromosomal DNA Extraction

Cell cultures were pelleted (1200 rpm, 20 min), chromosomal DNA extracted (Peterson *et al*, 1989; Sambrook *et al*, 1989), quantified using a spectrophotometer (Gene Quant II - Pharmacia, Biotech) and electrophoresed on 0.8% agarose gel (70 V/cm, 1-2 h). Chromosomal DNA extracted from McCoy cells infected with different genital ulcer specimens was intact, as seen by the presence of 1-kbp band (Plate 1).

3.6.4.3 Amplification for the Detection of *Chlamydia trachomatis*

Specific amplification of the cryptic plasmid and MOMP genes of *Chlamydia trachomatis* was undertaken using a set of divergent primers (synthesized by ß-cyanoethyl phosphoramide Model 8600, Milligen Biosearch) to amplify a conserved region of these genes. For the specific amplification of the cryptic plasmid gene yielding a 452 bp product, a sense 5' plasmid 24-mer that is homologous to nucleotides 2318-2341 (oDnaBL) and a 3' antisense primer homologous to

nucleotides 2770 2747 (oDnaBR) were used as described by Comanducci *et al* (1990) and Workowski *et al* (1993) (Table V). For the specific amplification of the MOMP gene yielding a 1128 bp product, a sense 5' MOMP 21 mer that is homologous to nucleotides 118-138 (o5MOMP) and a 3' antisense primer homologous to nucleotides 1113-1093 (oDVDIV), were used as described by Stephens *et al* (1987) (Table VI).

The experiments on 247 DNA samples from genital ulcer specimens were carried out in the laboratory of Professor Walter Stamm, Division of Allergy and Infectious Diseases, Department of Medicine, University of Washington, Seattle, USA. Control procedures were adopted as recommended in the proposed guidelines for molecular diagnostic methods (Newton, 1995) with unidirectional workflow and physical separation of reagent preparation, sample preparation, amplification and product detection. Each laboratory was equipped with dedicated equipment, and standard good laboratory practices were enforced.

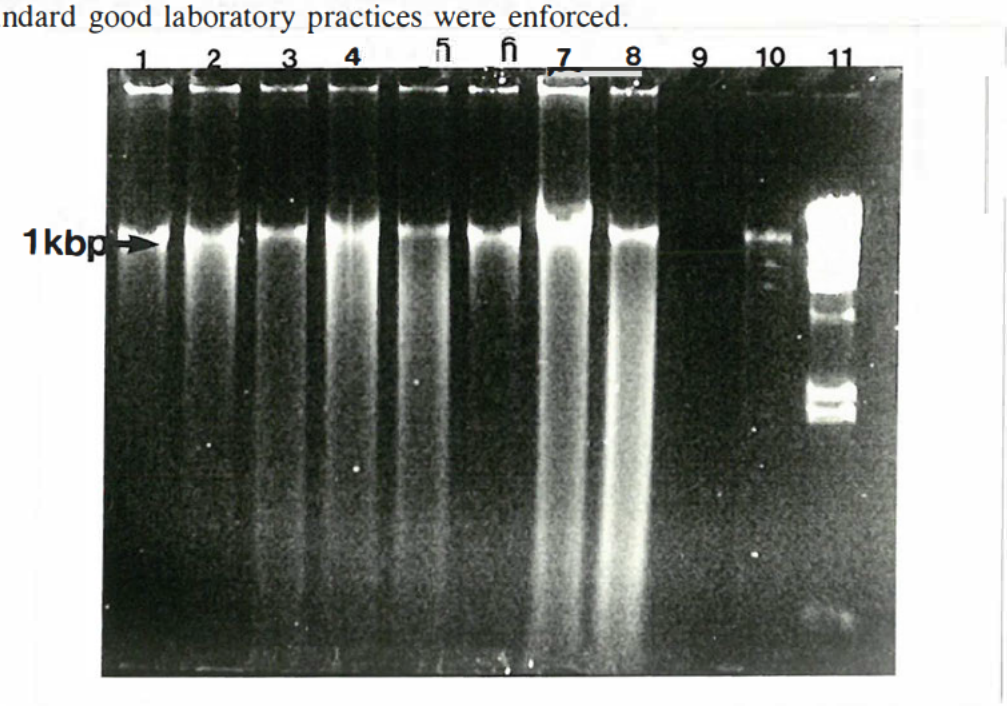


Plate 1. Chromosomal DNA (lanes 1-9) extracted from McCoy cells infected with genital ulcer specimens. Lane 9, PCR negative control. Lane 10, positive control (L2 DNA). Lane 11, molecular weight marker (M_rM) II (125 23130 bp; Boehringer).

Table V Location and sequences of the sense and antisense primers used to amplify a 452 bp fragment of the *C. trachomatis* plasmid gene

Primers	Sequence	Location
Sense		
oDnaBL[b]	5' CAA CAG CGT AGA GTT GGT TTC CTA 3'	2318-2341[a]
Antisense		
oDnaBR[b]	5' GCT GTC TCG CAA ATC TGA AAG CAT 3'	2770-2747[a]

[a]The primer pair was chosen in the highly conserved region of *C. trachomatis* cryptic plasmid (Comanducci *et al*, 1990).
[b]oDnaBL and oDnaBR primers amplify all known *C. trachomatis* serovars.

Table VI Locations and sequences of the sense and antisense primers used to amplify a 1128 bp fragment of the *C. trachomatis* MOMP gene

Primer	Sequence	Location
Sense		
o5'MOMP[a]	5' AGC CTT ATG ATC GAC GGA ATT 3'	118-138
Antisense		
oDVDIV[b]	5' AAT ACC GCA AGA TTT TCT AGA 3'	1113-1093

Primers derived from the MOMP sequences as published in Stephens *et al* (1987).
[a]o5'MOMP primer is found in the conserved region of all *C. trachomatis* strains at the amino-terminal end of the MOMP gene.
[b]oDVDIV acts as a PCR amplification primer for all serotypes and variants of *C. trachomatis*.

3.6.4.4 PCR Reaction Mixture

PCR reaction mixture (final volume 100 μl) comprised of 10 mM Tris hydrochloride (HCl), pH 8.0; 50 mM potassium chloride (KCl); 1.75 mM magnesium chloride ($MgCl_2$); 0.01 % gelatin; 200 μM each deoxynucleoside triphosphate (dNTPs); 1.0 μM of each primer; and 2.5 units *Thermus aquaticus* (*Taq*) polymerase (Amplitaq, Cetus); and 10 μl of DNA sample. The PCR reaction mixture was overlaid with 100 μl of mineral oil (Sigma), subjected to 40 cycles of amplification in a thermal cycler 480 (Perkin Elmer, Cetus) (1 min at 94^0C, 1 min at 67^0C, 1 min at 72^0C), following an initial denaturation at 94^0C for 4 min and were completed by an extension of 7 min at 72^0C. The same reaction mixture and conditions as described above, were repeated for the primer set for the specific amplification of the MOMP gene except that the annealing temperature was 55^0C (Lampe *et al*, 1993). PCR negative controls were provided by reactions containing all of the components without addition of the template. The products were analysed by electrophoresis on 0.8% ethidium bromide stained agarose gels, and photographed on a 302 nm ultraviolet transilluminator.

3.6.4.5 PCR Sensitivity for the Cryptic Plasmid and MOMP Genes

Ten fold serial dilutions (10^{-2} to 10^{-10}) of a known positive control (*C. trachomatis* L2 DNA) were tested for end point PCR sensitivity for the cryptic plasmid and MOMP genes, using their respective primers.

3.6.5 IMMUNOTYPING OF *CHLAMYDIA TRACHOMATIS* ISOLATES

The protocol for immunotyping of chlamydia isolates is the modified version of Barnes *et al* (1985) and Suchland and Stamm (1991). This indirect microimmunofluorescence (micro IF) serotyping assay employs a panel of monoclonal antibodies (MAb) which encompasses all 18 currently recognized serovars of *C. trachomatis*. The MAbs used in this study were provided by Dr M. Lampe (Division of Allergy and Infectious Diseases, Department of Medicine, University of Washington, Seattle, USA). Viable organisms are a requirement for this technique.

3.6.5.1 Isolation of *C. trachomatis*

The culture positive isolates were removed from 70^0C, quickly thawed (50^0C), and then inoculated (100 μl) onto cycloheximide-treated McCoy cell monolayers in 96 well microtitre plates. The isolation procedure, incubation steps and the conditions, were the same as described previously in 3.6.2.7 and 3.6.2.8. After 48 to 72 h incubation, chlamydial inclusions were detected by a direct fluorescein isothiocyanate conjugated (FITC) monoclonal antibody (MAb) to *Chlamydia* lipopolysaccharide (CF 2). Isolates with a lower initial inclusion count i.e $<50\%$ monolayer infection per well, were inoculated and serially passaged (two to five times) in track vials until 50 to 100% (i.e $\geq$ 20 inclusion forming units, IFU) of the monolayers were infected. Infection of the monolayers was observed by viewing the vials with an inverted microscope. Vials containing $>50\%$ infected monolayers as indicated by the presence of cytoplasmic refractile cellular inclusions characteristic of *C. trachomatis*, were used for chlamydial antigen preparation. These isolates, were stored at 70^0C until they were processed for typing.

3.6.5.2 Culture Preparation for Titration of Chlamydial Isolates

A flask containing confluent McCoy cell monolayer was trypsinized as described previously (3.6.2.3), and the cell numbers determined by the trypan blue exclusion method using a haemocytometer as previously described (3.6.2.4). The cells were diluted accordingly (depending on the number of microtitre plates needed) to get a final concentration of 3.5 X 10^5 cells per well of a 96 well microtitre plate. This cell concentration gives the required 90-95% cell confluency at 24 h. For example:

No. cells counted = 34 X 10^5 cells/ml
No. cells required = 3.5 X 10^5 cells/well
Require: 0.1 ml cell suspension/well
Therefore, for 96 well plate require: 96 X 0.1 = 9.6
To have more cell suspension, round it off to 12.0 ml

i.e $\frac{3.5 \times 10^5}{34 \times 10^5} \times 12.0 \text{ ml} = 1.24$

and: 12 1.24 = 10.76 ml

Therefore, to get the required number of cells per well, 1.24 ml of 34 X 10^5 cells/ml was transferred to a container and diluted with 10.76 ml of Chlamydia Complete media with Glucose plus Hepes (CMGH, pH 7.5) without cycloheximide (Appendix 2.4). A magnetic stirrer was placed inside the suspension container which was in turn placed on a magnetic plate to facilitate an even distribution of the cell suspension. Aliquots of cell suspension (100 μl) were added to each well and the microtitre plates incubated at 37^{0}C, in 5% CO_2, for 24 h.

3.6.5.3 Titration of Inclusion Forming Units

After 24 h incubation, each well was inspected for confluency (90 95%) and contamination, using an inverted microscope. All contaminated or overgrown microtitre plates were discarded and the remaining plates, placed in the incubator until ready to be inoculated.

Chlamydia stock suspensions were removed from 70^0C and thawed quickly at 37^0C. The suspensions were serially diluted to the desired concentration (10^0, 10^{-2} to 10^{-4}) with sterile sucrose-phosphate glutamate (SPG).

The microtitre plates were removed from the incubator, spent media removed with vacuum, and one drop ($\sim$60 μl) of CMGH medium containing NGV antibiotics (nystatin, 25 U/ml; gentamicin, 10 μg/ml; vancomycin, 25 μg/ml) and cycloheximide (1 mg/ml) added to the monolayer. From each dilution, 100 μl were added to two wells i.e in duplicates, and each plate was properly sealed before centrifuging at 2240 rpm for 1 h. Six isolates were inoculated per plate. After centrifugation, the suspensions were removed with vacuum, and 200 μl of CMGH (NGV and cycloheximide) were again, added to each well. Plates were incubated for 48 h at 37^0C in 5% CO_2.

After 48 h, the microtitre culture plates were examined under a microscope to ensure infection of McCoy cells by chlamydia. Following that, spent CMGH was removed by vacuum carefully without disturbing the monolayer. The monolayers were fixed with 100% methanol for 10 min, by placing the plate on a shaker. Methanol was removed and the plate allowed to dry for 5 min. When dry, the monolayers were washed with phosphate buffered saline with 0.05% Tween 20 (PBST) (Appendix 2.11) for 5 min.

Chlamydial inclusions in each well were detected by staining the monolayers with 10 μl of FITC MAb. The microtitre plate was placed on a shaker for 30 min at room temperature. Thereafter, the MAb stain was removed, the monolayers washed gently with distilled water, and then allowed to dry. When dry, one drop of FA mounting fluid containing 50% glycerol and 50% Tris buffer (pH 8.5) was added to each well, and the excess shaken off. Plates were read immediately using a fluorescence microscope (Olympus), or stored at 4^0C overnight. Fluorescence has been reported to be stable at 4^0C for up to 1 month.

Cell inclusions in each well (2 per isolate), were counted in three fields of view using x10, x16, and x40 objectives which have a conversion factor of 10, 25, and 150 respectively. Table VII shows an example of calculations derived in determining the titre of all chlamydial isolates. Plate 2 depicts the number of IFU at a dilution of 10^{-5} as viewed at x40 magnification.

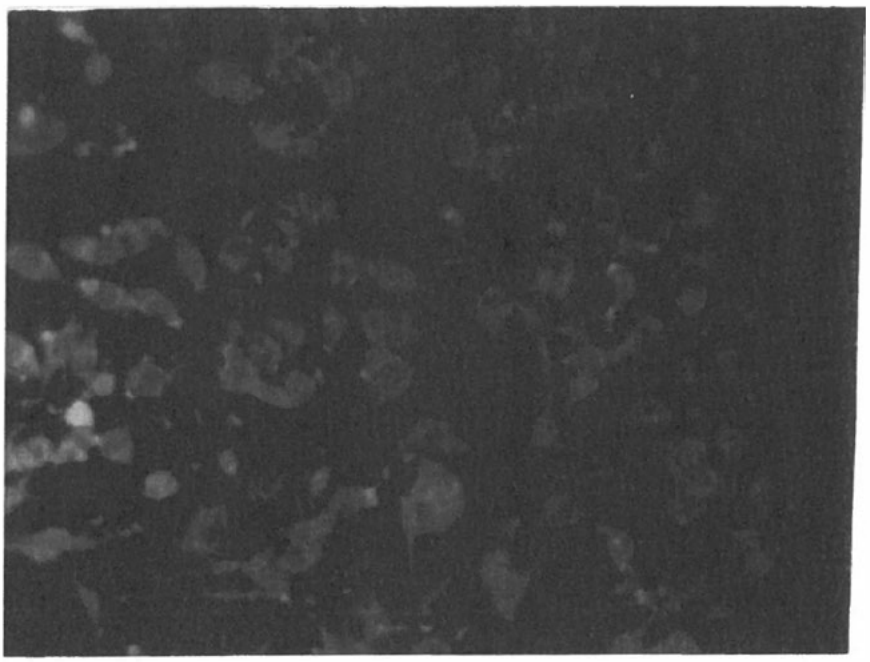


Plate 2. Immunofluorescence stained inclusions produced by *Chlamydia trachomatis* infection of McCoy cells in MIF test. 10^5 dilution at x40 magnification.

Table VII Titration of *C. trachomatis* serovar dilutions by MIF test

Dilution	Well	Objective	Results
10^{-2}	1	x10	Dilution too high to count
	2	x10	
10^{-3}	1	x40	Dilution too high to count
	2	x40	
10^{-4}	1	x40	Dilution too high to count
	2	x40	
10^{-5}	1	x40	13, 26, 19 = 58/3 = 19.3 (3 = fields counted)
	2	x40	15, 18, 18 = 51/3 = 17.0 } average = 18.1
10^{-6}	1	x10	34, 26, 31 = 91/3 = 30.3
	2	x10	25, 27, 31 = 83/3 = 27.6 } average = 28.95

MIF = microimmunofluorescence
Dilutions (100 μl) of each isolates (n=9) were infected in duplicates onto McCoy cell monolayers; 48 h post-infection, chlamydial inclusions were detected by a direct fluorescein isothiocyanate-conjugated (FITC) monoclonal antibody to *Chlamydia* lipopolysaccharide (CF-2).
Inclusions were counted in three fields of view using x10, x16, and x40 objectives which have a conversion factor of 10, 25, and 150 respectively.

To calculate the no. of IFU/ml:

1) 10^{-5} dilution

$= 18.1 \times 10 \times 150 \times 10^5$

$= \underline{2.7 \times 10^9}$

2) 10^{-6} dilution

$= 28.95 \times 10 \times 10 \times 10^6$

$= \underline{2.9 \times 10^9}$

AVERAGE = $\underline{2.8 \times 10^9 \text{ IFU/ml}}$ in the isolate

In the calculation: 150 and 10 = x40 and x10 lens conversion factors

10^5 and 10^6 = dilutions

Factor 10 = amount plated factor i.e 1000 μl/100 μl

1000 μl > total volume of serial dilutions prepared for IFU titration **and**

100 μl > amount of inoculum added to each well

3.6.5.4 Preparation of Chlamydial Antigen for Microtitre Typing

In order to determine the serotype of serovar, 50 70% cell confluency is required i.e approximately 1/ 3of the usual number of McCoy cells per well.

If 1.24 ml of 34 X 10^5 cells/ml were used to obtain 90 95% cell confluency by 24 h (4.10.2), then to get 75% required for typing: => $\frac{1.2 \times 75}{100}$

=> $\underline{0.9 \text{ ml of cell suspension}}$

Therefore: Add 12 0.9 = 11.1 ml of CMGH media to 0.9 ml of cell suspension.

After 24 h incubation of McCoy cells in a microtitre plate, the monolayers were inspected for contamination and for the required 50 70% confluency. Spent medium was removed with vacuum and 10 μl of CMGH (containing NGV + cycloheximide) were added. Following that, 10 μl of the last dilution where the

IFUs were too numerous to count, in this case the dilution was 10^4 (Table VII) were added to the monolayers.

Inoculated plates were centrifuged (Beckman J6-M centrifuge, 4.2 rotor) at 1.200 x *g* at 37^{0}C for 60 min. After centrifugation, the inoculum was aspirated from the wells, and then replaced with 200 μl of fresh medium containing 10% fetal bovine serum (BSA) and 1.0 μg/ml cycloheximide. This was followed by incubation at 37^{0}C in 5% CO_2 for 40 48 h. After incubation, spent medium was aspirated and the monolayers fixed with methanol for 10 min by placing the plate on a shaker, and then air dried (5 10 min).

