



**The molecular and serological detection of
Chlamydia trachomatis in women with reproductive
abnormalities.**

I hereby declare that no part of this thesis has previously been submitted in support of an application for any degree or qualification, of this or any other University or Institute of

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B.Sc. (Hons.)

**A thesis submitted for the degree of
Doctor of Philosophy**

**Section of Infection and Immunity,
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Declaration:

I hereby declare that no part of this thesis has previously been submitted in support of an application for any degree or qualification, of this or any other University or Institute of learning, unless otherwise acknowledged.

Dedication:

This thesis is dedicated to my family, especially my Granddad who has always been and will continue to be an inspiration to me.

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Summary

The primary aim of this study was to evaluate the role of *C. trachomatis* in women with ectopic pregnancy (EP), miscarriage and tubal factor infertility (TFI) using PCR, non-isotopic *in situ* hybridization (NISH) and serology when compared to controls. Also, the influence of polymorphisms in the TNF gene were investigated in a pilot study to determine if there was an association between chlamydial infection and the development of tubal pathology. The primary study included patients from the UK and Trinidad. A secondary study was also conducted to assess the impact of anti-chlamydial IgG on ovarian response to gonadotropin stimulation prior to *in vitro* fertilization (IVF).

Significant levels of anti-chlamydial IgG were found in the UK patients presenting with TFI, and a similar association was seen in the Trinidad EP group. In the case of miscarriage the detection of anti-chlamydial antibody was not significant. The IVF study showed that women undergoing IVF, who also had detectable levels of anti-chlamydial IgG were statistically more likely to respond poorly to gonadotropin ovarian stimulation prior to IVF.

The presence of chlamydial DNA, as determined by both PCR and ISH was significant in the UK TFI group. There was an increased detection of chlamydial DNA in both EP groups, although this association was found not to be significant. Statistically the presence of chlamydial DNA in the Trinidad miscarriage group was not suggestive of a role for *C. trachomatis* in the development of this condition.

Within the EP and TFI groups investigated, the presence of either anti-chlamydial IgG or chlamydial DNA was suggestive of tubal damage. In the case of the EP patients, a large number of patients had detectable levels of anti-chlamydial IgM and/or chlamydial DNA in serum and tissue samples respectively; whereas in the TFI group there was increased detection of anti-chlamydial IgG and/ or chlamydial DNA. From these data it is proposed that EP is positively associated with a concurrent *C. trachomatis* infection of the genital tract as supported by both serological evidence, and the detection of chlamydial DNA in genital specimens. In the case of tubal damage found in TFI patients the presence of anti-chlamydial IgG and/ or chlamydial DNA suggests that this condition is associated with a repeated past/persistent *C. trachomatis* infection as opposed to a current genital chlamydial infection.

List of Abbreviations

- AP-** alkaline phosphatase
APES- 3' aminopropyltriethoxysaline
ART- assisted reproductive technology
ATP- adenosine triphosphate
BCIP- 5'-bromo-4 chloro-3-indolyl phosphate
BGMK- buffalo green monkey kidney cells
bp- base pair(s)
BR- Bristol
BSA- bovine serum albumin
C DS1- chlamydial dual species serum 1
C DS2- chlamydial dual species serum 2
CCA- chlamydial cytheadhesin
CFT- complement fixation test
CHSP- chlamydial heat shock protein
CPN SP- *Chlamydia pneumoniae* specific serum
CT SP- *Chlamydia trachomatis* specific serum
DFA- direct fluorescent antibody
DMSO- dimethyl sulphoxide
DNA- deoxyribonucleic acid
dNTP- deoxynucleoside triphosphate(s)
DTH- delayed type hypersensitivity
dU- PCR product containing uracil
dUTP- uracil deoxynucleoside triphosphate
EB- elementary body
ECACC- European Collection of Animal Cell Cultures
ECL- enhanced chemiluminescence
EDTA- ethylenediaminetetraacetic acid
EIA- enzyme immunoassay
ELISA- enzyme-linked immunoassay
EP- ectopic pregnancy
F- forward PCR primer
FDA- Food and Drug Administration
FITC- fluorescein isothiocyanate
FSH- follicle stimulating hormone
GAG- glycosaminoglycan
GIFT- gamete intra-fallopian tube transfer
GM-CSF- granulocyte-macrophage colony stimulating factor
GPIC- guinea pig inclusion conjunctivitis
GRO- growth regulated oncogene