Each well was rinsed with a drop of PBST prior to adding approximately 200 μl of MAb-blocking and preservative (MABP) solution (2% BSA, PBST, 0.1% sodium azide) to the fixed wells and incubating for 4 h at 37^{0}C. At this stage, the plates were ready for staining and serotyping. If overnight storage was required, plates were held at 4^{0}C.

3.6.5.5 Serotyping of the Isolates by Microtitre Method

After 4 h incubation, MABP was removed and 25 μl of the MAb dilution (12 MAbs per row per plate) was added to the appropriate wells. Plates were agitated at a moderate speed on a shaker for 2 h. Thereafter, MAbs were carefully removed from the wells and then replaced in their original containers. Due to the unavailability of commercial MAbs, these MAbs had to be reused, therefore, extra care was taken when removing MAb from each well making sure that the correct MAb was placed into its correct tube.

The MAbs which were reacted with the fixed inclusions of each isolate, consisted of a panel of 17 *C. trachomatis* species , group , subgroup , and serovar-specific MAbs. The optimal titre of the MAbs for the detection of prototype strain inclusions had already been determined. Table VIII (Suchland and Stamm, 1991) shows the positions and titre of MAbs used for serotyping. This table was used as a guideline for determining serotypes of the isolates tested.

Using a pasteur pipette, wells were washed 3X with PBST, followed by addition of 20 μl of FITC goat antimouse immunoglobulin G (IgG) to each well. Plates were again placed on a shaker for 30 min at room temperature to allow the conjugate counterstain to react. The wells were washed once with PBST and finally with distilled water (dH_2O). Plates were inverted on paper towel to remove excess water. One drop of FA mounting fluid (50% glycerol, 50% Tris buffer pH 8.5) was added to each well, and the excess was shaken off. Plates were placed at 4^0C and read within 24 h.

Reading was done by inverting the microtitre plates and scanning each well with an epifluorescence microscope (Zeiss) at x100 magnification with a x10 Neofluor lens. Wells were scored according to the intensity of fluorescence of inclusions as: , $\pm$, (+), and +. Lack of or no fluorescence was recorded as , barely detectable fluorescence as $\pm$, light fluorescence as (+), and strong fluorescence as +. Serotype designation was assigned by numerically scoring positive wells and matching the isolate's numerical code to the numerical code of the assigned prototype antigen serotype.

Table VIII Microtitre serotyping monoclonal antibody (MAb) reaction patterns and scoring scheme cited from Suchland and Stamm (1991)

Group and complex [a]	Serovar	Reaction pattern for the following MT plate wells (MAb B complex/C complex [MAb titer])[b]												
		1[c] (LV-22 [1:40])	2 (LL-33 [1:30])	3 (GG-11 [1:30])	4 (BB-11 [1:40])	5[d] (BB-3 /PE-5 [1:40])	6 (DP-1/ AC-11 [1:40])	7 (LV-23/ LA-10 [1:40])	8 (LV-27/ CC-1 [1:40])	9 (KB-8 [1:40])	10 (JG-9 [1:40])	11 (DD-1/ KK-1 [1:40])	12 (FC-2 [1:40])	Numerical code designation
Group 3 C complex	C	+	(+)						+					**2-8**
	J	+	(+)	(+)					+					**2-3-8**
	I	+	(+)			+	+							**2-5-6**
	Ia	+	(+)	(+)		+								**2-5**
	I'	+	(+)	(+)										**2-pd7+**[f]
	A	+	(+)				+							**2-6**
	H	+	(+)	(+)				+						**2-3-7**
Intermediate	K	+	+			(+)						+		**2-5-11**
	L3	+	+	+										**2-3**
Group 2 F complex	G	+		+		(+)					+/-		+/-	**3-5**
	F	+				+/-					+/-		+	**12**
Group I B complex	B	+			+	+						+		**4-5-11**
	Ba	+			+	+		(+)			(+)			**4-5-7-10**
	L2	+			+	+		+	+					**4-5-7-8**
	L2a	+			+	+		+						**4-5-7**
	E	+			+			+		+				**4-7-9**
	Ea	+			+			+						**4-7**
	L1	+			+					+	+			**4-9-10**
	D	+			+		+	(+)/ +/-[e]			+/-			**4-6**
	Da	+			+			(+)/ +/_			+			**4-10**
	D'	+												**10**

[a] Group designations are based on amino acid homologies. Complex designations are based on serological relatedness.
[b] Scores for reaction patterns are as follows: +, strong fluorescence; (+), barely detectable fluorescence; -, no fluorescence. The B complex-C complex was made up of pooled MAbs.
[c] Well 1 (MAb LV-22) was common to every serovar and is not included in the code. Light type indicates a light-staining fluorescence reaction; bold type indicates a strong fluorescence reaction.
[d] MAb BB-3 is not included in the MA micro-IF serotyping kit. [e] ± or (+)/± reactions were not critical to scoring. [f] MAb PD-7 (not available for WRF) was used in our laboratory for confirmation of serovar I'.

3.7 ANALYSIS OF DATA

Where required, patient data were analysed using the Chi square (X^2) test with Yate's correction. The level of significance was set at 95% and recorded as $p \leq 0.05$.

The Kappa (K) statistic was calculated as follows:

$$K = \frac{2(ad - bc)}{P_1Q_2 + P_2Q_1}$$

CHAPTER 4

RESULTS

4.1 INTRODUCTION

This study comprised a part of the Department of Medical Microbiology's epidemiological survey of genital ulcer disease. The methodology used for the diagnosis of GUD other than LGV, are not part of this study and are therefore, not discussed. All 247 specimens were designated codes i.e specimen codes (P001 045 and U001 202) in the order of specimen collection and laboratory codes (randomly from 1 to infinity). This was done to distinguish one specimen from the other, and to avoid introduction of any form of bias while performing various laboratory tests. Results of different tests were recorded and analyzed separately. Results obtained are represented in both the specimen and laboratory codes.

4.2 DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF THE POPULATION STUDIED

During the period 3 July 1995 to 22 August 1995, a total of 247 patients consented to participate in the study. They were all adult males and predominantly of African race, with a mean age of 25.7 (range, 15 to 54 years). The majority of the patients were unmarried 215 (87%). The mean age at first intercourse was 15.4 years, and the mean number of sexual partners in preceding 6 months was 3 (range, 1 20). Of the 247 men, 141 (57,1%) had a history of a previous STD.

A total of 18 (7.3%) patients were circumcised. On examination of the genitalia, 91 (37%) had single lesions and 156 (63.2%) multiple lesions. These ulcers were located on the penile shaft, coronal sulcus, prepuce (outside and inside), and glans. Phimosis was present in 26 (10.5%). Lymphadenopathy (either unilateral or bilateral) was detected in 107 (43.3%) of the patients, 11 (4.5%) of whom had buboes. Data obtained from patients are depicted in Table XI.

4.3 OVERALL LABORATORY FINDINGS

As this study was part of a comprehensive departmental genital ulcer study, the microbial agents isolated from the patient population studied are presented in Table XII. The diagnosis of the conditions was essentially based on culture except for syphilis which was based on serology. *Haemophilus ducreyi* was the most prevalent and was isolated from 83 (34%) of the specimens. Syphilis, defined as positive RPR and TPHA, was diagnosed in 70 (28.3%) patients, while genital herpes, granuloma inguinale and lymphogranuloma venereum (LGV) were detected in 28 (11.3%), 15 (6.1%) and 13 (5.3%) respectively. The 13 (5.3%) which were positive for LGV are based on culture without immunotyping. In 38 (15.4%) patients, none of the diseases investigated for, were diagnosed. Mixed infections were detected in 35 (14.2%) of the patients, 25 of whom had syphilis. LGV was detected in 2 patients as a single infection and in the remaining 11, as mixed infections. Four of these patients were infected concurrently with syphilis, 4 with chancroid, and 3 with granuloma inguinale. Triple infections with syphilis, chancroid and granuloma inguinale were detected in 2 patients (Table XIII).

Of the endourethral swab specimens (n=247), *Neisseria gonorrhoeae* was detected in 21 (8.5%) patients by culture. A total of 128 (52.2%) patients were seropositive for human immunodeficiency virus (HIV).

Table IX Demographic, history and clinical characteristics of patients presenting with genital ulcers (n=247) at the City Health STD clinic, Durban

Characteristics	Number (mean/%)
Age in years	
Range (mean)	15-54 (25.7)
Age at first intercourse	
Range (mean)	13 21 (15.4)
Marital status	
Single	215 (87%)
Married	32 (13%)
Sexual partners (past 6 months)	
Range (mean)	1-20 (3.0)
Previous history STDs	
Present	141 (57.1%)
Absent	106 (42.9%)
Circumcision status	
Circumcised	18 (7.3%)
Uncircumcised	229 (92.7%)
Phimosis	
Present	26 (10.5%)
Number of lesions	
Single	91 (36.8%)
Multiple	156 (63.2%)
Inguinal lymphadenopathy	
Present*	107 (43.3%)
Buboes	11 (4.5%)

* 11 (4.5%) of the 107 (43.3%) patients also had buboes.

Table X A breakdown of sexually transmitted diseases detected in patient population presenting with genital ulcers (n=247)

	Single	Mixed	Total
Chancroid	62	21	83 (33.6%)
Syphilis*	45	25	70 (28.3%)
Genital herpes	21	7	28 (11.3%)
Granuloma inguinale	7	8	15 (6.1%)
Lymphogranuloma venereum	2	11	13 (5.3%)
None of the above detected	-	-	38 (15.4%)

* All the above diseases but Syphilis, were detected using culture methods. Syphylis was diagnosed on the basis of positive syphilis serology.

Table XI Microbiological diagnosis in 35 patients with mixed infections presenting with genital ulcers at the City Health STD clinic, Durban

Infections	No. of patients
Double infections	
Chancroid + Syphilis	12
Chancroid + Genital herpes	3
Chancroid + **LGV**	4
Granuloma inguinale + **LGV**	3
Syphilis + Granuloma inguinale	3
Syphilis + **LGV**	4
Syphilis + Genital herpes	4
Triple infections	
Syphilis + Chancroid + Granuloma inguinale	2

LGV = Lymphogranuloma venereum
Microbiological diagnosis of the diseases was based on the specimens collected from 247 patients who presented with genital ulcers at the City Health STD Clinic, Durban.

4.4 GENITAL ULCER SPECIMEN RESULTS

4.4.1 Results of *C. trachomatis* Culture

Culture of genital ulcer lesions for *C. trachomatis* was performed on all 247 patients; for the first 200 specimens, this was done within 24 hours of specimen collection and for the last 47 specimens, after a week of storage at 70^0C. After the initial evaluation following 48 hours incubation, *C. trachomatis* inclusions were detected in 10 (4%) of the specimens by fluorescent monoclonal antibody staining (Plate 3). No non specific clusters of fluorescent particles were found in these 10 and free extracellular fluorescent particles were seen only rarely. A further 47 (19%) of the specimens were categorised as doubtful because free apple green fluorescent particles could be visualised. These specimens were subjected to a blind passage together with the 190 (76.9%) negative specimens in which no inclusions were detected.

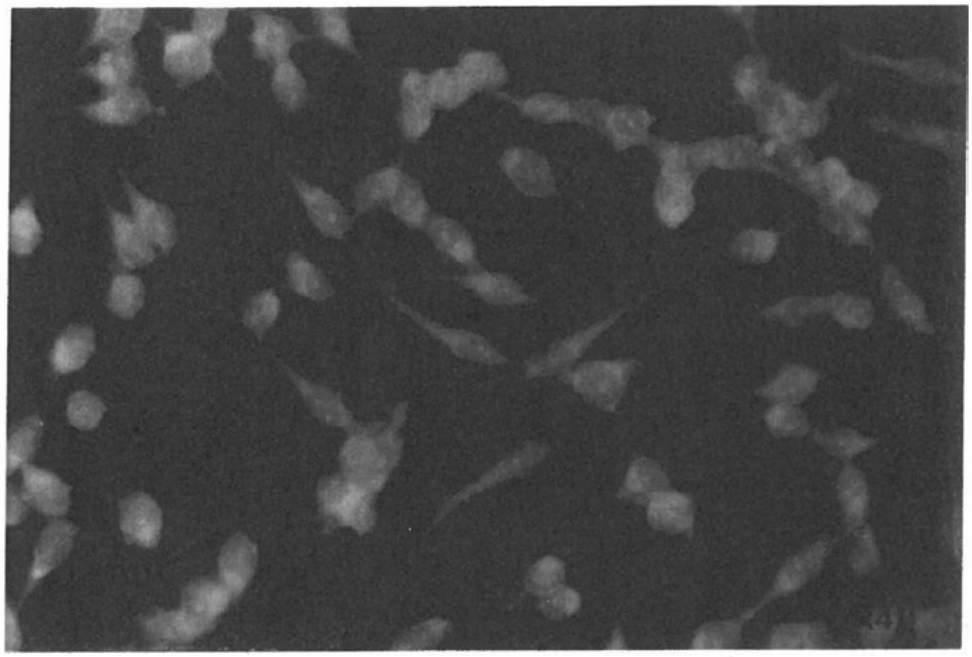
Following blind passage and a 48 h incubation, a further 3 specimens developed the characteristic apple green inclusion forming units (Plate 4, 5, 6,7). None of the clinical specimens which were stored at 70^0C for a week prior to inoculation, produced inclusion bodies when evaluated initially, nor after subjection of blind passage. In total, *C. trachomatis* was successfully isolated from cell cultures in 13 (5.3%) of the specimens tested and the remaining 234 (94.7%) cultures were negative. Table XIV shows the number of inclusions recorded per positive sample.

Table XII Number of inclusion forming units (IFU) per coverslip from cycloheximide-treated McCoy cells infected with genital ulcer specimens

Passage	Specimen Number	Number of Inclusions*
First passage	P 016	$\geq$ 200
	P 039	10 100
	P 042	$\geq$ 200
	U 041	$\geq$ 200
	U 090	$\geq$ 200
	U 113	10 100
	U 119	101-200
	U 128	101-200
	U 135	101 200
	U 145	$\geq$ 200
Second passage	P 010	< 10
	U 144	< 10
	U 149	< 10

Cultures grown on coverslips were fixed with methanol and stained with fluorescein-labeled monoclonal antibodies specific for *C. trachomatis* detection.

* Inclusions per coverslip were counted 48 h (first passage) and 72-96 h (second passage) post-infection. 234 isolates did not produce inclusions even after several passages.



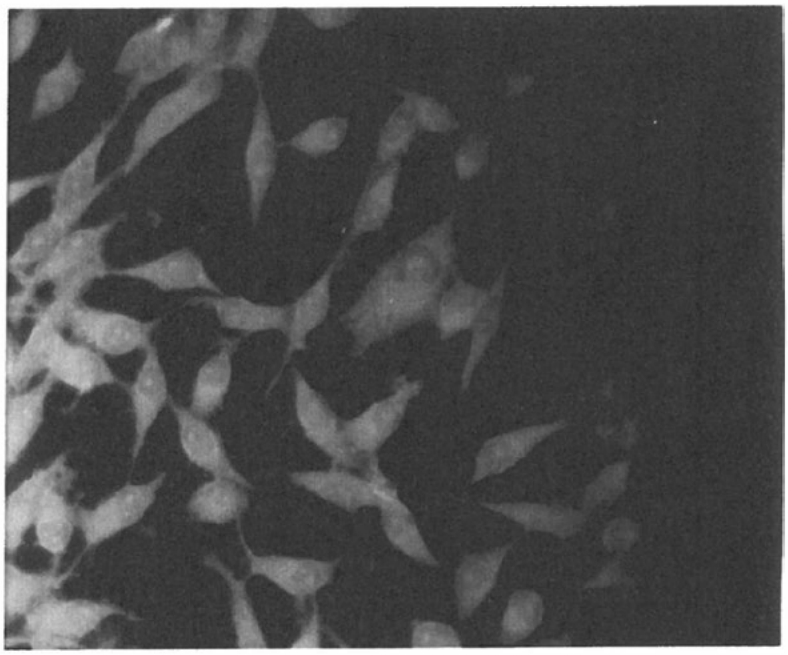
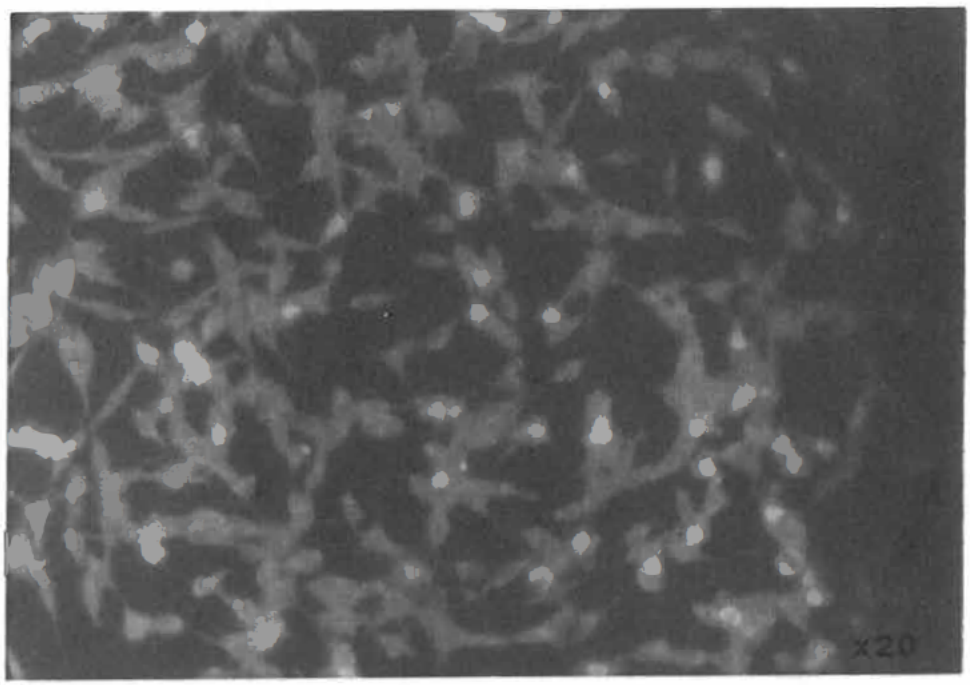


Plate 3. Uninfected McCoy cells showing the characteristic spindle shape of fibroblast cells. The cells appear red in colour due to Evans blue counterstain. Arrows show artifacts (light green fluorescing particles) often caused by residual culture media.