GUM- genitourinary medicine
H&E- haematoxylin and eosin
HLA- human leukocyte antigen
hMG- human gonadotropin
HRP- horse radish peroxidase
HSP- heat shock protein
ICAM-1- intercellular adhesion molecule-1
IDO- indoleamine 2,3-dioxygenase
IF- immunofluorescence
IgA- immunoglobulin A
IgG- immunoglobulin G
IgM- immunoglobulin M
IL- interleukin
IFN γ - interferon gamma
iNOS- inducible nitric oxide synthase
ISH- *in situ* hybridization
IU- international units
IVF- *in vitro* fertilization
JHW- Jessop Hospital for Women
Kdo- 3 deoxy-D-manno-octulosonate
LCR- ligase chain reaction
LGV- lymphogranuloma venereum
LH- luteinizing hormone
LPS- lipopolysaccharide
LT- lymphotoxin
MC- miscarriage
MHC- major histocompatibility complex
MIF- microimmunofluorescence
MOMP- major outer membrane protein
MoPn- mouse pneumonitis agent
NASBA- nucleic acid sequence based amplification
NBT- nitroblue tetrazolium
NGU- non-gonococcal urethritis
NISH- non-isotopic *in situ* hybridization
nt- nucleotide(s)
OD- optical density
PAGE- polyacrylamide gel electrophoresis
PBS- phosphate buffered saline
PCR- polymerase chain reaction
PGU- post-gonococcal urethritis
PID- pelvic inflammatory disease

PMN- polymorphonuclear leukocytes
R- reverse PCR primer
RB- reticulate body
RNA- ribonucleic acid
rRNA- ribosomal ribonucleic acid
RT- reverse transcriptase
SARA- sexually acquired reactive arthritis
SD- standard deviation
SDS- sodium dodecyl sulphate
SSC- saline sodium citrate buffer
ssDNA- single stranded deoxyribonucleic acid
STD- sexually transmitted disease
TBE- tris-borate EDTA buffer
TEMED- n,n,n',n'-tetramethyl-ethylenediamine
TFI- tubal factor infertility
Th1- type 1 helper
TMA- transcription mediated amplication
TNF- tumor necrosis factor
Tr- Trinidad
UK- United Kingdom
UNG- uricil N-glycosylase
USA- United States of America
UV- ultraviolet
VD- variable domain
WHO- World Health Organization
WIF- whole inclusion fluorescence

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Web Sites

<http://www.ncbi.nlm.nih.gov/> - National Center for Biotechnology Information (NCIB).

<http://www.ncbi.nlm.nih.gov/BLAST> - NCIB search tool designed to support the analysis of nucleotide and protein data bases.

<http://chlamydia.www.berkeley.edu:4231/> - Chlamydia Genome Project. Chlamydial DNA sequences found at this site can also be accessed through NCIB.

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Chapter 1.0

General Introduction

1.1 Historical perspectives of *Chlamydia*

In 1877, two years before the description of the gonococcus by Albert Neisser, Emil Noeggerath published findings that linked sexuality, genital infection and infertility suggesting that approximately 90% of sterile women were married to men who had suffered from gonorrhoea. During the latter part of the last century *Neisseria gonorrhoeae* was documented as a causative agent of urethritis, epididymitis, cervicitis and salpingitis. Post-infectious infertility was described as a result of both gonococcal salpingitis and bilateral gonococcal epididymitis (cit. Oriel, 1994) and during the turn of the century only 6% of women who had had gonococcal salpingitis conceived (Forssner, 1907).

The oculogenital association of ophthalmia neonatorum was initially confirmed by the work of Piringer in 1841, who showed that the gonococcus could infect the human eye (cit. Duke-Elder, 1965). In 1884, Kroner published a report on a non-gonococcal manifestation of ophthalmia neonatorum, it was suggested that the infective organism was transmitted from the genital tract of the neonate's mother. In 1907, the causative agent of trachoma (a long recognised eye disease) was identified by Halbertaedter and von Prowazek, who originally suggested the name Chlamydozoa, derived from the Greek chlamys meaning 'mantle' describing the halo effect observed surrounding Giemsa stained inclusions.

Other investigators found similar inclusions in conjunctival scrapings from neonates, and from urogenital tract samples from their mothers suggesting a link between the cause of the inclusions and sexually transmitted disease (Halbertaedter and von Prowazek, 1909; Stargardt, 1909; Heymann, 1910; Lindner, 1910). Fritsch *et al.* (1910) in a series of experiments infected the conjunctivae of monkeys with cervical secretions from mothers and urethral secretions from the fathers of infected neonates. The monkeys went on to develop inclusion conjunctivitis irrespective of the original source of the inoculum.