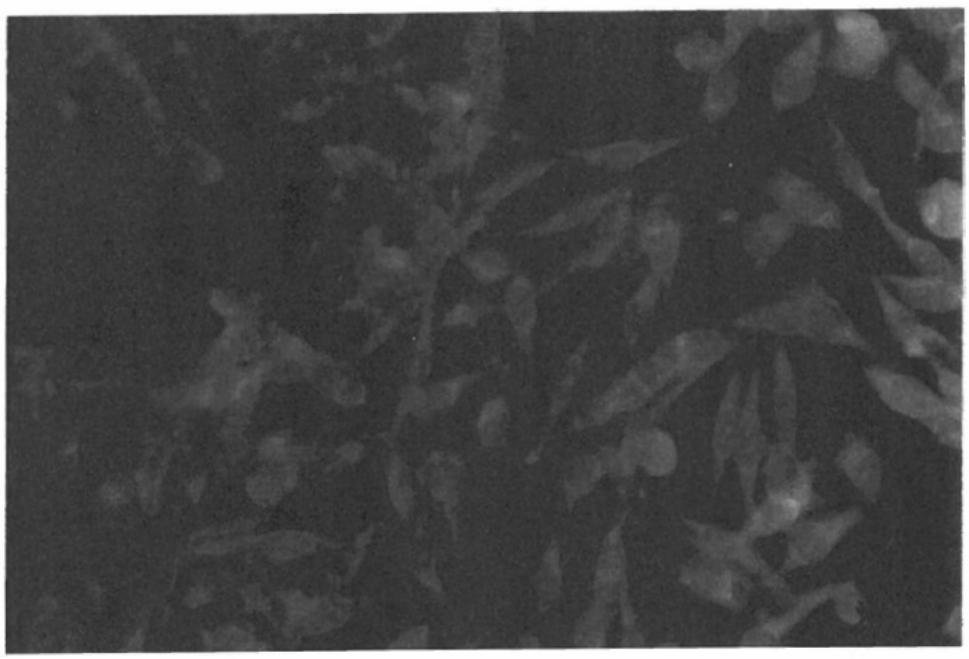


Plate 4. *C. trachomatis*-positive culture showing apple-green fluorescent inclusions contrasted against the red background of the Evans-blue counterstained cells. Plate above shows inclusions are clearly visible even at low magnification (x20). In plate below, Elementary bodies (EBs) and/or reticulate bodies (RBs) can be seen inside inclusions at x40 magnification.

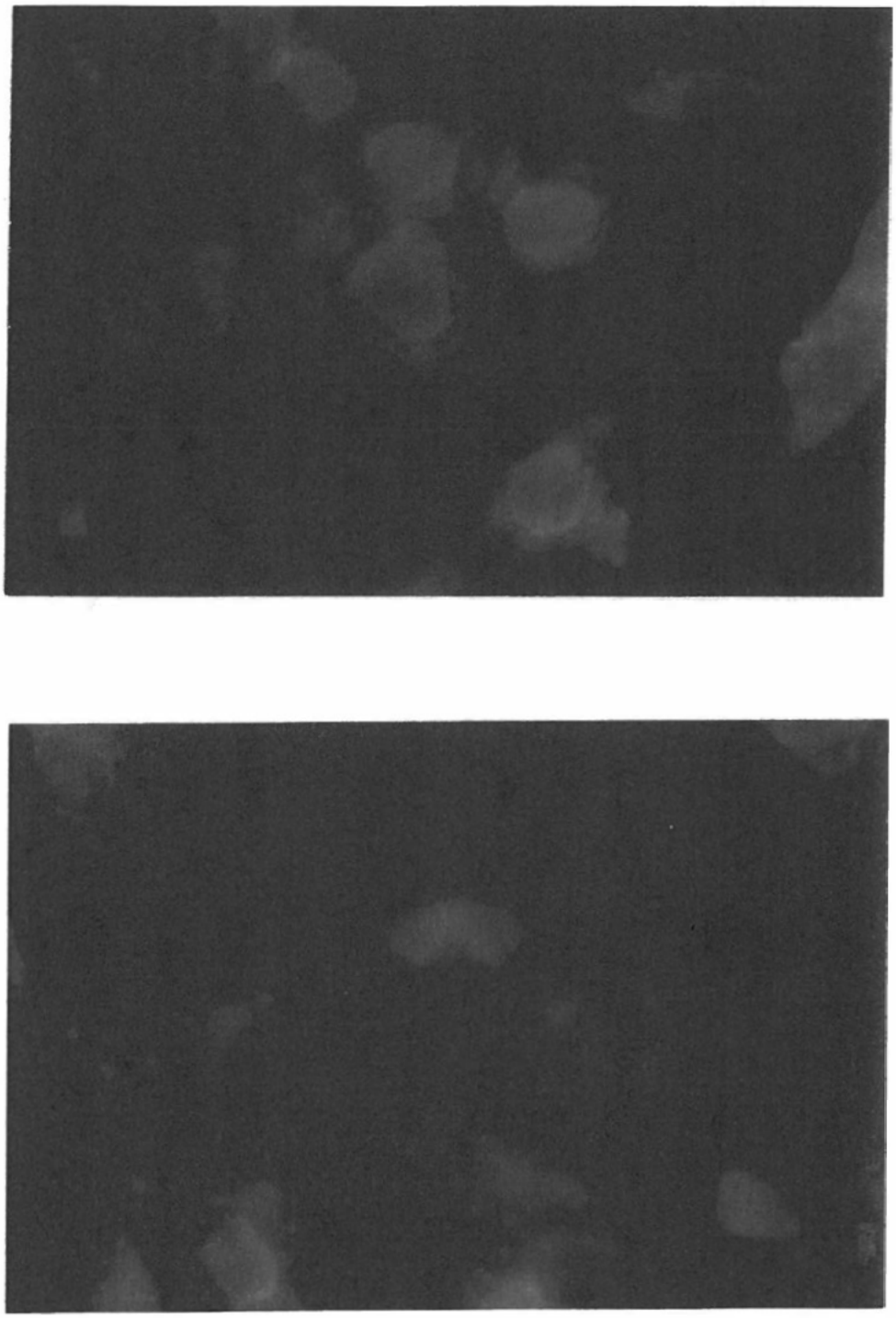


Plate 5. Inclusions produced by *Chlamydia trachomatis* infection of McCoy cells. Monolayers stained with fluorescent monoclonal antibody stain (x100, oil objective). Two inclusion bodies inside host cell, push host cell nucleus towards one side. Arrows show host cell nucleus.

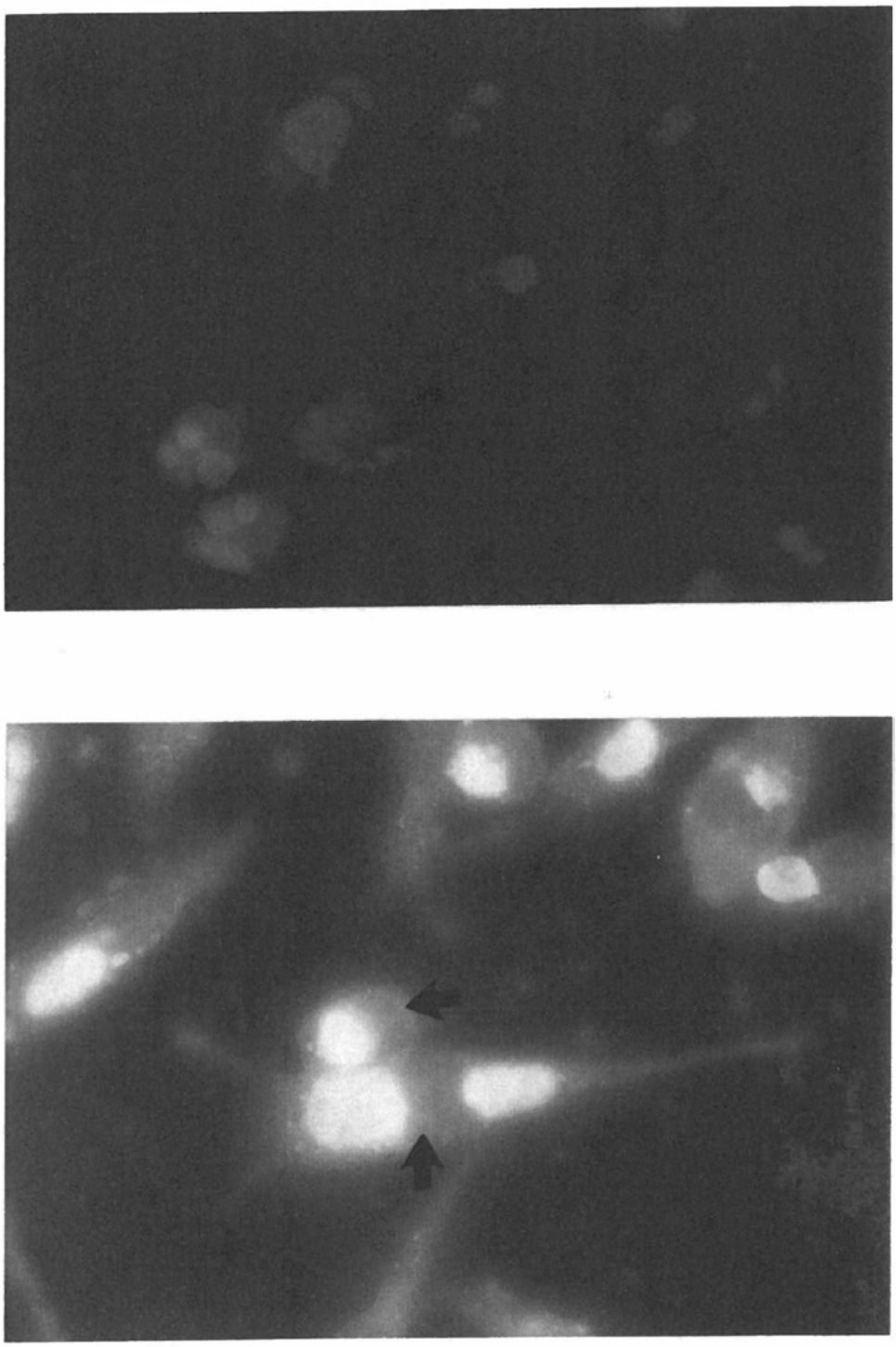
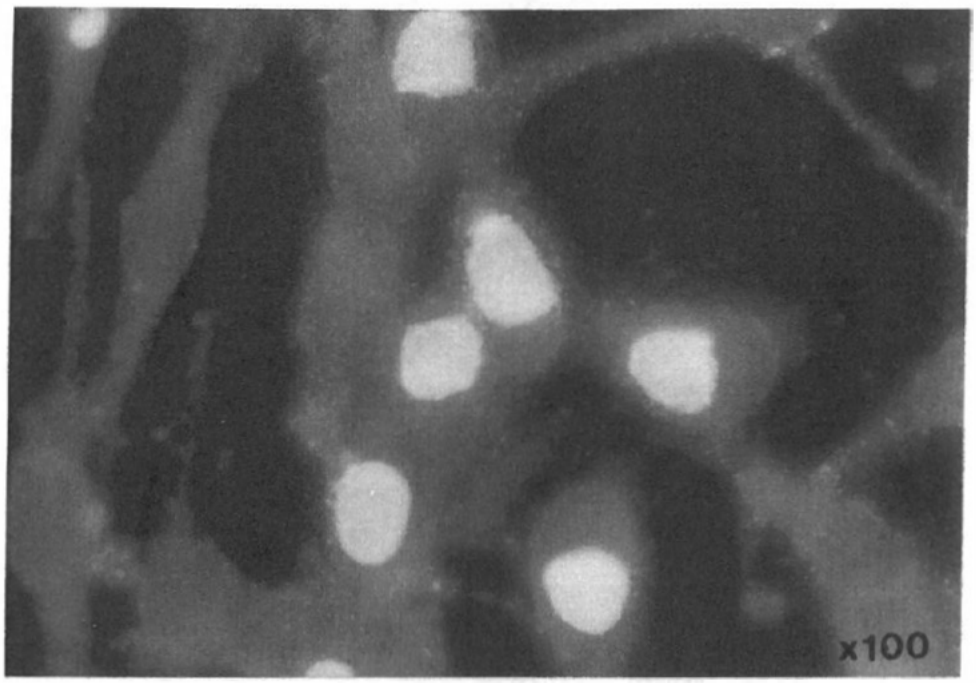


Plate 6. Photographs showing multiple inclusion bodies inside host cells (x100, oil objective). Arrows show the nucleus of the host cell. The bottom photograph shows a spindle-shaped McCoy cell with two inclusions sandwiching host cell nucleus, and another infected cell is attached to it.



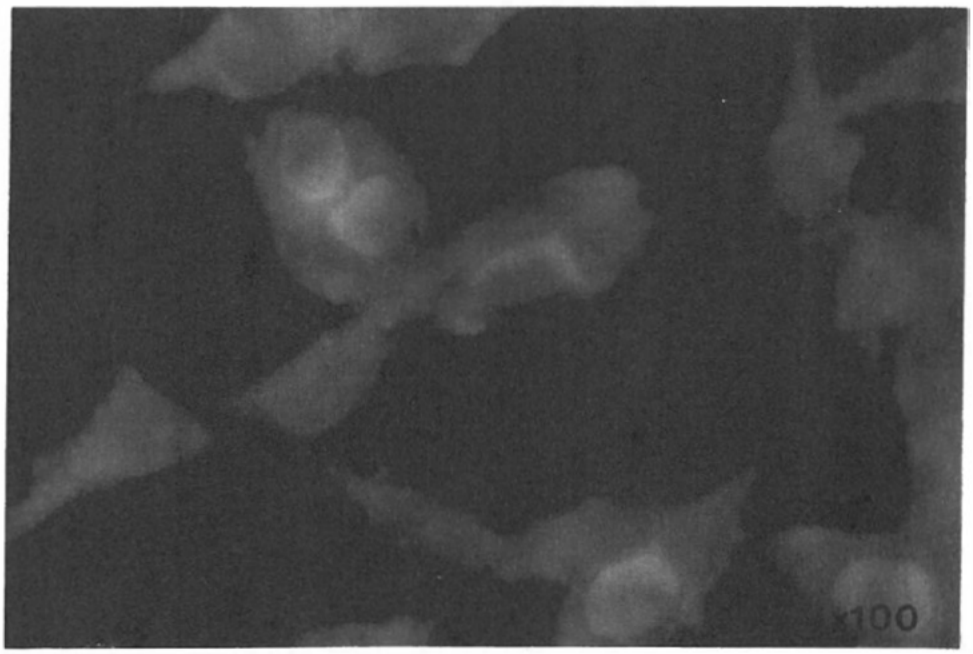


Plate 7. Free fluorescing *C. trachomatis* EBs are visualised outside McCoy cells 48 h post infection with genital ulcer specimens. At the end of the cycle (48 96 h), inclusions release their EBs at various times to infect other cells.

4.4.2 Results of Specific Amplification of Cryptic Plasmid and MOMP Genes by PCR

4.4.2.1 PCR Sensitivity using *C. trachomatis* L2 DNA

Initial experiments were performed on the positive control, *C. trachomatis* L2 DNA (from University of Washington, Seattle) with the two primer sets (plasmid and MOMP) at annealing temperatures of 67^0 and 55^0C, respectively. Serial dilutions of 10^{-2} to 10^{-10} were prepared and the resulting amplified products were analysed on a 1.5% agarose gel stained ethidium bromide. As anticipated, using primers for the cryptic plasmid and MOMP genes, a 452 base pair (bp) and 1128 bp product was amplified respectively. Higher sensitivity was obtained with primers for the endogenous plasmid (10^{-10} dilution) as compared to the MOMP gene (10^{-7}) of *C. trachomatis* L2 DNA (Plate 8).

4.4.2.2 PCR with culture-positive and -negative material

The 247 genital ulcer patient specimens cultured onto McCoy cell monolayers were used as templates for amplification of the endogenous plasmid by PCR. All 13 genital ulcer specimens which successfully infected McCoy cells also yielded the 452 bp product after amplification of the endogenous plasmid DNA by PCR (Plate 9), while only 10 yielded the 1128 bp product specific for the MOMP gene (Plate 10). The intensity of the 452 bp product decreased with decreasing numbers of IFU of the culture sample and the corresponding ethidium bromide stained gel is shown in Plate 10. The results are summarized in Table XIII.

When the 234 samples that were negative by culture were subjected to specific amplification of the cryptic plasmid gene, the expected 452 bp fragment was obtained in 42 (17%) (Plate 11 and 12). Sixteen (38%) of the samples (n=42) yielded products specific for the MOMP gene. The remaining 26 samples which were negative by culture yielded notably weak signals in the specific amplification of the weak plasmid gene.

Hence, overall, specific amplification of the 452 bp product of the *C. trachomatis* cryptic plasmid DNA by PCR was detected in 55 (22.3%) of the samples as compared to 26 (10.5%) for the 1128 bp of the MOMP gene.

As culture and PCR (using primers specific for amplification of endogenous plasmid gene) were used to diagnose *C. trachomatis* in 247 genital ulcer specimens collected, comparison of the results obtained by the two techniques are shown in Table XIV. Both tests together, detected *C. trachomatis* in 55 (22.3%) specimens. Culture was unable to detect 42 (17.0%) of the specimens that were detected by PCR. There were 13 (5.3%) true positives and 192 (77.7%) true negatives. As suggested by other workers, PCR and culture were considered as an expanded gold standard and the former had a 100% detection rate while culture detected 25% of the infections. To measure the agreement between PCR and culture results, Kappa statistic (K) was calculated; it was found to be 0.485 indicating a moderate agreement.

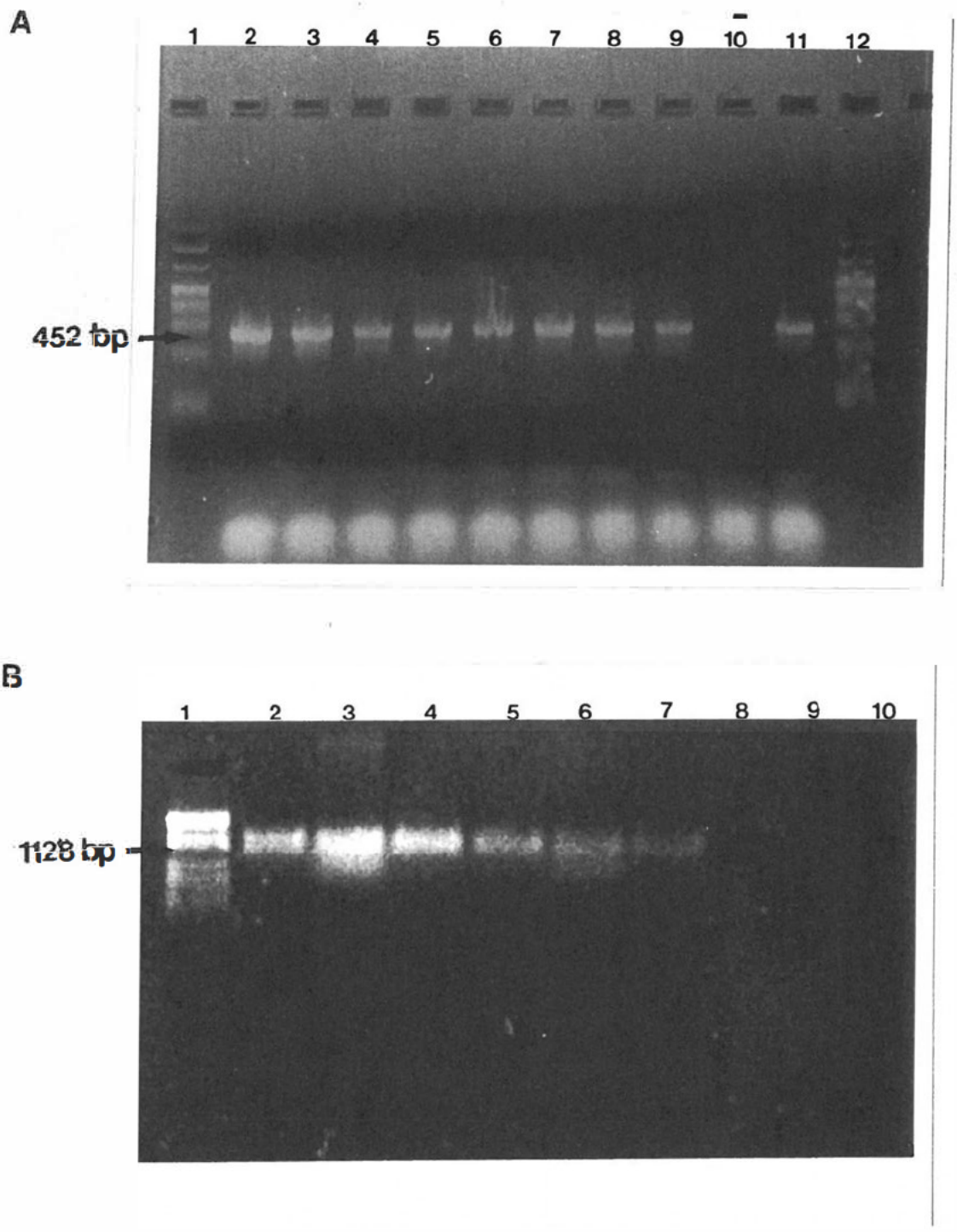


Plate 8. PCR on a 10-fold dilution series (10^{-2} to 10^{-10}) of *C. trachomatis* L2 DNA with **(A)** oDnaBL and oDnaBR primers and **(B)** o5'MOMP and oDVDIV primers, for specific amplification of the endogenous plasmid DNA and the MOMP gene by PCR, respectively. **A and B**: lanes 1, molecular weight marker (M,M) VI (154-2176 bp, Boehringer); lanes 2-9, 10^{-2} to 10^{-9} dilutions; lanes 10, PCR negative controls; lanes 11, 10^{-10} dilution.

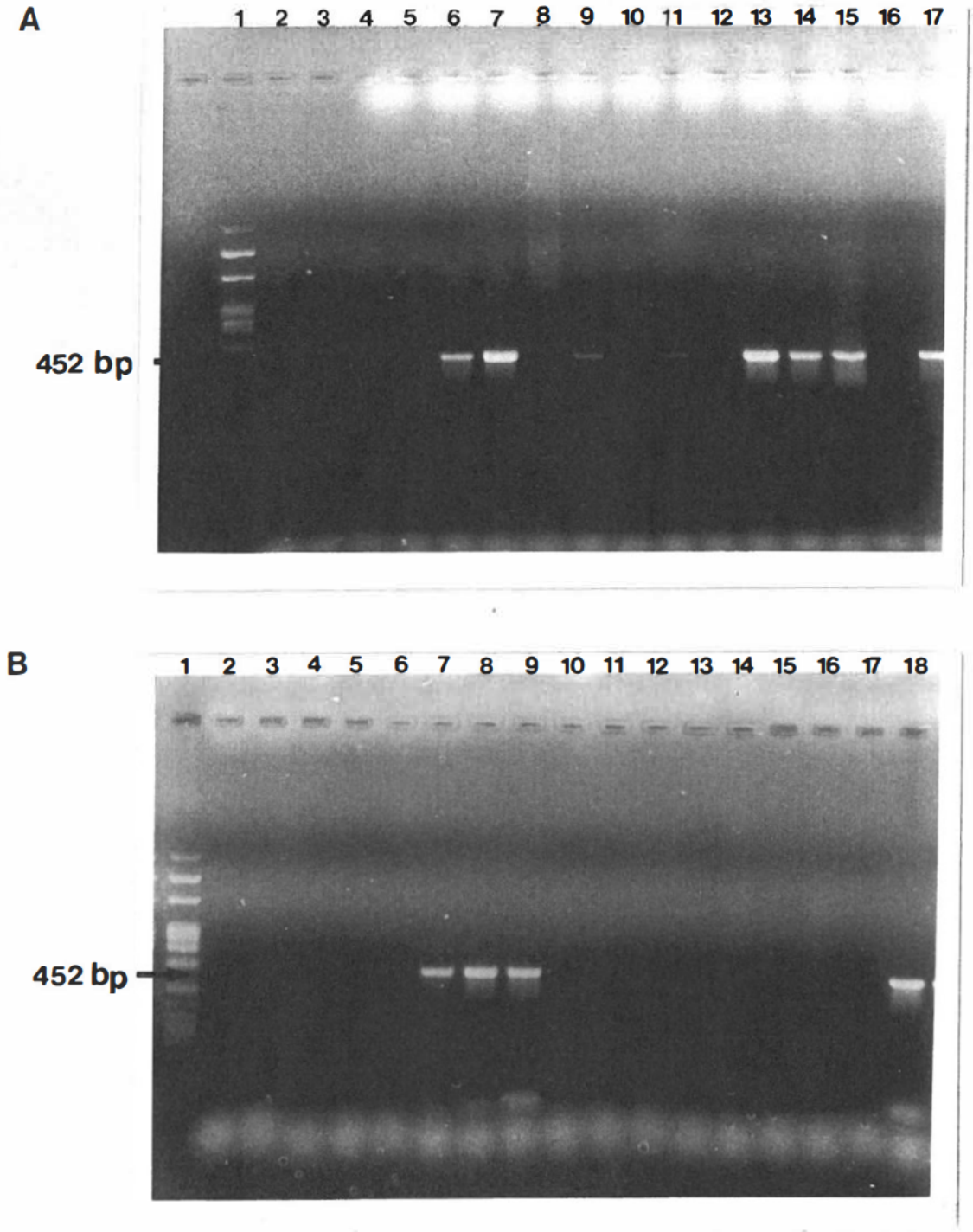


Plate 9. Agarose gel (1.5% w/v) electrophoresis analysis of PCR products by using oDnaBL and oDnaBR primers specific for amplification of *C. trachomatis* plasmid DNA from the 13 samples that were positive by culture. The 452 bp fragment obtained with plasmid primers. **A)** Lane 1, M_rM VI (154 2176 bp, Boehringer); lanes 3-5, culture-negative samples; lanes 6-11, 13-15, 17, culture positive samples (U113, U145, P010, P039, U149, U144, P016, U119, U128, U041); lanes 2, 12, 16, PCR negative controls. **B)** Lane 1, M_rM VI (Boehringer); lanes 3 5, 10, 11, 13 15, culture-negative samples; lanes 7-9, culture positive samples (U135, P042, U090); lane 17, PCR negative control; lane 18, positive control (L2 DNA).

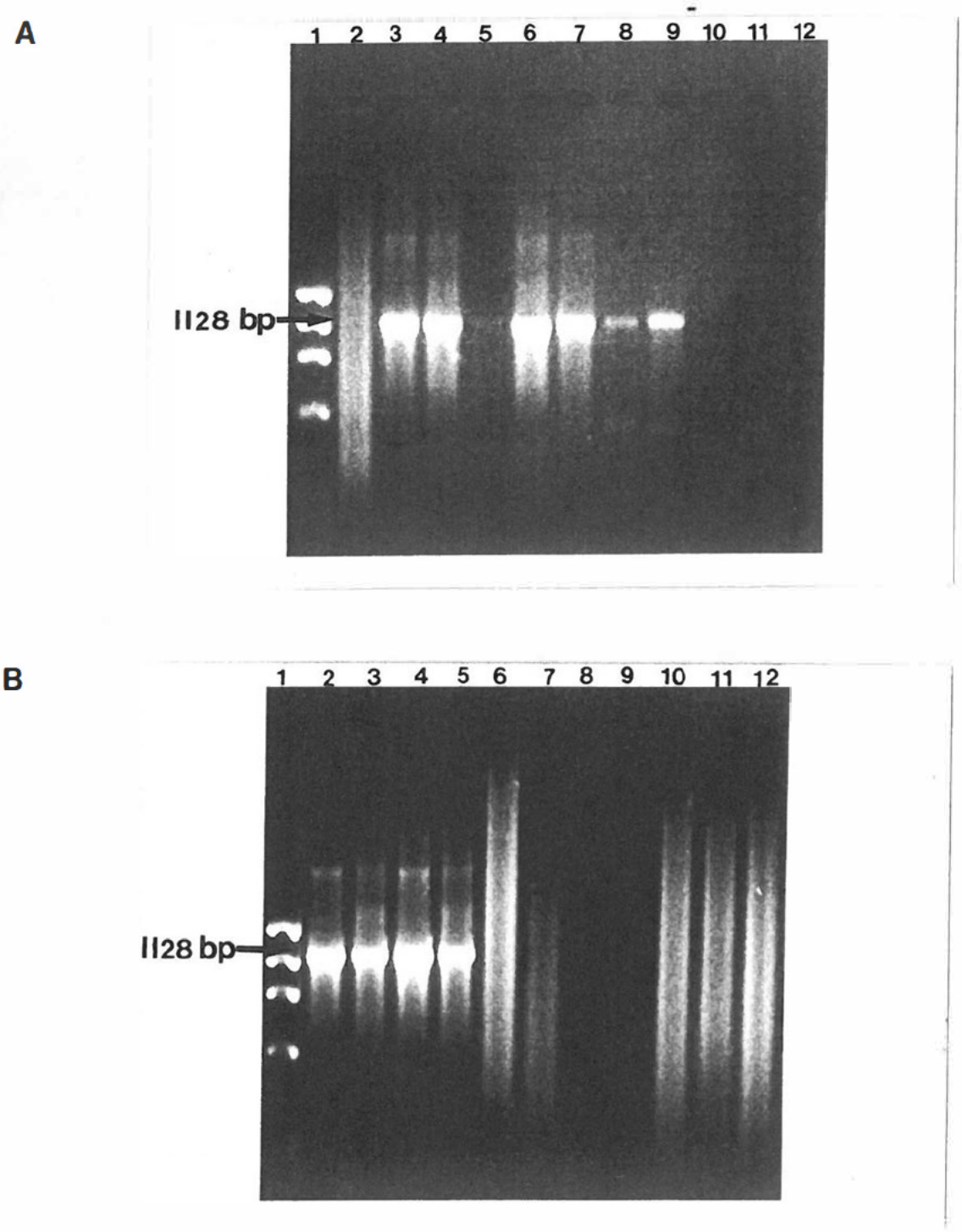


Plate 10. Electrophoretic analysis of PCR products after specific amplification of the MOMP gene from the 13 *C. trachomatis* culture positive genital ulcer specimens. The 1128 bp fragment obtained by PCR with primers o5'MOMP and oDVDIV. **A)** Lane 1, M_rM IX (72 1353 bp, Boehringer); lanes 2 8, culture positive samples (P010, P016, P042, P039, U041, U090, U113); lane 9, positive control (L2 DNA); lane 10, PCR negative control. **B)** Lane 1, M_rM IX (Boehringer); lanes 2 7, culture positive samples (U119, U128, U145, U135, U149, U144); lanes 8, 9, PCR negative controls; lanes 10-12, culture negative samples.

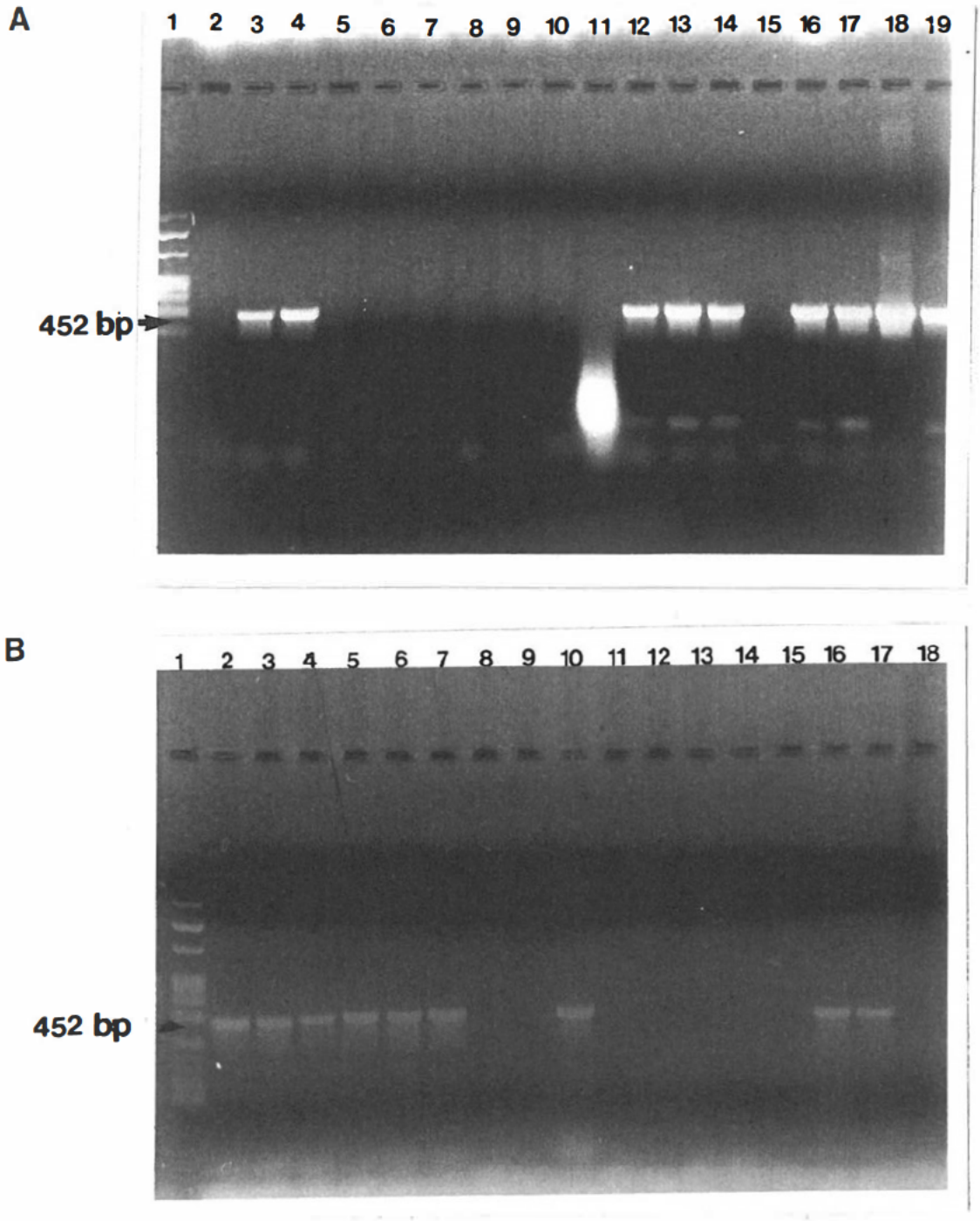


Plate 11. Detection of *C. trachomatis* plasmid DNA in culture negative genital ulcer samples: analysis of the PCR products by agarose gel (1.5% w/v) electrophoresis. Arrows indicate the size (452 bp) expected to be amplified from the *C. trachomatis* genome using primers oDnaBR and oDnaBL. **A)** Lane 1, M_rM VI (154 2176 bp, Boehringer); lane 2, PCR negative control; lanes 3 18, culture negative samples (P034, P005, P014, P021, P011, P019, P023, P036, P043, U083, U120, U161, U060, U200, U048, P001); lane 19, positive control (L2 DNA). **B)** Lane 1, M_rM VI (Boehringer); lanes 2 8, culture-negative samples (U165, U175, U103, U039, U162, U057, U061); lane 9, 18, PCR negative controls; lanes 10 17, culture negative samples (U012, U073, U078, U001, U094, U009, U098, U174).