Due to the difficulty of culturing the causative agent of inclusion conjunctivitis there was an extended delay in the study of chlamydiae until the end of the 1930s with the identification of the agent of psittacosis (Bedson *et al.*, 1930) and Lymphogranuloma venereum (Hellerstrom and Western, 1930). In 1932, Bedson and Bland described the two distinct forms of chlamydiae, the elementary body and the reticulate body, and demonstrated the chlamydial developmental cycle.

In 1957 T'ang and co-workers successfully isolated the causative agent of trachoma using the yolk sac of fertilized hens eggs. This was followed in 1965 with the introduction of tissue culture by Gordon and Quan using irradiated McCoy cells.

Jones *et al.* (1959) isolated *Chlamydia trachomatis* from a neonate with inclusion conjunctivitis and from the cervix of the mother thus confirming the original findings of investigators at the beginning of the century. *C. trachomatis* was subsequently isolated from urethral secretions of men who had had previous sexual contact with women with inclusion conjunctivitis, and from women diagnosed as having non-gonococcal genital tract infection (Jones, 1964; Dunlop *et al.*, 1964; Holt *et al.*, 1967). But it was not until the 1970s that the importance of *C. trachomatis* as a cause of non-gonococcal urethritis (Hilton *et al.*, 1974; Swanson *et al.* 1975; Oriel and Ridgway, 1982), and acute salpingitis and pelvic inflammatory disease (Mardh *et al.*, 1977; Punnonen *et al.*, 1979; Mardh *et al.*, 1981) was established.

1.2 Classification and taxonomy of *Chlamydia*

The taxonomic classification of the order Chlamydiales reflects their unique obligate intracellular biology. Chlamydiae have been placed in their own order, Chlamydiales, containing one family *Chlamydiaceae* and a single genus *Chlamydia* (Moulder *et al.*, 1984). Although chlamydia were originally thought to be protozoa, or viruses, they were later found to have properties that classified them as bacteria (Sarov and Becker, 1971). It was confirmed using rRNA analysis that *Chlamydia* were in fact eubacteria but with very little relatedness to other eubacterial orders (Weisburg *et al.*, 1986).

The genus *Chlamydia* presently consists of four species, *Chlamydia trachomatis*, *Chlamydia psittaci*, *Chlamydia pneumoniae* and the newest member of the genus *Chlamydia pecorum*. DNA homology between chlamydial species is reported to be less than 10% (Kingsbury and Weiss, 1968; Campbell *et al.*, 1987; Fukushi *et al.*, 1992), although structurally and biochemically they appear to be similar, with amino acid homology between analogous proteins being high.

Studies of the phylogenetic structure of the genus *Chlamydia* by analysis of the 16S rRNA gene show a phylogenetic tree with a distinct line of descent within the family *Chlamydiaceae* (Pettersson *et al.*, 1997). This descent being built around two main clusters, labelled the *C. pneumoniae* cluster (human *C. pneumoniae* isolates and the equine strain N16), and the *C. psittaci* cluster (*C. psittaci*, *C. pecorum* and *C. trachomatis*).

Similar work by Pudjiatmoko *et al.* (1997) also using analysis of the 16S rRNA gene sequences gave two clusters comprising eight groups, each cluster corresponding to a phenotype as defined by inclusion morphology, glycogen content, sulfadiazine sensitivity, and the number of rRNA gene loci. One cluster coincided with *C. trachomatis*, which forms vacuolar inclusions, contains glycogen in its inclusions, is sulfadiazine sensitive and has two rRNA loci. The second cluster included *C. psittaci*, *C.*

pneumoniae and *C. pecorum* which form dense inclusions that do not contain glycogen, are sulfadiazine resistant and only have one rRNA locus.

Gaydos *et al.* (1993) by the analysis of the 16S ribosomal DNA sequences of *C. pneumoniae*, *C. trachomatis* and *C. psittaci* showed that *C. pneumoniae* was more closely related to *C. psittaci* than *C. trachomatis*. From an evolutionary standpoint it has been suggested that N16 and/or *C. psittaci* carry ancestral features of the original species, and that *C. trachomatis* although not newly described is the most recently evolved species within the genus. It may be that studies on the phylogentic analysis of the 16S rRNA gene sequences may eventually re-define the structure of the family *Chlamydiaceae* (Adam *et al.*, 1998).