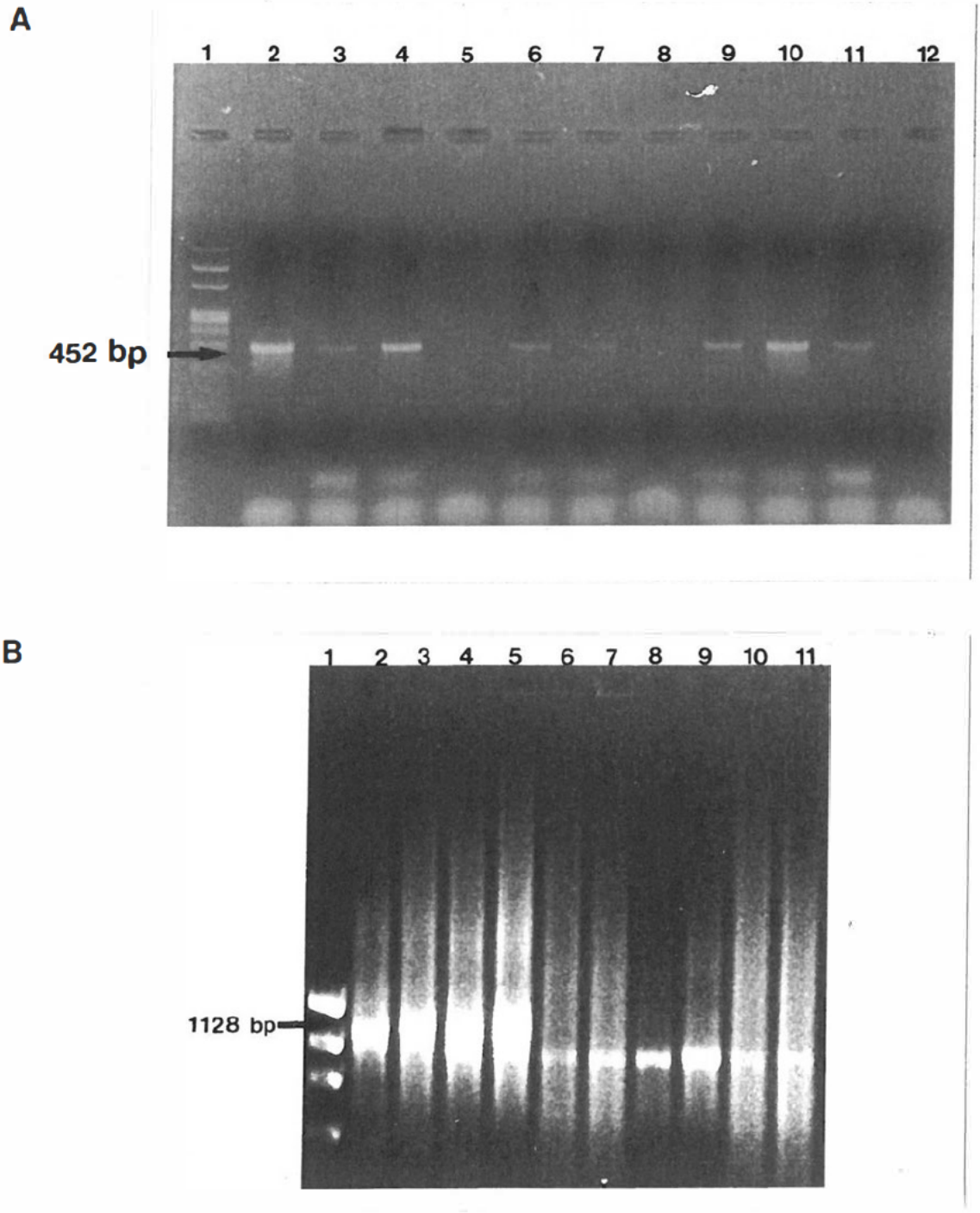


Plate 12. Investigation of a series of culture-negative genital ulcer specimens by PCR. **A)** Specific amplification of the 452 bp fragment of the *C. trachomatis* plasmid gene using oDnaBR and oDnaBL primers. Lane 1, M_rM VI (154 2176 bp, Boehringer); lane 2, positive control (L2 DNA); lanes 3 11, culture-negative samples (U086, U154, U106, U153, U163, U047, U093, U185, U067); lane 12, PCR negative control. **B)** Specific amplification of the 1128 bp fragment of the MOMP gene by PCR using primers o5'MOMP and oDVDIV. Lane 1, M_rM IX (72-1353 bp, Boehringer); lane 2, positive control (L2 DNA); lanes 3 11, culture-negative samples (U200, P005, U057, P017, U027, U029, U193, U121, U136); lane 12, PCR negative control.

Table XIII Detection of *C. trachomatis* in culture-positive specimens (n=13), by PCR with primers for the specific amplification of the MOMP and the endogenous plasmid genes

Specimen Number	PCR results	
	Plasmid Gene	MOMP Gene
P 010*	±	—
U 144*	±	—
U 149*	±	—
U 041	+	+
P 016	+	+
P 039	+	+
U 113	+	+
U 119	+	+
U 128	+	+
P 042	+	+
U 145	+	+
U 090	+	+
U 135	+	+

PCR, polymerase chain reaction
MOMP, major outer membrane protein
±, represents weakly positive products at gel level; +, positive; -, negative.
* Specimen positive on sub-culture only.

Table XIV Comparison of cell culture and PCR for the detection of *C. trachomatis* in genital ulcer samples (n=247)

	PCR		
CULTURE	POSITIVE	NEGATIVE	TOTAL
POSITIVE	13	0	13
NEGATIVE	42	192	234
TOTAL	55	192	247

PCR, polymerase chain reaction
Results of PCR with plasmid primers carried out on culture samples
When specific amplification of the cryptic plasmid gene by PCR and culture used as an expanded gold standard for detection of *C. trachomatis* in genital ulcer specimens, the former had a 100.0% detection rate while the latter had 25%. Kappa statistic (K) = 0.485

4.4.3 Results of Microtitre Typing of Chlamydial Isolates

The 13 culture positive samples which had been stored were revived on McCoy cell lines for immunotyping by the micro immunofluorescence (MIF) test. In 7 of the samples viable organisms were recovered after initial isolation and two further isolates were recovered after 3 passages. The remaining 4 samples were not recoverable and MIF could not be performed as viability is neccesary for titration and serotyping. These 4 isolates are assumed to have died during storage and transport. Titres varying from 1.4×10^3 to 5.3×10^6 were obtained for the nine viable isolates as shown in Table XV. Inclusion forming units (ifu) in dilutions higher than 10^{-3}, were not detected in most of the isolates.

Serotyping was performed on the 9 recoverable culture positive clinical isolates. The titre used in the serotyping, was the one that gave countable numbers of inclusions i.e the highest dilution appearing in Table XV for each of the samples tested. Table VIII. in Chapter 3, was used as a guideline in determining the serovars of the group of chlamydial strains characterized. Serovar designations were compared once all the isolates (n=9) (inclusive of those that had to undergo 3 passages prior to the test) had been completely typed. A positive MAb LV 22 reaction was included in the test to verify growth of isolate (which were used as antigens) on the microtitre plate and also, as a measure of confirmation that the strain being tested does indeed belong to the species *C. trachomatis*.

The reaction patterns of the monoclonal antibodies (MAbs) against the 9 chlamydial strains are represented in Table XVI. All the 9 isolates reacted with the *C. trachomatis* species specific MAB LV 22 (well 1). Strong fluorescence (+) was given by all isolates but two. MAbs LL-33 (well 2) and GG 11 (well 3) reacted only with the two strains designated as Ia, while KB 8 (well 9) was common to sample 1, 8, and 9 designated as serovars L1 and E. Well 4 (BB-11), well 7 (LV 23/LA 10) and well 8 (LV-27/CC 1), distinguished the L2 strains from the others. Mab KB 8 (well 9) reacted strongly with serovars L1 and E.

Of the infecting isolates, 7 belonged to the B complex [serovar L1, 1 (11.1%); serovar L2, 4 (44.4%); serovar E, 2 (22.2%)], and 2 belonged to the C complex (serovar Ia, 2). Five of the isolates characterized, belonged to the lymphogranuloma venereum (LGV) biovar (1 of L1, 4 of L2). Table XVII shows specimen and sample numbers with the corresponding serovars.

Table XV Titration of chlamydia strains in the micro-immunofluorescence test

Sample Number	Dilutions			Titre Results
	10^{1}	10^{2}	10^{-3}	
1	TNTC	5.25×10^{6}	5.33×10^{6}	5.29×10^{6}
2	7.8×10^{4}	3.4×10^{5}	TFTC	2.1×10^{5}
3	6.73×10^{4}	TFTC	TFTC	6.73×10^{4}
4	2.06×10^{5}	TFTC	TFTC	2.06×10^{5}
5	1.0×10^{5}	4.75×10^{5}	1.25×10^{6}	6.08×10^{5}
6	1.4×10^{3}	TFTC	TFTC	1.4×10^{3}
7	TNTC	1.13×10^{6}	1.33×10^{6}	1.23×10^{6}
8	N/D	N/D	N/D	N/D
9	N/D	N/D	N/D	N/D
10	N/D	N/D	N/D	N/D
11	N/D	N/D	N/D	N/D
12	3.08×10^{3}	TFTC	N/D	4.47×10^{4}
13	3.08×10^{4}	1.08×10^{5}	TFTC	6.94×10^{4}

TNTC: too numerous to count; TFTC: too few to count; N/D: not done
This table shows the results of the three dilutions as these were suitable for serotyping.

Table XVI Microtitre serotyping monoclonal antibody (MAb) reaction patterns and scoring scheme for *Chlamydia trachomatis* culture positive specimens

Specimen No:	Immunofluorescent Reaction[a] with *C. trachomatis* genital ulcer strains													
	1 (LV-22 [1:40])	2 (LL 33 [1:30])	3 (GG-11 [1:30])	4 (BB-11 [1:40])	5 (BB-3 /PE-5 [1:40])	6 (DP-1/ AC-11 [1:40])	7 (LV-23/ LA-10 [1:40])	8 (LV-27/ CC-1 [1:40])	9 (KB-8 [1:40])	10 (JG-9 [1:40])	11 (DD-1/ KK-1 [1:40])	12 (FC-2 [1:40])	Numerical code designation[b]	Serovar
1	+			+	(+)				+	+			**1-4-5 9-10**	L1
2	+	+	+		+								**1 2-3-5**	Ia
3	+			+			+	+					**1-4-7-8**	L2
4	+			+	(+)		+	+					**1-4-5-7-8**	L2
5	+	+	+		+								**1-2-3 5**	Ia
6	+			+	+		+	+					**1-4-5-7-8**	L2
7	+			+			+		+				**1-4-7 9**	E
8	+			+	+		+	+					**1-4-5-7-8**	L2
9	+			+			+		+				**1-4-7 9**	E

[a] +, bright fluorescence; (+) light fluorescence; blank square, no reaction.

[b] Titres and serovars are designated numerically and alphabetically.

Light type indicates a light-staining fluorescence reaction, and bold type indicates a strong fluorescence reaction.

LV-22 (well 1) which is species-specific, was common to all serovars.

The MAbs reacted with fixed inclusions of each isolate consisted of a panel of 17 *C. trachomatis* species-, group-, subgroup-, and serovar specific MAbs.

Table XVII Results of serotyping of the *C. trachomatis* isolates (n=9) by monoclonal micro-immunofluorescence (MIF)

Specimen Number	Sample Number	Serovar*
U 041	1	L1
U 090	2	Ia
U 113	3	L2
U 119	4	L2
U 128	5	Ia
U 135	6	L2
P 016	7	E
P 039	8	L2
P 042	9	E

Only 9 of the 13 culture-positive isolates survived shipment to University of Washington, Seattle.
* determined by MIF test
5 isolates belonged to the LGV biovar (1 of L1, 4 of L2)

4.5 RESULTS OF ENDOURETHRAL SPECIMENS

4.5.1 Chlamydial Antigen Detection By Direct Immunofluorescence (DIF) Test

Endourethral swab specimens for direct chlamydial antigen testing were obtained from 230 of the 247 patients recruited. The remaining 17 patients had severe phimosis which made it difficult to collect endourethral swab specimens. All 230 endourethral swab specimens obtained were evaluated by DIF, 36 (15.6%) were positive on first reading. A further 11 specimens showed non specific flourescence and were re evaluated. All 11 were found to be negative when compared with the positive control which had been tested in parallel with the specimens. Most of the *C. trachomatis* positive slides demonstrated a considerable number of fluorescent elementary bodies (EBs) and the number of EBs for every positive slide was recorded.

The majority of the DIF specimen smears (n=24) had EBs in the range of 2 15, while 10 had EBs in the 11 50 range, and only 2 smears had between 51 and 100 EBs. There were no slide smears with $\geq$ 100 EBs (Plate 13).

4.5.2 Results Of Ligase Chain Reaction (LCR) Testing

First catch urine specimens were obtained from all 247 patients and were stored at 20°C until testing. All urine specimens were tested in January 1996. Reactions were read the day following the LCR procedure. One specimen gave an invalid reading due to an air bubble and had to be repeated. A reading above the cut off value (positive determining factor) was recorded in 46 of the 247 specimens tested; thus LCR on first catch urine samples detected chlamydial DNA in 18.6% of patients.

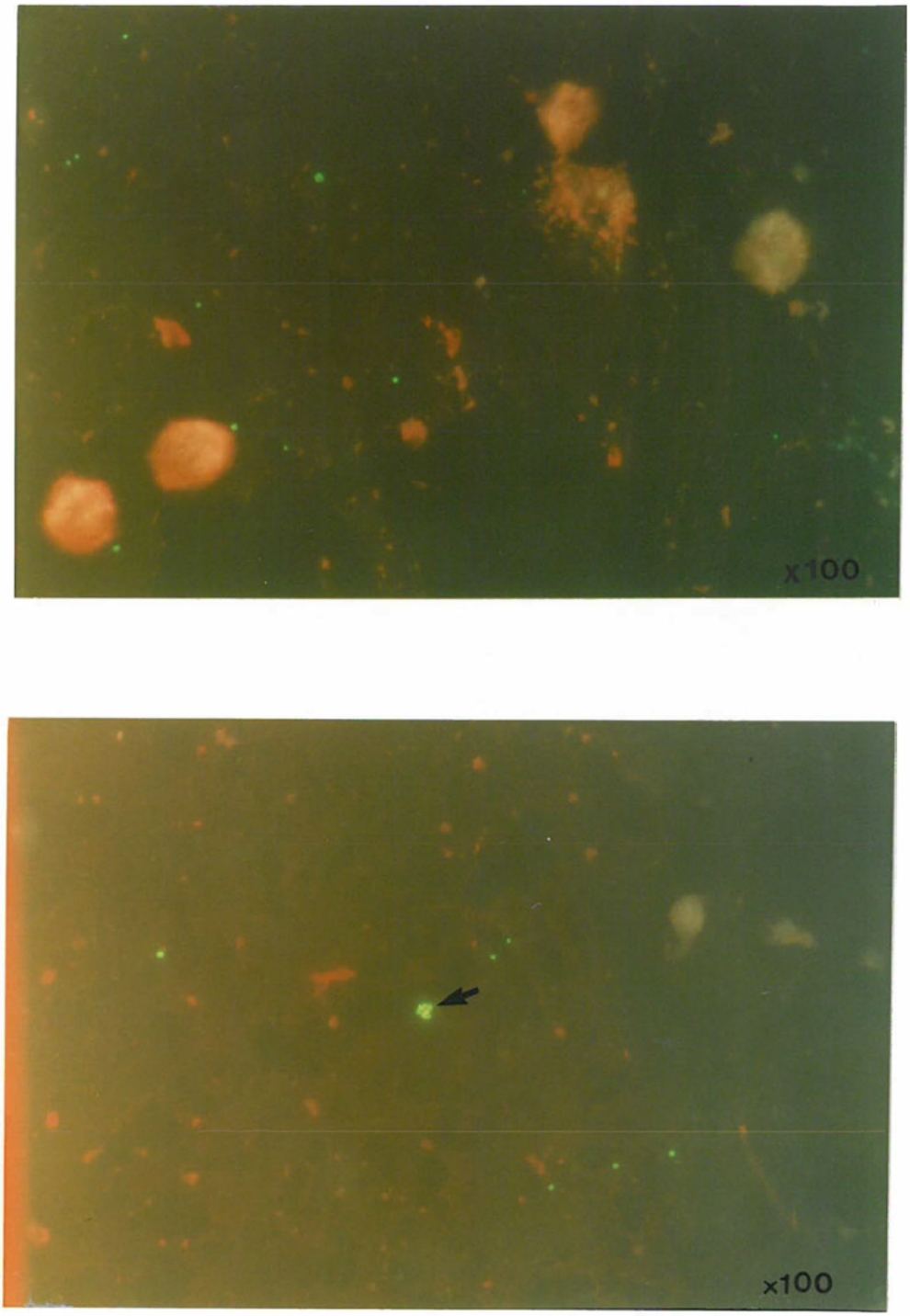


Plate 13. Several *C. trachomatis* elementary bodies (EBs) can be seen in this fluorescein-labeled monoclonal antibody stained endourethral swab smear. At a higher magnification (x100, oil objective), the apple green EBs (positive identification) appear evenly fluorescing, smooth edged and disc shaped. Arrow shows a cluster of EBs with a contrasting reddish brown background of counterstained cells.

Comparison of the results obtained by DIF and LCR was done for the reciprocal 230 endourethral swab specimens and first catch urine specimens (shown in Table XVIII). Twenty three (10%) specimens were positive by both DIF and LCR, an additional 13 (5.65%) were detected by DIF only, while 18 (7.83%) were positive by LCR only. When DIF and LCR are taken as the expanded gold standard for the detection of chlamydial urethritis in the 230 specimens tested, DIF and LCR had a successfull detection rates of 66.6% and 75.9%, respectively.

4.6 COMPARISON OF ULCER AND ENDOURETHRAL SPECIMEN RESULTS

The use of culture on genital ulcer specimens yielded 13 isolates. A comparison of antigen detecting tests and molecular techniques was made. Of the 13 specimens which were positive by culture, 4 (30.7%) were positive by all techniques viz. DIF, PCR, LCR and culture; all by both culture and PCR; 6 by all three tests viz. culture, LCR, and PCR. None of the culture positive specimens were negative by the other three tests (PCR, LCR, and DIF). The results are summarized in Table XIX.

The urethra was considered to be infected by *C. trachomatis* when specimens were positive by either DIF and/or LCR tests. An ulcer was considered to be infected by when specimens were positive by culture and/or PCR. Twenty four (44.4%) of the 55 genital ulcer specimens from which *C. trachomatis* was detected, had concurrent urethral colonisation. Fourteen (25.5%) of the above 24 were positive by DIF only, 19 (34.5%) by LCR only, while 31 (56.4%) were negative by both techniques. Four patients had phimosis and endourethral swab specimens were not obtained; of these, 1 was positive by LCR. Two of the 5 patients with ulcers, who had proven L strains immunotyped by the MIF test, also had *Chlamydia* in the urethra confirmed by DIF and LCR. Three of the 4 patients with ulcers from whom non LGV strains were isolated, also had *Chlamydia* in the urethra. The results are shown in Table XX.

Comparison of the results obtained in the two categories (ulcer and urethritis), are shown in Table XXI. Of the 193 patients that did not have *Chlamydia* in their urethral specimens, 16.1% had *C. trachomatis* in the ulcers. Twenty four (44.4%) of the 54 patients in whom the urethra was infected, had *C. trachomatis* was also cultured from their ulcer specimens. The significant association (p value) and the relative risk (RR) between genital ulcer and urethritis was found to be 0.001 and 2.75, respectively. This means that patients who had either urethritis or genital ulcers are more likely to be positive for the other, and that patients with urethritis are 2.75 times more likely to have genital ulcers than those patients who did not have urethritis.

Table XVIII Comparison of DIF and LCR for the detection of *C. trachomatis* in endourethral specimens (n=230)

	LCR		
DIF	POSITIVE	NEGATIVE	TOTAL
POSITIVE	23	13	36
NEGATIVE	18	176	194
TOTAL	41	189	230

DIF, direct immunofluorescence
LCR, ligase chain reaction
DIF and LCR had detection rates of 66.6% and 75.9%, respectively. Results are based on the urines and endourethral swab smears of the patients from whom, both specimens were collected. The number excludes the 17 patients who had phimosis and on whom, endourethral swab specimens could not be collected.

Table XIX Analysis of results of culture, DIF, LCR, and amplified endogenous plasmid DNA by PCR, on culture positive samples (n=13) for the detection of *C. trachomatis*

Specimen No.	DIF	Culture	LCR	PCR
P 010	—	+	—	+
P 016	+	+	+	+
P 039	—	+	+	+
P 042	—	+	+	+
U 041	+	+	+	+
U 090	+	+	+	+
U 113	—	+	—	+
U 119	—	+	—	+
U 128	—	+	—	+
U 135	—	+	—	+
U 143	+	+	—	+
U 145	+	+	+	+
U 149	—	+	—	+

DIF, direct immunofluorescence
LCR, ligase chain reaction
PCR, polymerase chain reaction
+ = positive
- = negative
DIF and LCR carried out on urine and endourethral swab specimens, respectively.

Table XX Concurrent urethral colonisation in patients presenting with genital ulcer(s) in which *C. trachomatis* was detected (n=55)

Patient No.	Ulcer	Urethritis		Patient No.	Ulcer	Urethritis	
	Cult/PCR	DIF	LCR		Cult/PCR	DIF	LCR
P 001	+	-	-	U 103	+	+	-
P 010	+	-	-	U 107	+	+	+
P 015	+	-	+	U 113*	+	-	-
P 016[a]	+	+	+	U 119*	+	-	-
P 034	+	-	+	U 121	+	-	-
P 039*	+	-	+	U 128[b]	+	-	-
P 042[a]	+	-	+	U 130	+	-	-
U 027	+	+	+	U 135*	+	-	-
U 037	+	-	-	U 136	+	-	-
U 041*	+	+	+	U 138	+	-	-
U 048	+	-	-	U 142	+	-	-
U 050	+	+	-	U 143	+	+	-
U 057	+	-	+	U 145	+	+	+
U 067	+	-	-	U 149	+	-	-
U 083	+	-	-	U 159	+	-	+
U 084	+	PH	-	U 161	+	-	-
U 090[b]	+	+	+	U 162	+	-	-
U 096	+	+	+	U 163	+	-	-
U 097	+	+	+	U 165	+	-	-

Table XX continued

Patient No.	Ulcer	Urethritis	
	Cult/PCR	DIF	LCR
U 166	+	-	-
U 170	+	-	-
U 174	+	-	+
U 175	+	-	+
U 176	+	-	+
U 178	+	PH	-
U 183	+	+	+
U 185	+	PH	-
U 188	+	-	-
U 191	+	+	-
U 193	+	PH	+
U 194	+	+	-
U 196	+	-	-
U 198	+	-	-
U 199	+	-	-
U 200	+	-	-
U 202	+	-	-

+, positive; -, negative.
PH = Phimosis (endourethral swab specimens were not collected).
PCR, polymerase chain reaction (carried out on culture specimens).
LCR, ligase chain reaction (performed on first void urine samples).
DIF, direct immunofluorescence (performed on endourethral swab specimens).
* LGV biovar strains (1 of L1, 4 of L2); [a,b] oculogenital strains ([a] 2 of E, [b] 2 of Ia).

Table XXI The association of *C. trachomatis* detection in genital ulcer and endourethral specimens by PCR and/or culture, and/or DIF and/or LCR, respectively (n=247)

	GENITAL ULCER[b]		
URETHRA[a]	POSITIVE	NEGATIVE	TOTAL
POSITIVE	24	30	54
NEGATIVE	31	162	193
TOTAL	55	192	247

PCR, polymerase chain reaction (results based on specific amplification of the 452 bp product of the endogenous plasmid DNA from culture-positive samples).
DIF, direct immunofluorescence
LCR, ligase chain reaction
[a] DIF and LCR used as expanded gold standard for the detection of *C. trachomatis* from endourethral specimens. Positive identification made when specimen was positive by either or both tests.
[b] PCR and culture used as expanded gold standard for the detection of *C. trachomatis* from genital ulcer specimens. Positive identification made when specimen positive by either or both tests.
P-value = 0.001; relative risk = 2.75

CHAPTER 5

DISCUSSION AND CONCLUSION

This cross sectional study was part of a comprehensive departmental genital ulcer survey, which was performed in order to establish the prevalence of genital ulcer diseases (GUD) in patients attending the City Health STD clinic in Durban. This project sought to detect the common aetiological conditions associated with GUD viz. chancroid, lymphogranuloma venereum (LGV), syphilis, granuloma inguinale and genital herpes. It was shown that chancroid was the most prevalent at 34% (n=83), with syphilis being the second commonest at 28.3% (n=70). These findings are consistent with what is reported from most developing countries and furthermore, are in agreement with studies performed in South Africa, where chancroid and syphilis were ranked as the two most prevalent GUD (Crewe Brown *et al*, 1982; Coovadia *et al*, 1985; O'Farell *et al*, 1991; O'Farell *et al*, 1994). As expected, conditions such as genital herpes and granuloma inguinale were detected less frequently i.e in 28 (11.3%) and 15 (6.1%) patients respectively. Concurrent infections were diagnosed in 35 (14.2%) of the patients, 25 of whom had syphilis. Triple infections were detected in only 2 patients and in both, syphilis, chancroid and granuloma inguinale were diagnosed. This high prevalence of mixed infections has implications for choosing antimicrobial therapy.

A large study population (247 subjects) was enrolled for this study. This number allowed for appropriate laboratory analysis for determining the aetiology of GUD and compares favourably with other studies of this nature in South Africa (Crewe Brown *et al*, 1982; Coovadia *et al*, 1985; O'Farell *et al*, 1991; O'Farell *et al*, 1994). The latter studies reported sample sizes of only 100 patients.

The high prevalence of STDs in developing countries has been attributed to a number of factors. In this study, most patients were unmarried men and this may reflect the greater

promiscuity of single men, as well as the socio economic class, because in the African culture, a man needs to pay a bride price (lobola) which is likely to be beyond the reach of men attending this type of clinic. The age at first intercourse was low and the reasons are largely unknown and not within the scope of this study. The mean number of sexual partners in the preceding 6 months was 3, which is surprisingly low. In a study of urethral discharge conducted ten months earlier at the same STD clinic, the mean number of sexual partners was found to be 8 (Small and Maleka, unpublished data). This difference is unlikely to be due to the difference in behaviour, as both studies looked at the same population and were conducted within the same time frame. One explanation could be that urethritis patients mainly spread their STD organism through sexual networking while, ulcer patients attend to commercial sex workers (CSW). However, in an area that is highly endemic for both syndromes, it is unlikely that the organisms would be distributed differently within sub-populations. It could also be related to a possible difference in infectivity of the microbes. This would mean that urethritis organisms would be less infective than the microbes that cause genital ulcers. Another reason for this lower figure in ulcer patients could be the difference in health seeking behaviour, as those with genital ulcers might tend to seek medical help much quicker compared to those with urethritis. The fact that more than half of the patients had a history of a previous STD could reflect the failure of the health services in implementing preventative strategies.

Clinical findings in the patients were consistent with typical genital ulcer patients and there was nothing exceptional noted. Circumcision was noted in a low percentage of patients (7.3%) and this is consistent with previous studies done in Durban which have also shown low rates of circumcision (Coovadia *et al*, 1985; O'Farell *et al*, 1991). This reflects Zulu custom in that it is not part of the tradition for men to undergo circumcision. Our findings are in contrast with other African studies such as in Ghana (Mabey *et al*, 1987) where 98.1% were circumcised. As it is generally accepted that minor trauma is essential in the pathogenesis of GUD, it has been hypothesised that uncircumcised as compared to circumcised men develop ulcers more frequently. There was no significant difference in clinical findings between the chlamydia negative and positive patients. This is expected

as it is well known that the clinical features of genital ulcers cannot be used to make a microbial aetiological diagnosis (von Zeissl, 1882; cited by: Sturm 1981; Sturm *et al*, 1987).

The diagnosis of the ulcerative condition LGV, was based on the expanded reference standard of positive cell culture and PCR. The specimens were centrifuged onto McCoy cell monolayers pretreated with cycloheximide to enhance the efficiency of infection. Samples that were positive by culture were immunotyped by the microimmunofluorescence (MIF) test. For molecular analysis, DNA was extracted from *C. trachomatis* infected and uninfected McCoy cells by the classical phenol/chloroform extraction method (Peterson *et al*, 1989; Sambrook *et al*, 1989). The DNA extracts were subjected to specific amplification of the *C. trachomatis* plasmid gene by PCR and those which yielded a 452 bp band were further subjected to specific amplification of the major outer membrane protein (MOMP) gene. Immunotyping revealed L-strains (L1 and L2) which cause LGV, as well as non-LGV strains i.e the oculogenital strains (D to K) responsible for urethritis and other genital infections. Possible explanation for the isolation of these oculogenital strains in the ulcer specimens could be that these strains do somehow cause LGV or that there was contamination of the ulcers from a subclinical urethral infection.

The use of currently available diagnostic technology (explained above) showed a prevalence of 22.3% for *C. trachomatis* in ulcer specimens. This figure for LGV is markedly increased from previous studies in Durban, which were restricted in their diagnostic methods. This figure is similar to the 24% reported for Madagascar (Harms *et al*, 1994), but is considerably higher than the 7% reported in The Gambia (Mabey *et al*, 1987), 0.55% in Nigeria (Ghatak, 1992), and 8% in Zimbabwe (Tswana *et al*, 1995). Only Mabey *et al* (The Gambia, 1987) cultured the specimens on McCoy cells and found a prevalence percentage which is comparable with ours by means of culture. Harms *et al* (Madagascar, 1994) and Ghatak *et al* (Nigeria, 1992), used direct immunofluorescence test (MicroTrak, Syva) to diagnose *C. trachomatis*, while Tswana *et al* (Zimbabwe, 1995) used

Chlamydiazyme test (Abbott Diagnostics) for *Chlamydia* detection. Due to the different methodology employed in all five studies, comparison and interpretation of the results are difficult. However, the similarity between the culture results from The Gambia and this study suggests similar prevalence rates.

Using culture alone, 5.3% of the LGV infections were detected in the specimens obtained from genital ulcers. Previous studies done in Durban have shown rates of chlamydial (LGV) infection of 1%, 7%, and 1.5% by Coovadia *et al* (1985), O'Farell *et al* (1991), and O'Farell *et al* (1994), respectively. The first study used clinical criteria only, while the latter studies used direct immunofluorescence (DIF) for the detection of chlamydial antigen in ulcer exudates. These figures are in sharp contrast to those obtained in this study and show the obvious problems related to difference in methodology.

The use of molecular methodology which was previously unavailable, does improve detection. However, if the increase in chlamydial infection is not only due to improved diagnostic techniques, one could speculate that improved access to health care facilities and subsequent antibiotic therapy that does not cover chlamydia, may account for some of the increase in prevalence of chlamydial infections. Future studies using the more advanced techniques employed in this study will most likely shed more light on the trends in GUD including those caused by *C. trachomatis* (L1-3) in this population.

Although culture is considered the gold standard in chlamydia diagnosis despite its relative low level of sensitivity (Leonardi *et al*, 1992; Bass *et al*, 1993; Chernesky *et al*, 1994; Schachter *et al*, 1994; Thejls *et al*, 1994), there were still a large number of false negatives in this study when compared to molecular techniques. This is in accordance with recent studies performed for the diagnosis of urethritis with other amplification techniques or studies that used multiple immunoassays in which it was suggested that culture as the gold standard may have a sensitivity of only 55 to 65% (Leonardi *et al*, 1992; Bass *et al*,

1993; Chernesky *et al*, 1994; Schachter *et al*, 1994; Thejls *et al*, 1994). For the diagnosis of LGV, this low sensitivity of culture creates a problem of interpretation because presently, culture is the only technique by which typing can be done. The possibility of the use of amplification techniques with subsequent utilisation of restriction enzymes may offer an alternative for detecting different strains.

The factors that could lead to false negative *Chlamydia* cultures are inadequate specimen collection, transportation of the specimens to the laboratory, and delay in processing of the specimens (Mardh *et al*, 1981; Kuo *et al*, 1983). In our case, false negativity in cultures could have been due to transportation of specimens at room temperature instead of 4^0C, a factor that is known to reduce the sensitivity of the cell culture assay. For maximal results, it is imperative that adequate specimens be collected, and since the object is to obtain a representative sample of epithelial cells, it is recommended that the specimens be collected with some vigour either by swabbing or scraping. Swabbing, however, is the method advised by the Centers for Disease Control and Prevention (CDC) for the collection of chlamydia specimen using dacron swabs on metal or plastic shafts recommended for recovery of this fastidious organism (Mardh and Zeeberg, 1981; CDC, 1993). In this instance, vigorous scraping under the edges of the ulcer(s) was done using a disposable plastic loop so as not to subject patients to multiple sampling for our comprehensive study. Furthermore, owing to the painful nature of most ulcer(s), the yield of the material in subsequent scrapings is likely to be compromised.

In this study, a suspension of ulcer scraping was made in 0.5 ml of phosphate buffered saline (PBS) before being aliquoted (100 μl) into various transport media (2 3 h after collection) for the isolation of the microorganisms. Although the specimen collection procedure was patient friendly and solved the problem of sequence sampling for the different techniques, it could however, have compromised the yield as far as chlamydia cultures are concerned. Preserving chlamydial infectivity in a clinical specimen is the obvious prerequisite for successful culture diagnosis, and thus, the use of PBS and not

direct inoculation into chlamydia transport medium (CTM), could have contributed to the low yield in culture and hence, the high positive rate when using amplification of plasmid DNA by PCR. The considerable variation of chlamydial strains in their infectivity for cell culture could also explain the successful isolation of some specimens; notably LGV strains grow much easier than the oculogenital strains (Schachter and Wyrick, 1994). Therefore, in view of the above statement, specimens in this study which were negative by culture but positive by PCR, could reflect the presence of the latter.

The 50 specimens which were processed a week after sampling (due to excessive workload which had accumulated from the first 200 specimens) and after being stored in transport media at 70^0C, were all negative by culture. This may have been due to death of the organisms during storage, which could have been aggravated by specimen transportation at room temperature prior to storage. The recommendation that specimens be stored at 70^0C if delay in inoculation is expected stems from the knowledge that the viability of any chlamydiae present is quickly lost upon storage at 70^0C or if the specimen is kept for more than 24 h at 4^0C. This is substantiated by the existence of various reports on the reduction of chlamydial infectivity after a freeze-thaw cycle. The ability of PCR for the specific amplification of the endogenous plasmid gene to detect *Chlamydia* in 22 of the last 50 samples, could possibly be that storage at 70^0C may have resulted in the rupturing of the membranes of *Chlamydia* organisms and release of the DNA, therefore, enhancing their detection by PCR. This however, is only speculative. The problems encountered highlight the difficulties in collecting specimens from subjects for which various fastidious organisms are to be cultured from the same specimen. Adequate pre planning of the method of collection and processing is essential to ensure the optimal isolation of all organisms sought.

The culture procedure employed, was similar to that used by many other laboratories. By using tissue culture technique, *C. trachomatis* was successfully cultured from 13 (5.3%) of the 247 specimens collected. Ten specimens were positive on initial isolation and a

further 3 specimens became positive after a second passage. Initial cultures of the latter, did show extracellular fluorescing particles suggesting EB release. Therefore, the reason for the increase in the number of positive specimens after a blind passage, was likely not due to the fact that multiple passaging increases chlamydial recovery, but because the specimens were originally positive and that the *Chlamydia* replication cycle could have been longer than 48 h. Extracellular EBs cannot positively be identified as such. Another reason could be that the specimens had low copy number of infectious particles which could have been aggravated by the inoculation of most or all the infectious particles into the second track vial (culture done in duplicates), thereby giving a negative result upon staining. Since different isolates may have slightly different growth rates, any set incubation time is a compromise. A single blind passage was undertaken on initial negative culture as further passaging does not increase the number of positives (Schachter and Martin 1987).

The high number of inclusions (> 200) in the cultures which were initially positive reflects the bacterial load in these specimens and it indicates that getting adequate specimens can increase the positivity rate. It is possible that if the study was only done for the detection of chlamydial infection, the positive yield of cultures would have been greater as the procedures followed in this study could have compromised the chlamydia culture.

For improving the detection of chlamydiae in specimens which were either positive or negative by culture, DNA extracts from all 247 specimens were subjected to specific amplification of the endogenous plasmid by PCR. A 452 bp fragment was obtained from 55/247 (22.3%) specimens [i.e all 13 (5.3%) specimens positive by culture plus an additional 42 (17%) specimens negative by culture. The reason for the higher sensitivity by PCR of plasmid DNA compared to culture may be that non viable chlamydiae were present in the samples, or that the chlamydiae in the sample, were in their reticulate body state which can be detected by PCR and not by tissue culture, as they are non infectious.

A second PCR assay using primer pair oDVDIV and o5'MOMP specific for the amplification of the MOMP gene (Lampe *et al*, 1993), was employed to compare its sensitivity to plasmid DNA by PCR. Only 26 (47.3%) of the 55 samples yielded products specific for the MOMP gene. About 27% of the samples in which no amplified products were obtained for the MOMP gene, corresponded to those yielding weak signals in the specific amplification of the endogenous cryptic plasmid DNA by PCR. Comparison of PCR with the different primer sets on the 13 culture positive samples especially those with <10 IFU, showed the plasmid primer set to be the most sensitive. This is in accordance with the results obtained on the sensitivity of the test performed on the prepared dilution series of *C. trachomatis* L2 DNA. Specific amplification by PCR of L2 DNA was a thousand fold less sensitive using primers for the MOMP gene as compared to the endogenous plasmid. This can be explained by the presence of multiple copies of plasmid in each chlamydial cell, as serovar L2 contains 10 plasmid copies per chromosomal DNA equivalent (Palmer and Falkow, 1986).

Several investigators have determined the sensitivity of the PCR in a model system. Purified elementary bodies (EBs) were used in all instances, and one copy of target DNA could be detected by specific amplification of both the MOMP gene (Palmer *et al*, 1991) and the plasmid gene (Ostergaard *et al*, 1990). However, results in this study are based on the infectivity of *C. trachomatis* serovar L2 in McCoy cells, and are therefore, more closely related to the clinical situation.