Many *Chlamydia*-like organisms have been described in the literature, including the *Simkania* sp. strain "Z" (Kahane *et al.*, 1993; Kahane *et al.*, 1995) which was originally described as a contaminant of laboratory cell cultures. By analysis of the 16S rRNA gene sequence Pudjiatmoko *et al.* (1997) have suggested that "Z" may belong to a second genus of the family *Chlamydiaceae*, and that it may cause/contribute to community acquired pneumonia (Lieberman *et al.*, 1997). Amann and colleagues (1997) have also described a chlamydia-like organism (BN9) which is an endoparasite of *Acanthamoebae*. The 16S rRNA gene was shown to have between 86-87% sequence homology to chlamydial 16S rRNA.

1.2.1 *Chlamydia pecorum*

C. pecorum is the most recently described chlamydial species and consists of bovine, ovine and swine strains that have been previously classified as *Chlamydia psittaci* (Fukushi and Hirai, 1992; Fukushi and Hirai, 1993). Chlamydial strains belonging to *C. pecorum* have been isolated from cattle with encephalomyelitis, from synovial fluid samples from sheep and calves with polyarthritis, and also from conjunctival and intestinal samples of ruminants. *C. pecorum* can also cause inapparent enteric infection, and animals can become carriers after recovery from infection.

Known phenotypes of *C. pecorum* are indistinguishable from *C. psittaci* in that they form oval and dense inclusions in cell culture, do not deposit glycogen in inclusions, and are resistant to sulfadiazine. The percentage DNA homology between *C. pecorum* and other *Chlamydia* has been shown to be less than 30%, and between strains of *C. pecorum* greater than 88%.

1.2.2 *Chlamydia pneumoniae*

The first isolates of *C. pneumoniae* were obtained during the trachoma studies of the 1960s; TW-183 was isolated from the eye of a child with suspected trachoma in Taiwan, and IOL-207 was isolated from the eye of a child with trachoma in Tehran; it was shown that IOL-207 and TW-183 were antigenically similar by the micro-immunofluorescence (MIF) test (Dwyer *et al.*, 1972). During 1983 a similar isolate was recovered from a college student with pneumonia in Seattle and named AR-39. AR-39 was shown to be antigenically similar to TW-183 (Grayston *et al.*, 1986). The acronym TWAR reflects the geographic site of the initial isolate (Taiwan-TW), and the clinical manifestation of the latter (acute respiratory disease-AR).

Initially TWAR was thought to be a *C. psittaci* strain on the basis of inclusion morphology and staining characteristics, but subsequent analysis demonstrated that it was a distinct organism which has now been recognized as a separate chlamydial species (Grayston *et al.*, 1989; Grayston *et al.*, 1990). Restriction endonuclease pattern analysis and nucleic acid hybridization studies have shown that *C. pneumoniae* isolates have less than 10% homology with *C. psittaci* and *C. trachomatis*, and between *C. pneumoniae* isolates greater than 95% homology.

Unlike *C. trachomatis*, different serovars of *C. pneumoniae* have not been identified, and it has been suggested that all *C. pneumoniae* organisms represent a single strain (Campbell *et al.*, 1990; Grayston, 1992). To date the *omp1* gene of nine *C. pneumoniae* strains have been sequenced and have been found to be 100% identical, suggesting the gene is conserved (Carter *et al.*, 1991; Melgosa *et al.*, 1991; Jantos *et al.*, 1997).

However, Jantos *et al.* (1997) showed that even though the *omp1* gene was identical in the *C. pneumoniae* isolates studied, antigenic diversity did exist possibly within a 65kDa protein.

1.2.3 *Chlamydia psittaci*

C. psittaci comprises a plethora of isolates from many different mammals, birds and amphibians. *C. psittaci* is an important pathogen of domestic ruminants, and can induce abortion in cattle, goats and sheep. It can also cause respiratory disease, enteritis, conjunctivitis, arthritis and reproductive disorders, but latent infections (eg. intestinal colonization) without clinical symptoms are a common feature.

C. psittaci can also cause zoonotic disease in man, being responsible for respiratory infection where the host has had contact with infected birds (psittacosis). *C. psittaci* is spread via the inhalation of aerosols derived from animal faeces or other animal products and causes predominantly pneumonia in man. Disease in humans can vary from a mild flu-like to severe generalised illness with symptoms ranging from a fever, to anorexia, and photophobia. In severe cases nausea, vomiting, and diarrhoea may be seen. Pregnant women who come into contact with infected placental material from domestic animals can suffer abortion (Herring *et al.*, 1987).