Comparison of DNA based methods (using *C. trachomatis* plasmid and MOMP genes) with culture has been reported by several workers. Claas *et al* (1990), reported 26/156 urethral samples to be positive by culture. These 26 samples and an additional two, gave positive results with primers specific for the amplification of both the plasmid and ribosomal DNA by PCR. In a study by Holland *et al* (1990), 14 cervical samples were tested, two of which were weakly positive, five strongly positive, and seven negative in culture. The weakly positive samples gave visible bands in gels only after specific

amplification of the MOMP gene by PCR, and the culture negative samples remained negative. Bobo *et al* (1990), cultured 104 cervical samples and all the 46 culture positive samples were found to be positive by PCR with primers for the specific amplification of the MOMP gene. Ostergaard *et al* (1990), tested 223 clinical samples (endourethral, endocervix and conjunctival), of which 26 were positive by culture. These and 14 culture negative samples gave positive results after amplification of plasmid DNA by PCR. Of the culture negative samples, 12 (86%) were confirmed as positive by ELISA (IDEIA, Boots Celltech). None of these authors, except Bobo *et al* (1990), report on the number of inclusion forming units (IFU) of *C. trachomatis* in the patient samples. This study has shown that PCR assays (depending on the primer set used) can give negative results on samples that are weakly positive (<10 ifu) by culture for *C. trachomatis* (L2 strain).

Results of this study with regard to the sensitivity of PCR, corroborate findings by others (Mahony *et al*, 1993; Loeffelholtz *et al*, 1992; Ossewaarde *et al*, 1992) although different primers were used from the ones in this study, these workers have shown that specific amplification of the plasmid DNA by PCR was more sensitive than that of the chromosomal DNA, detecting up to 25% more positives. In contrast, Bobo *et al* (1990) found all the weakly culture positive samples to be positive following amplification of the MOMP gene by PCR. This group used primers for the specific amplification of the MOMP gene by PCR only, and therefore, no conclusions can be drawn about the relative sensitivity of this PCR in comparison with that of specific amplification of the plasmid gene.

Comparison of the detection of *C. trachomatis* by culture and that of specific amplification of the plasmid gene in genital ulcer specimens, the Kappa (K) statistic (0.485) revealed a moderate agreement. Specific amplification of plasmid DNA by PCR and culture (either singly or combined), managed to detect *C. trachomatis* in 55 (22.3%) of the samples. Culture was unable to detect 42 (17%) of the samples detected by PCR. Between the two tests, 13 (5.3%) were true positives while, 192 (77.7%) were true negatives.

Although culture is the "gold standard", this study where the detection rate of 25% was observed, emphasizes the fact like elsewhere, that culture has a sensitivity of up to 55 to 65% (Leonardi *et al*, 1992; Bass *et al*, 1993; Chernesky *et al*, 1994; Schachter *et al*, 1994; Thejls *et al*, 1994).

Of the 13 culture positive specimens which were to be immunotyped using micro immunofluorescence (MIF), 4 were lost during storage and/or transport, the major factor being a breakdown of the 70^0C freezer. In keeping with the typical LGV serovars 5/9 of the strains were of the LGV biovar. Two of the isolates belonged to the recently described serovar Ia and these together with the two E serovar isolates represent non LGV isolates. This is the first time that the serovars L2, E and Ia have been reported from South Africa. In keeping with the predominance of B complex isolates worldwide, this study confirmed this, as 77.7% of the isolates were of class B. The dominance of class B isolates (L2, L2a, E, D, Da, B, Ba) may indicate some biological advantage over other serovars.

Only one of the strains belonged to the L1 serovar which was the predominant serovar in the only other South African study which used MIF. In the study reported by Hayes *et al* (1994), 18 isolates were characterised by MIF, of which 11 (61%) belonged to the L1 serovar and the remaining 7 (39%) showed no reaction. In Durban, a far greater variety of serovars was found and this would indicate the circulation of many different strains within the community as opposed to fewer strains in the Johannesburg study. This may indicate a more endemic pattern of spread in Durban as opposed to a possible epidemic spread of single strains in Johannesburg.

Endourethral swab specimens and first void urine (FVU) specimens were collected for the diagnosis of concurrent urethral infection. Chlamydial antigen detection was performed on endourethral swab specimens by direct immunofluorescence (DIF) using MicroTrak

slides (MicroTrak, Syva). This test uses species specific monoclonal antibodies that bind to MOMP. A molecular technique, a plasmid based ligase chain reaction (LCR) was used on the FVU specimens. Studies done worldwide, as well as those done in South Africa, have employed a variety of diagnostic tools for the detection of *C. trachomatis* urethritis. Almost all studies have used tissue culture together with other tests such as the DIF antibody test, enzyme immunoassays (EIAs), serology, and LCR (Alexander *et al*, 1985, Bentsi *et al*, 1985; Stamm and Mardh, 1990; Bassiri *et al*, 1995; Van der Pol *et al*, 1995). Pham Kanter *et al* (1996), have summarized all the results of studies conducted in South Africa and various tests used in the diagnosis of chlamydial urethritis. They reported *C. trachomatis* to be responsible for 30 40% of non gonococcal urethritis (NGU).

Of the 247 patients recruited, DIF was performed on 230 endourethral specimens thereof. In the remaining 17 patients, it was not possible to perform DIF as the patients had phimosis and endourethral swab specimens were unobtainable. This is a problem when testing for chlamydial infection with DIF alone as can be seen in this study where 5 of the 17 patients (29.4%) with phimosis were positive by LCR. The positive signal in the 5 urine specimens, could be due to the *Chlamydia* originating from either the urethra or intraprepusal ulcers. Thirty six of the 230 (15.6%) urethral specimens obtained, had the characteristic fluorescent pinpoint apple green elementary bodies (EBs). However, none of the slides had EBs $\geq$ 100. The majority (n=24) of the DIF slides had between 2 15 EBs, probably reflecting the subclinical nature of urethral infection. This could also be the reflection of the asymptomatic nature of chlamydial urethritis. The 11 specimens which showed non specific fluorescence and which were subsequently confirmed to be negative when compared to positive control slides illustrates the subjectivity with which fluorescence microscopy is fraught and also, the importance of using control slides when doing diagnostic immunofluorescence microscopy. These patients were also all negative when their urines were tested by the LCR.

Urine analysis for the detection of endourethral infection has for the last few years, been the focus of the new STD testing strategies. It is felt to be the specimen of choice because of the non invasive nature of specimen collection. Testing of both male and female FVU specimens for *C. trachomatis*, has been recently evaluated using LCR (Chernesky *et al*, 1994a, 1994b; Schachter *et al*, 1994; Bassiri *et al*, 1995; Lee *et al*, 1995). LCR appears to be highly sensitive and specific for the detection of chlamydial urethritis (Chernesky *et al*, 1994a, 1994b). FVU contains exfoliated urethral epithelial cells and is therefore, a good diagnostic sample for urethritis. The ease of specimen collection and the automated test procedure makes this technique user friendly as well as rapid, highly sensitive (87% to 98%) and specific (99.8% to 100%).

LCR detected 46/247 (18.6%) of the urogenital infections, thus giving a detection rate of 75.9%. To our knowledge, this is the first time that LCR has been used to detect the presence of chlamydial infection in urine specimens of patients with genital ulcer diseases. Radebe *et al* (conference proceedings) used LCR to detect *C. trachomatis* urethral infections from male first catch urine. They did not perform LCR for *C. trachomatis* on all the specimens collected (234 men), but tested only the 70 FVU specimens which were negative for gonococcal culture. In a study by Dille *et al* (1993), a dilution panel of *C. trachomatis* serovar L2 prepared from stocks grown in McCoy cells using both LCR and specific amplification of the cryptic plasmid DNA by PCR, were able to consistently amplify amounts of DNA equivalent to three *C. trachomatis* EBs. However, Dille's study is not comparable as it used a stock strain whereas in this study, patient specimens were utilised.

The number of urethral specimens that were positive by DIF and/or LCR or both totalled 54/230 (23.5%). DIF as compared to LCR identified 36/54 chlamydial infections while LCR identified 41/54. There were 23 (10.0%) specimens positive by both tests. Overall, 46/247 FVU specimens were positive by LCR alone. This shows that DIF would have been as sensitive as LCR, had it not been for the 17 patients who had phimosis and

endourethral swabs could not be collected. The low sensitivity of the DIF and LCR test for chlamydia as compared to PCR, may be due to their dependency upon the amount of inflammation in the urethra. How this relates to ulcer scrapings is unknown. Since the main site of infection was the outer genitalia (penis), there may not have been concurrent chlamydial infection in the urethra or the infection titres were extremely low. These results indicate as expected, that FVU cannot be used to diagnose the ulcerative chlamydial disease.

Four techniques were used to diagnose both LGV and urethral infections. Culture and PCR were used for the detection of LGV while DIF and LCR were used for the detection of urethritis. Specimens were categorised as positive for genital ulcer or LGV, if they were positive by culture and/or PCR, and as positive for urethritis if they were positive by DIF and/or LCR. Tissue culture being the "gold standard", detected only 13/247 (5.3%) of the specimens tested. Of these, 4 were positive by all tests used viz. culture, PCR, LCR, and DIF; 6 by culture, LCR and PCR, and none were negative by PCR, LCR, and DIF. Specific amplification of plasmid DNA by PCR of ulcer specimens, was found to be the most sensitive method detecting 55/247 (22.3%) of the infections for all categories analyzed, with LCR [detecting 46/247 (18.6%) infections] and DIF [detecting 36/230 (15.6%) infections] being less sensitive.

Of the 247 patients enrolled in the study, *C. trachomatis* was not detected in urethral specimens (n=193) by both DIF and LCR. From these 193, 16.1% had *C. trachomatis* isolated from their ulcer specimens. Of the 54 that had chlamydial urethritis (i.e from whose endourethral specimens *C. trachomatis* was cultured), 44.4% had *Chlamydia* in their ulcer(s). When considering the 55 genital ulcer specimens in which *C. trachomatis* (PCR and/or culture confirmed) was detected, 24 (44%) had concurrent urethral colonisation, while 31 (56.4%) had no *Chlamydia* in the urethra. Of the 5 patients with proven L strains diagnosed by immunotyping, 2 also had urethritis; 3/4 persons from whom non LGV strains were isolated, also had urethritis. Of the 13 specimens from

which chlamydia was successfully cultured, 11 had mixed infections i.e with syphilis (n=4), chancroid (n=4) and granuloma (n=3). Despite the drawbacks, this data shows that genital ulcer infections are underdiagnosed and on the increase. There are two possibilities to these concurrent infections; either non LGV strains such as E and Ia, can cause genital ulcers or that there could have been contamination of the ulcers by the urethral discharge, thereby leading to the isolation of non-LGV strains from ulcers. Since PCR is highly sensitive, it would be interesting to know whether specific amplification of the endogenous plasmid DNA by PCR reflects only the presence of plasmids of dead Chlamydiae or the presence of low copy numbers of still viable chlamydiae. In the case of the latter, the patient might still be infectious. Either way, this has to be evaluated further. There was a significant association (RR 2.75, p=0.001) in patients with *C. trachomatis* colonising either the urethra or ulcer(s), i.e chlamydial infection detected in the urethra was also likely to be present in ulcers and vice versa. Furthermore, those who had chlamydial urethritis are 2.75 times more likely to have chlamydia isolated from their genital ulcers than those patients who did not have urethritis.

From the above data, four groups of patients can be identified. The first being those from whose ulcer(s), LGV strains (5/9) were isolated; these can be diagnosed as definite LGV. The second group includes patients in whom the presence of *C. trachomatis* was detected by PCR in the ulcer(s) but not in the urethra, as well as those, from whose ulcer(s) oculogenital strains were isolated; this group can be considered as probable LGV. The third group includes patients whose ulcers contained an oculogenital strain and *C. trachomatis* was also detected in the urethra, plus those patients from whose genital ulcer specimens *C. trachomatis* was not detected by PCR but the endourethral specimens were positive by DIF and/or LCR; this group can be considered as inconclusive. The fourth and last group is the non LGV group, which encompasses all specimens in which *C. trachomatis* was not isolated from the ulcer but in the urethra, and those specimens that were negative by all tests.

Certain conclusions regarding the epidemiological characteristics, spread and risk factors of LGV in Durban, Kwazulu Natal, can be drawn. There is no national data in South Africa on the prevalence of chlamydial diseases especially GUD, because most infections are neither revealed nor reported officially. There is a lack of knowledge as far as the disease is concerned and a lack of proper diagnostic techniques in various centres and chlamydial treatment in most clinics. It is clear that chlamydial infections have been increasing in the Durban region and transmission has been facilitated by the fact that chlamydial urogenital infections are often asymptomatic. Our data also shows that a more relaxed attitude towards sexual activity associated with the low age at first intercourse and higher frequency of intercourse allow an increased possibility of infection, which contribute to this high frequency of chlamydia positive cases.

Regular well planned screening programmes of this nature should be continued so that the prevalence of chlamydia infection can be monitored and these, can guide management policies when it comes to drawing up syndromic management protocols. As far as chlamydial diagnosis of genital ulcers is concerned, plasmid PCR (despite its inability to differentiate between the oculogenital and the LGV serotypes) is the method of choice above culture alone. However, further investigations of the significance of the oculogenital strains in the pathogenesis of ulcer disease are required.

CHAPTER 6

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CHAPTER 7

APPENDICES: PROTOCOL FOR MEDIA AND REAGENT PREPARATION

Note: All autoclaving was performed at 121^{0}C for 15 min.
Water used was sterile triple distilled water.
For sterility checking media was incubated at 37^{0}C in 5% CO_2 for 24 hours (h)

APPENDIX 1: MEDIA, ANTIBIOTICS AND SOLUTIONS USED FOR CULTURE

1.1 Reagents obtained from Bio-Whittaker (Durban, South Africa)

- Minimum Essential Media (MEM) Eagle with Earle's salts (# 12-611)
- MEM Non Essential Amino Acids (NEAA) (# 13-144)
- Chlamydia Cycloheximide Overlay Base Media (# 12 712)

1.2 Antibiotics used

Amphotericin B	0.05 g
filtered water	10.00 ml

The above component was dissolved; 0.6 ml thereof, was aliquoted into 1.5 ml cryotubes and stored at -20^{0}C. 0.5 ml/500 ml MEM was used to obtain a final concentration of 5 μg/ml.

Gentamicin	0.1 g
filtered water	10.0 ml

The above component was dissolved and 0.6 ml aliquots were dispensed into 1.5 ml cryotubes and stored at -20^{0}C. 0.5 ml/500 ml MEM was used to get to a final concentration of 10 μg/ml.

Vancomycin	0.1 g
filtered water	10.0 ml

The above component was dissolved and 0.6 ml aliquots were dispensed into 1.5 ml cryotubes and stored at 20^0C. 0.5 ml/500 ml MEM was used to get to a final concentration of 10 μg/ml.

1.3 Chlamydia Growth/Culture Medium for McCoy Cells

E MEM with L glutamine	500.0 ml
NEAA (100 X)	5.0 ml
10% Foetal calf serum (FCS)	25.0 ml
Gentamicin (10 μg/ml)	0.5 ml
Vancomycin (10 μg/ml)	0.5 ml
Amphotericin B (5 μg/ml)	1.0 ml

The above components were mixed and filter sterilized. The medium was stored at 4^0C.

1.4 Chlamydia Maintenance Medium for McCoy Cells

E MEM with L-glutamine	500.0 ml
NEAA (100X)	5.0 ml
FCS (5%)	12.5 ml
Gentamicin (10 μg/ml)	0.5 ml
Vancomycin (10 μg/ml)	0.5 ml
Amphotericin B (5 μg/ml)	1.0 ml

These components were mixed, filter sterilized and stored at 4^0C.

1.5 Chlamydia Transport Medium (CTM)

E MEM with L glutamine	500.0 ml
FCS (10 %)	50.0 ml
10 % Glucose	25.0 ml
Gentamicin (10 μg/ml)	0.5 ml
Vancomycin (10 μg/ml)	0.5 ml
Amphotericin B (5 μg/ml)	1.0 ml

The above components were mixed and filter sterilized. 2 ml aliquots of the transport medium were added into cryotubes and stored at 20^0C until required.

1.6 *Chlamydia trachomatis* monoclonal antibody culture stain

The lyophilized stain was resuspended with 3.0 ml of diluent provided with the kit. The stain was then mixed properly and left to stand at room temperature for 15 min prior to use. (Syva # 8H019)

1.7 *Chlamydia trachomatis* monoclonal antibody direct slide stain

The lyophilized stain was reconstituted with 2.0 ml of diluent provided with the kit. The stain was mixed properly and allowed to stand for 15 min prior to use. (Syva # 8H139)

1.8 EDTA Solution (0.02 %)

1 M EDTA solution (pH 8)	5.0 ml
Phosphate buffered saline (PBS)	88.0 ml

The components were added into a 100 ml schott bottle, mixed and stored at room temperature.

1.9 Freezing Medium

Solution 1 = E MEM + 10% FCS
Solution 2 = Solution 1 + 10% Glycerol

To prepare solution 1, 180.0 ml of E-MEM were mixed with 20.0 ml of FCS. 18 ml aliquots from solution 1 were added to universal bottles and 2.0 ml of glycerol were added to each bottle to get solution 2 with a final concentration of 10% Glycerol. These were stored at 20^0C.

1.10 Glucose Solution (10%)

D Glucose	10.0 g
E MEM	80.0 ml

The above components were dissolved and the volume brought up to 100.0 ml with E MEM. The solution was filter sterilized using a 0.2 μm membrane filter and aliquoted into 25.0 ml volumes. These were stored at 4^0C.

1.11 Disinfectant for hood (METHS)

Absolute ethanol (SVR)	334.0 ml
Methanol	15.0 ml
Water	151.0 ml

This 500.0 ml mixture was prepared in a squeeze bottle and stored in the hood.

1.12 Phosphate Buffered Saline (PBS)

5 PBS tablets were added to each 500 ml sterile Schott bottle. The tablets were dissolved in 500.0 ml of water and autoclaved. The solutions were stored at 4^0C.

1.13 0.4 M Sucrose-Phosphate Buffer (4-SP)

sucrose	68.460 g
Anhydrous dipotassium hydrogen phosphate (K_2HPO_4)	1.044 g
Anhydrous potassium dihydrogen phosphate (KH_2PO_4)	0.544 g

The sucrose was dissolved in about 250.0 ml of water and the potassium phosphates in about 50.0 ml. The two solutions were mixed in one 500 ml measuring cylinder and the volume adjusted to 500.0 ml with water. If necessary, the pH was adjusted to 7.0 with dilute HCl or NaOH solution. 4 SP was dispensed into 100 ml bottles and autoclaved. These were stored at 4^0C.

1.14 Trypan Blue

trypan blue	0.2 g
PBS	50.0 ml

The above component was dissolved in PBS to prepare a 0.4% stock solution. 10.0 ml of the stock solution was added into a glass bottle and diluted with 40.0 ml of PBS. This was stored at 4^0C.