Restriction endonuclease pattern analysis and DNA hybridization studies have shown that *C. psittaci* isolates have between 14-95% DNA homology within the species, between 1-8% with *C. pneumoniae* isolates and below 33% with *C. trachomatis* isolates. Sequence analysis of restriction enzyme profiles of DNA amplified from the MOMP gene of *C. psittaci* indicate that ruminant abortion strains, guinea pig inclusion conjunctivitis (GPIC), and feline isolates have relatively homogenous MOMP sequences when compared to *C. pecorum* isolates.

1.2.4 *Chlamydia trachomatis*

C. trachomatis has been subdivided into three biovariants (biovars); trachoma, lymphogranuloma venereum (LGV) and murine pneumonitis agent. DNA homology studies of the genome of the three *C. trachomatis* biovariants, and direct comparison of the DNA sequences of specific genes have shown that trachoma and the LGV biovars are basically identical, and that the murine biovar is more distantly related (Weiss *et al.*, 1970; Peterson and de la Maza, 1988). The trachoma and LGV biovars have distinct clinical features, LGV biovars can cause systemic infection and proliferate in lymphatic tissue, compared to the trachoma biovars which primarily infect the columnar epithelial cells of the mucous membranes.

Currently the trachoma biovar consists of 14 serovars designated by the letters A to K including serovariants Ba, Da, D*, Ga, Jv, Ia, and I⁻ (Wang and Grayston, 1991; Dean *et al.*, 1992; Lampe *et al.*, 1993; Dean *et al.*, 1994; Ossewaarde *et al.*, 1994a; Morre *et al.*, 1998). The LGV biovar consists of 4 serovars L1, L2, L2a, and L3 (Wang and Grayston, 1970; Wang *et al.*, 1985). In addition three serogroups independent of biovar have also been described; the B complex (serovars B, Ba, D, Da, E, L1, L2, and L2a), the C complex (serovars A, C, H, I, Ia, J, K, and L3), and the intermediate complex (serovars F and G) (Caldwell *et al.*, 1982a; Wang *et al.*, 1985; Yuan *et al.*, 1989).

The major serovar, subspecies, and species specific antigens have been attributed to the major outer membrane protein (MOMP) (Caldwell *et al.*, 1981; Stephens *et al.*, 1982; Baehr *et al.*, 1988; Stephens *et al.*, 1988). The polymerase chain reaction (PCR) amplification of the MOMP gene (*omp1*) and restriction enzyme digestion of PCR products have been used to differentiate the *C. trachomatis* serovars on the basis of restriction fragment length polymorphism (Frost *et al.*, 1991; Lan *et al.*, 1993; Rodriguez *et al.*, 1993; Lan *et al.*, 1994). Serovars A to C predominate in ocular trachoma, whereas serovars D-K are responsible for oculogenital infections.

1.3 Biology of *Chlamydia*

1.3.1 General characteristics

Chlamydiae are obligate intracellular bacteria, characterized by a biphasic developmental cycle consisting of two developmental forms the elementary body (EB), and the reticulate body (RB) (Figure 1). The EB is extracellular, and an infectious form characterized by an osmotically resistant outer membrane and a highly condensed chromosome.

Following attachment and entry into a host cell the EB transforms into the RB, this transition involving structural changes in the outer membrane organization and the relaxation of the condensed nucleoid. The RB is the intracellular, replicative form of chlamydiae. Chlamydiae have been termed energy parasites, and are incapable of generating a net gain of ATP and therefore rely on the host for ATP. Due to this, cell-free growth of chlamydia has not been achieved, and culture in cell culture systems or yolk sac are needed for their *in vitro* growth.

Another unique feature of chlamydiae is the envelope structure which is Gram negative in that it includes an inner membrane and an lipopolysaccharide-containing outer membrane. The chlamydial envelope apparently lacks peptidoglycan although chlamydiae possess penicillin binding proteins (Barbour *et al.*, 1982), and are sensitive to drugs that inhibit peptidoglycan synthesis such as penicillin G and D-cycloserine.

Chlamydiae also have unique 18 to 22 regularly spaced dome shaped surface projections per elementary body that protrude approximately 20nm above the outer membrane (Matsumoto, 1981; Nichols *et al.*, 1985). The projections are also found in a less ordered array in the reticulate body (Gregory *et al.*, 1979; Matsumoto, 1988). Studies have suggested that the projections extend through the domes and possibly through the membrane within which the reticulate bodies divide (Matsumoto, 1981; Matsumoto, 1982; Matsumoto, 1988; Chang *et al.*, 1997). The question still remains as to the purpose of the projections, they may serve as an anchor within the inclusion to bring the chlamydiae into close contact with the host cytoplasm; or they may actively participate in

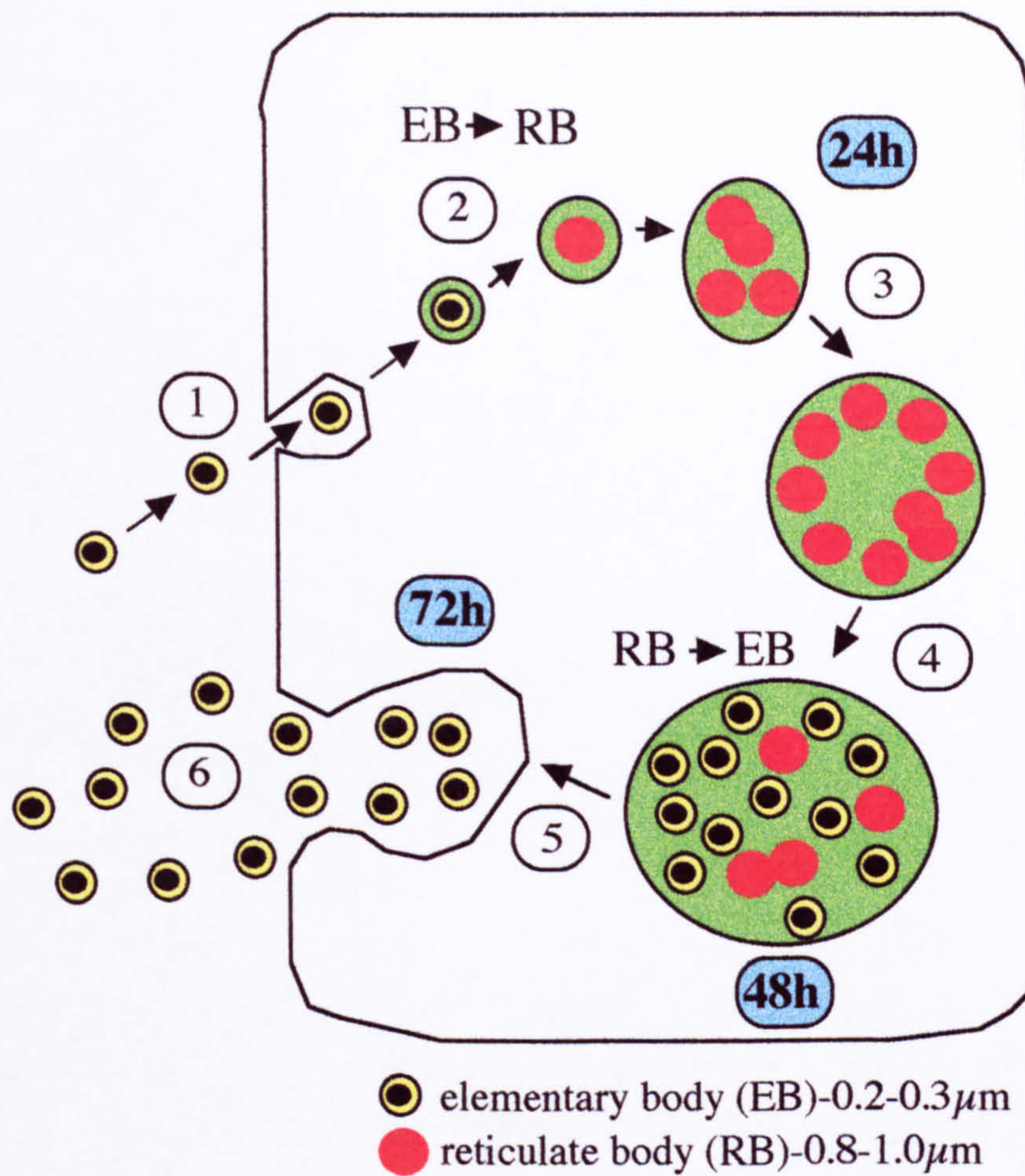


Figure 1. Schematic representation of the chlamydial developmental cycle.