1.15 Trypsin (0.25 %)

Trypsin (2.5%)	20.0 ml
PBS (pH 7.5)	180.0 ml

A bottle of 2.5% Trypsin (20.0 ml) was thawed, poured into a 75 cm^3 culture flask, then topped up to 200.0 ml with PBS (180.0 ml). 1.0 ml of the solution was added per track vial and stored at 20^0C.

APPENDIX 2: MEDIA AND SOLUTIONS USED FOR MICROIMMUNOFLUORESCENCE (MIF) TYPING

2.1 Antibiotics [Nystatin, Gentamycin, and Vancomycin (NGV-stock solutions)]

Nystatin

8 bottles (20 ml each), 10.000 Units (U)/ml

Total volume required: 160 ml = 1.600.000 U

Gentamycin

8 vials (2 ml each), 40 mg/ml

Total volume required: 16 ml = (640 mg)

Vancomycin

2 vials (10 ml each), 1 g/vial

Total volume required: 16 ml = 1600 mg

8 bottles of Nystatin (20 ml each) were dispensed into a 250 ml Erlenmeyer flask. A sterile magnet was placed inside the flask which was in turn, placed on a magnetic stirrer. The flask was covered with foil at all times.

Metal caps were removed from each Gentamycin vial which were first swabbed with ethanol soaked gauze and then flamed. 2 ml of Gentamicin were drawn into a 5 ml syringe and then added to the flask.

Metal caps were removed from each Vancomycin vial which were first swabbed with ethanol soaked gauze and then flamed. 5 ml of tdH_20 were drawn, and using a syringe, the water was injected into the vial for resuspension. The solution was well mixed, and 4 ml of the resuspended Vancomycin was drawn and added to the Erlemeyer flask. The whole procedure was repeated with all the vials. 3.2 ml of NGV stock solution were aliquoted into sterile track vials, and stored at 20^0C until required.

2.2 APMEM: Autoclavable powdered Minimal Essential Medium with glucose added

APMEM (SIGMA # M0769)	
1x10 Litre bottle	
glucose (dextrose)	36.0 g
water	9.6 L

APMEM and glucose were dissolved in distilled water using a magnetic stirrer. 420.0 ml of media were aliquoted per 500 ml serum bottle, then autoclaved at 121^0C for 30 min. Media was stored at 4^0C.

2.3 Bovine Serum Albumin (BSA) Stock, 10 mg/ml

BSA	1.0 g
PBS (pH 7.6)	100.0 ml

The above components were mixed thoroughly (without heating) on a magnetic stirrer until dissolved. The reagent was filter sterilized through a 0.2 micron nalgene disposable filter unit; 0.8 ml aliquots were dispensed into cryotubes and stored at 20^0C for less than 6 months.

2.4 CMGH (Chlamydia - Complete Media with glucose plus HEPES) pH 7.5

GAPMEM	420.0 ml
Foetal Bovine serum	50.0 ml
HEPES (1 M)	10.0 ml
$NaHCO_2$ (2.8 %) (sodium bicarbonate)	15.0 ml

Foetal bovine serum, HEPES and sodium bicarbonate were added to GAPMEM, mixed well and the pH adjusted to 7.5 using 10N NaOH. The CMGH was incubated at 35^0C for 7 days to check for sterility. The bottles were then, transferred to 4^0C refrigerator.

Prior to use: 5.0 ml of L glutamine, cycloheximide and NGV (Amphotericin B, Gentamicin, Vancomycin) were added to the CMGH.

2.5 Chlamydia group specific, monoclonal antibody culture stain [CF-2]

Dilution	1:40 (8ml)	1:50 (8ml)	1:60 (8ml)
CF2 stock	200.0 μl	160.0 ml	135.0 ml
BSA (stock)	0.8 ml	0.8 ml	0.8 ml
PBS + Evans blue + sodium azide + glycerol stock	7.0 ml	7.0 ml	7.1 ml

All ingredients were mixed into the BSA amber 2 dram vial, then vortexed. The stain was stored at 4^0C.

Warning: Evans blue is an animal carcinogen; mask and gloves were worn and appropriate precautions taken.

2.6 Cycloheximide (1.0 mg/ml)

Cycloheximide	100.0 mg
water	100.0 ml

Cycloheximide (Sigma # C 6255) was dissolved in water in an erlenmeyer flask on a magnetic stirrer. The mixture was then filter sterilized through a 0.22 μm filter and 1 ml aliquots dispensed into cryotubes and stored at 70^0C.

2.7 HEPES Buffer (1 M, pH 7.5)

HEPES	714.9 g
water	3.0 L
NaOH (10 N)	

HEPES (Sigma #H9136) was added to water on a magnetic stirrer to dissolve. The solution was refrigerated overnight to equilibrate. The pH was adjusted to 7.5 using 10N NaOH and then filter sterilized through a 22.0 μm membrane filter. The filter flasks containing the solutions were incubated for 1 week to check for contamination. 150 ml aliquots were dispensed into 200 ml bottles and stored at room temperature.

2.8 Monoclonal Antibody Blocking Solution

BSA	15.0 g
NaN_3 (sodium azide)	1.0 g
Tween-20	0.5 ml
(Polyoxyethylene Sorbitan Monolaurate)	
PBS (pH 7.5) to	1.0 L

The above components were mixed thoroughly on a magnetic stirrer (without heating) until dissolved. The solution was then filter sterilized into 250 ml schott bottles using a 0.2 micron nalgene disposable filter unit. The solution was stored at 4^0C for 6 months.

2.9 PBS/Evans Blue/Sodium Azide diluent for monoclonal antibody stain (25 X EB stock)

PBS (pH 7.6)	250.00 ml
evans blue (0.5%)	1.25 g
sodium azide (5.0%)	12.50 g

The above components were dissolved thoroughly on a magnetic stirrer by applying very low heat. The stain was filter sterilized using a 0.2 micron nalgene disposable filter unit and then stored at room temperature.

2.10 PBS/Evans Blue/Sodium Azide diluent for monoclonal antibody stain (1 X stock)

PBS (pH 7.6)	200.0 ml
glycerol	25.0 ml
25 X stock (evans blue)	25.0 ml

Glycerol and 25X Evanss Blue (EB) stock were mixed into PBS and stored at room temperature for no more than six months.

2.11 PBS, pH 7.4 (0.05 % Tween) (1 X)

1 X PBS, pH 7.4	1.0 L
Tween-20	0.5 ml

The above components were mixed and stored at room temperature. PBS T was added to the label.

2.12 Sucrose-Phosphate-Glutamate Freezing Media (SPG)

Sucrose	75.00 g
KH_2PO_4	0.52 g
$Na_2HPO_4.7H_2O$	2.30 g
Glutamic acid	0.72 g
water to	1.00 L

The above components were combined together with 800.0 ml of water, and mixed thoroughly on a magnetic stirrer. The pH was adjusted to 7.5 using 10 M NaOH or HCl as required. The volume was then adjusted to 1000.0 ml. The solution was filter sterilized using a 22.0 micron filter and stored at 4^0C for $\leq$ 12 months.

APPENDIX 3: SOLUTIONS USED FOR DNA EXTRACTION

3.1 Absolute Ethanol cold

50.0 ml of absolute ethanol (BDH) was poured into a 50 ml plastic tube and stored at 20^0C.

3.2 Chloroform-isoamyl alcohol (24:1)

24 parts of $CHCl_3$ were mixed with 1 part of isoamyl alcohol in a dark bottle. The bottle was then stored at 4^0C.

3.3 Cold Ethanol (70 %)

70.0 ml of absolute ethanol and 30 ml of water were added, mixed and stored at 20^0C in a plastic bottle.

3.4 Ethidium bromide (10 mg/ml)

ethidium bromide	0.2 g
water to	20.0 ml

To prepare a 10 mg/ml stock solution, the above component was dissolved in 20.0 ml of water and stirred on a magnetic stirrer for several hours to ensure that the dye had dissolved. The container was wrapped in foil and stored at 4^0C. For every 100.0 ml of agar, 100.0 μl of ethidium bromide solution were added i.e 1.0 μl/ml.

3.5 1 M EDTA (pH 8)

disodium EDTA.$2H_20$	93.05 g
water to	250.00 ml

The above component was dissolved in 200.0 ml of water by stirring vigorously on a magnetic stirrer. The pH was adjusted with approximately 10.0 g of NaOH pellets and the volume adjusted to 250.0 ml with water. The solution was autoclaved and stored at room temperature.

3.6 0.5 M EDTA (pH 8)

disodium EDTA.$2H_20$ (Sigma # ED255) 46.53 g
(disodium ethylenediaminetetraacetate.$2H_20$)

The above reagent was dissolved in 200.0 ml of water by stirring vigorously on a magnetic stirrer. The pH was adjusted to 8 with approximately 6.0 g of NaOH pellets. The volume was adjusted to 250.0 ml with water, then aliquoted in 20 ml amounts, autoclaved and stored at room temperature.

3.7 1 M Hydrochloric Acid (HCl)

20.0 ml of concentrated HCl was diluted in 220.0 ml of water. The solution was stored at room temperature, in a dark bottle.

3.8 5 M NaCl

NaCl (Sigma # S 9625)	73.05 g
water to	250.00 ml

The solution was heated at low temperature while stirring to dissolve the chemical. The volume was adjusted to 250.0 ml with water, dispensed into 20 ml aliquots, autoclaved, and stored at room temperature.

3.9 Phenol/Chloroform/iso-amyl alcohol (25:24:1)

25 parts of phenol and 24 parts of $CHCl_3$ were mixed with 1 part of iso amyl alcohol in a dark bottle. The bottle was then stored at 4^0C.

3.10 Proteinase K (Boehringer Mannheim)

To prepare a stock solution of 20 mg/ml, 100 mg were weighed out into a sterile bijou bottle and dissolved in 5 ml of water. 250 μl aliquots were dispensed into 0.5 ml Eppendorf tubes and stored at 20^0C.

3.11 RNAse stock solution (10 mg/ml)

pancreatic RNAse	0.2 g
sodium acetate (0.01 M)	20.0 ml

The pancreatic RNAse was dissolved in 20.0 ml of sodium acetate solution (pH 5.2) and boiled at 100^0C for 15 min. The solution was allowed to cool slowly to room temperature, then the pH was adjusted to 7.4 by adding 0.1 volumes of 1 M Tris.HCl. The solution was dispensed into 2 ml aliquots and stored at 20^0C.

3.12 3 M sodium acetate (pH 5.2 and pH 7.0)

sodium acetate.$3H_20$	102.03 g
water to	250.00 ml

The above component was dissolved in water and the pH adjusted to 5.2 with glacial acetic acid or adjusted to pH 7.0 with dilute acetic acid. The volume was adjusted to 250.0 ml with water,dispensed into 20 ml aliquots, autoclaved and stored at room temperature.

3.13 Sodium dodecyl sulphate 10% (SDS)

SDS	25.0 g
(sodium lauryl sulphate)	
water to	250.0 ml

SDS was dissolved in 200.0 ml of water and the final volume was adjusted to 250.0 ml. The solution was dispensed into aliquots and stored at room temperature.

NB! The solution was not autoclaved nor was the pH adjusted.

3.14 10 X Tris-EDTA (TE) Buffer (pH 8.0)

1 M Tris HCl (pH 8.0)	10.0 ml
0.5 M EDTA (pH 8.0)	2.0 ml
water	88.0 ml

The buffer was stored at room temperature.

3.15 0.1 X TE Buffer

10 X TE Buffer	1.0 ml
water	99.0 ml

The buffer was stored at room temperature.

3.16 1 M Tris-HCl (pH 8)

Tris base (Sigma # T 8524)	30.3 g
water to	250.0 ml

The above component was dissolved in 200.0 ml of water. The pH was adjusted to 8 by adding approximately 10.0 ml (drop by drop) of concentrated HCl, thereafter, the volume was brought up to 250.0 ml. The solution was aliquoted into working solutions, autoclaved and store at room temperature.

3.17 2 M Tris-HCl (pH 9.0)

Tris base (Sigma # T 8524)	30.3 g
water to	250.0 ml

The above component was dissolved in 200.0 ml of water. The pH was adjusted to 9.0 by adding approximately 10.0 ml (drop by drop) of concentrated HCl, thereafter, the volume was brought up to 250.0 ml. The solution was aliquoted into working solutions, autoclaved and stored at room temperature.

APPENDIX 4: REAGENTS USED FOR PCR

4.1 Deoxynucleoside Triphosphate (dNTP) Mix

dATP (10 mM)	25.0 μl
dCTP (10 mM)	25.0 μl
dGTP (10 mM)	25.0 μl
dTTP (10 mM)	25.0 μl
Filtered water	100.0 μl

The above components were added into a 1.5 ml sterile Eppendorf tube, vortexed and stored at 20^0C.

4.2 10 X PCR Buffer

1 M Tris HCl (100 mM), pH 8.0	1.00 ml
1 M KCl (500 mM)	5.00 ml
gelatin (0.1 %)	0.01 g
water	4.00 ml

The above components were mixed, aliquoted into 2.0 ml Eppendorf tubes and stored at 20^0C.

4.3 Reagents obtained from University of Washington Chemistry Lab.

- 10 X magnesium chloride ($MgCl_2$) 1.75 mM for plasmid PCR
  10 X $MgCl_2$ 100 mM for MOMP PCR
  Primers (Plasmid): ODnaB L (20 μM)
  ODnaB R (20 μM)
  Primers (MOMP): oDVD IV (20 μM)
  o5'MOMP (20 μM)

APPENDIX 5: REAGENTS AND BUFFERS USED IN ELECTROPHORESIS

5.1 Acrylamide 30%

Acrylamide	29.0 g
N,N' methylene bis acrylamide	1.0 g
water to	100.0 ml

The solution was heated to 37^0C to dissolve the chemicals. The solution was adjusted to 100.0 ml with water and filter sterilized through a Nalgene filter (0.45 micron pore size). The pH was checked to make certain that it was 7.0 or less, and the solution was stored at room temperature in dark bottles.

5.2 Ammonium persulphate 10%

Ammonium persulphate	1.0 g
water to	10.0 ml

This solution was stored at 4^0C for several weeks.

5.3 6 X Gel-loading Buffer

bromophenol blue	0.25 %
xylene cyanol FF	0.25 %
glycerol in water	30.00 %

The above components were mixed and stored at 4^0C.

Bromophenol blue migrates through agarose gels approximately 2.2 fold faster than xylene cyanol FF, independent of the agarose concentration.

This gel-loading buffer serves three purposes:

- **it increases the density of the sample, ensuring that the DNA drops evenly into the well,**
- **it adds colour to the sample, thereby simplifying the loading process, and**
- **it contains dyes that, in an electric field, move toward the anode at predictable rates.**

5.4 Molecular Size Markers

1 kbp (GIBCO BRL)
Marker II (Boehringer)
Marker V (Boehringer)
Marker VI (Boehringer)
Marker IX (Boehringer)

These were all stored in the 20^0C freezer.

5.5 Restriction enzymes (Boehringer Mannheim)

Alu I (# 239 275)
Cfo I (# 688 541)
Dde I (# 835 293)
EcoRI (# 703 737)
Fok I (# 1004 824)
HinfI (# 779 652)

These were all stored in the 20^0C freezer.

5.6 5 X TBE buffer (Tris-borate-EDTA)

tris base	54.0 g
boric acid	27.5 g
0.5 M EDTA (pH 8)	20.0 ml
water to	1000.0 ml

The solution was diluted to working concentration with water and stored at room temperature.

5.7 1 X TBE Buffer

5 X TBE Buffer	200.0 ml
water	800.0 ml

A 1:5 (50.0 ml 5X TBE buffer + 200.0 ml water) dilution was prepared. The solution was stored at room temperature.

5.8 0.5 X TBE buffer

5 X TBE Buffer	100.0 ml
water	900.0 ml

The buffer solution was mixed and stored at room temperature.

APPENDIX 6: GENITAL ULCER STUDY QUESTIONNAIRE

GENITAL ULCER STUDY 1995

1. STUDY NO _____
2. NAME _____ CLINIC NO_____
3. AGE IN YEARS _____
4. MARITAL STATUS SINGLE =1
   MARRIED =2
   STABLE PARTNER =3
5. CIRCUMCISED YES =1
   NO =2

6. SEXUAL HISTORY
   A. AGE AT FIRST INTERCOURSE_____
   B. NO. OF SEXUAL PARTNERS (SINCE JANUARY 1995)_____
   C. TOTAL NO. OF LIFETIME PARTNERS_____
   D. PREVIOUS EPISODES OF STD_____
   E. LAST SEXUAL CONTACT (DAYS)_____
   F. DURATION OF LESION_____
   G. INTERCOURSE WHILE LESION PRESENT_____

7. LYMPHADENOPATHY YES =1
   NO =2
   IF YES: LEFT =1
   RIGHT =2
   BILATERAL =3
   TENDER =1
   NON-TENDER =2
   DESCRIPTION OF LYMPHADENOPATHY:
   SHOTTY =1

FIRM	=2
INDURATED	=3
BUBO/SUPPURATIVE	=4
GROOVE SIGN	=5
ULCERATED	=6
OTHER	=7

8. ULCER SITE:

PENILE SHAFT	=1
GLANS	=2
PREPUCE INSIDE	=3
PREPUCE OUTSIDE	=4
CORONAL SULCUS	=5
SCROTUM	=6
FRENULUM	=7
OTHER	=8

9. NO. OF LESIONS_____

10. TYPE OF LESION:

PAPULE	=1
VESICLE	=2
PUSTULE	=3
EROSION	=4
ULCER	=5

11. PAINFUL:	YES	=1
	NO	=2

12. BASE:	SUPERFICIAL	=1
	DEEP	=2

13. EDGE:	IRREGULAR	=1
	REGULAR	=2
	UNDERMINED	=3
	SMOOTH	=4
	RAISED	=5
	OTHER	=6
14. BLEEDING:	YES	=1
	NO	=2
15. CONCURRENT URETHRAL DISCHARGE:		
	YES	=1
	NO	=2
16. CLINICAL DIAGNOSIS:		
	PRIMARY SYPHILIS	=1
	SECONDARY SYPHILIS	=2
	CHANCROID	=3
	LGV	=4
	HERPES GENITALIS	=5
	GRANULOMA INGUINALE	=6
	WARTS	=7
	OTHER	=8
17. TREATMENT:	PENICILLIN	=1
	ERYTHROMYCIN	=2
	TETRACYCLINE	=3
	CEFTRIAXONE	=4
	OFLOXACIN	=5
	CO TRIMOXAZOLE	=6
	ACYCLOVIR	=7
	BACTRIM	=8
	OTHER	=9