- 1) EB adherence/attachment to the host cell, followed by internalization.
- 2) Inhibition of phagolysosomal fusion, and primary differentiation EB- \rightarrow RB.
- 3) RB multiplication by binary fission.
- 4) Asynchronous re-differentiation RB- \rightarrow EB.
- 5) EB release from host cell.
- 6) Continuation of chlamydial developmental cycle.

nutrient acquisition or be ion channels (Stephens, 1993). An alternative function of the surface projections has been proposed by Bavoil and Hsia (1998) based on the recent finding of type III (contact-dependent) genes in chlamydiae (Hsia *et al.*, 1997). Bavoil and Hsia have postulated that the surface projections and the associated fimbrial projections represent chlamydial type III secretion machinery and the associated virulence effector proteins.

1.3.2 Chlamydial developmental cycle

1.3.2.1 Attachment and entry mechanisms

The infectious process commences with the contact of the chlamydial elementary body (EB) to the mucosal epithelial cell surface of the host. The identity of chlamydial adhesins (ie. the determinants of the initial EB/cell interactions) is a matter of conjecture but a number of putative adhesins have been proposed which include the chlamydial outer membrane protein (MOMP) (Su *et al.*, 1990; Su *et al.*, 1996), chlamydial cytoadhesin (CCA) (Joseph and Bose, 1991), a heparan sulphate-like glycosaminoglycan (GAG) (Zhang *et al.* 1992), chlamydial heat shock protein 70 (HSP70) or a HSP70-genetically linked product (Schmiel *et al.*, 1991; Raulston *et al.*, 1993; Raulston *et al.*, 1998a), a 28kDa *grpE* protein (Schmiel *et al.*, 1996), and the cysteine-rich outer membrane protein OMP2 (Ting *et al.*, 1995). The precise mechanism by which EB uptake is mediated is not clear but proposed mechanisms include receptor-mediated endocytosis in clathrin-coated pits, pinocytosis in non-coated pits, and parasite-specified phagocytosis.

Zhang *et al.* (1992) and Stephens *et al.* (1994a, 1994b) using LGV demonstrated that chlamydial heparan sulphate attached to an undefined chlamydial surface ligand could bind the infecting EB to heparan sulphate receptors located in coated pits found on the surface of HeLa cells facilitating attachment and entry. Trachoma serovar infectivity has also been shown to use the same heparan sulphate-like mechanism (Chen and Stephens, 1994).

Kuo *et al.* (1996) have identified a glycosylated component of MOMP which has been shown to bring the EB in close contact with the surface of HeLa cells. It has also been shown that after conformational changes in the EB envelope, the substrate binding domain of envelope-associated chlamydial heat shock protein 70 (CHSP70) becomes selectively exposed. The exposed CHSP70 can either directly interact with a host cell ligand or present a separate chlamydial ligand which may trigger entry into the cell by an energy dependent process (Raulston *et al.*, 1998a).

1.3.2.2 Growth and multiplication

Once the chlamydiae have entered the host cell they remain within a vacuole termed the inclusion which does not fuse with lysosomes (Moulder, 1991) due to the early synthesis of chlamydial proteins (Scidmore *et al.*, 1996). Primary differentiation of EB to RB involves the reductive cleavage of MOMP and the other outer membrane proteins, and the relaxation of the chlamydial chromosome.

Sphingomyelin from the host's trans-Golgi network is specifically transported to the inclusion membrane, and it has even been detected in chlamydiae (Hackstadt *et al.*, 1995). Evidence has also suggested that chlamydiae re-model the inclusion membrane by the insertion of chlamydial proteins (Rockey *et al.*, 1995; Taraska *et al.*, 1996; Bannantine, 1998).

The RB replicates by binary fission within the inclusion with the resulting progeny remaining closely associated with the inclusion membrane until later in the developmental cycle, suggesting that such an intimate interaction may be required for intracellular growth (Matsumoto, 1981).

1.3.2.3 Release from host cell

Between 48-72h post-infection the asynchronous process of RB to EB differentiation begins. It has been suggested by Hackstadt *et al.* (1997) that RB detachment from the

inclusion membrane may be a signal for differentiation. RB to EB differentiation involves the oxidation of the outer membrane cysteine containing proteins, and the recondensation of the chlamydial chromosome.

The actual mechanism by which chlamydiae are released from the host cell, and the triggering mechanism remains unclear. It has been proposed that the chlamydial inclusion membrane fuses with the host cell membrane, releasing EBs, or that there may be a gradual disintegration of the inclusion releasing EBs into the host cytoplasm which then leave the cell.

1.3.3 Chlamydial persistence

The importance of re-infection in the development of chlamydial disease is well documented. In both ocular and genital tract infection progression to disease involves a stage of chronic immunopathology and scarring during which chlamydiae are rarely isolated. There has been indirect evidence in cases of genital tract and ocular infection that supports the existence of persistent chlamydial forms (Schachter *et al.*, 1988; Holland *et al.*, 1992; Campbell *et al.*, 1993; Patton *et al.*, 1994b).

The concept of persistent chlamydial infection is not new (Meyer *et al.*, 1933).

Persistence *in vitro* has been induced via nutrient deficiency, antimicrobial agents and immunologically. Penicillin and ampicillin when added to chlamydial culture inhibit the redifferentiation of RB to EB by affecting the synthesis of the cysteine rich 60kDa outer membrane protein (Cevenini *et al.*, 1988; Sardinia *et al.*, 1988).

In vitro chlamydial persistence induced by nutrient depletion is characterized by smaller inclusions, and a reduction in the number of infected cells (Coles *et al.*, 1993). The specific depletion of cysteine results in the development of morphologically and metabolically abnormal chlamydiae (Allen *et al.*, 1985), possibly due to the requirement of cysteine in the biosynthesis of MOMP and other cysteine rich outer membrane proteins.

Beatty *et al.* (1993) demonstrated interferon gamma (IFN- γ) mediated chlamydial persistence *in vitro*, characterized by enlarged morphologically abnormal developmental forms similar to those induced by penicillin and nutrient depletion (Matsumoto and Manire, 1970; Kramer *et al.*, 1971; Coles *et al.*, 1993). In persistently infected cells aberrant chlamydiae are non-infectious, however viability is maintained as shown by the recovery of infectious progeny following the removal of IFN- γ (Beatty *et al.*, 1995). The development of persistence has been postulated to be related to the induction of host indoleamine 2,3-dioxygenase (IDO), and the subsequent depletion of cellular tryptophan coupled with reduced synthesis of MOMP, 60kDa outer membrane protein and lipopolysaccharide and the continued/up-regulated synthesis of chlamydial heat shock protein 60 (Beatty *et al.*, 1994).

1.3.4 Chlamydial genetics

1.3.4.1 Chlamydial genome

The genome of *C. trachomatis* has been measured at 1045kb, and as such is one of the smallest prokaryotic genomes being approximately a quarter the size of the *E. coli* genome (Birkelund and Stephens, 1992). The biochemical and/or physical events that trigger and mediate chlamydial differentiation from elementary body to reticulate body, and back again are unknown.

The chlamydial elementary body displays a highly condensed genome (Costerton *et al.*, 1976) containing stage specific DNA binding proteins (Wager and Stephens, 1988) which have been shown to be analogous to eukaryotic histone protein H1 (Hackstadt *et al.*, 1991; Perara *et al.*, 1992; Brickman *et al.*, 1993).

Early in the transition from elementary body to reticulate body the supercoiled state of the DNA is relaxed which is then compatible for transcription and replication. RNA synthesis by chlamydial DNA-dependent RNA polymerase is an essential part of chlamydial differentiation (Sarov *et al.*, 1971). Data suggest that the chlamydial DNA-

dependent RNA polymerase is activated within minutes after the elementary body enters the host cell.

1.3.4.2 Plasmid

Chlamydial plasmids have been isolated from nearly all chlamydial species and isolates except *C. pneumoniae* (Campbell *et al.*, 1987). Many strains of *C. psittaci* carry plasmids ranging in size from 6.2-7.9kb. *C. pecorum* encephalomyelitis strains have been shown to contain a plasmid (Timms *et al.*, 1988; Hugall *et al.*, 1989) that differs from the plasmids of *C. psittaci* and *C. pecorum*.

Lovett *et al.* (1980) were the first to describe the presence of a 7.5kb plasmid in three serovars of *C. trachomatis*. The 7.5kb plasmid of *C. trachomatis* is not essential for growth or disease, and three isolates have been described that lack the plasmid (Peterson *et al.*, 1990; An *et al.*, 1992; Farencena *et al.*, 1997).

Tam *et al.* (1994) used the 7.5kb plasmid of *C. trachomatis* to construct a chimeric plasmid for the introduction of recombinant DNA into *C. trachomatis* by electroporation, this method proved to be unsuccessful in that stable transformants were not produced. Recently O'Connell *et al.* (1998) described a successful method for the introduction of foreign DNA into *C. psittaci* using shuttle plasmids carrying chloramphenicol resistance.

1.3.4.3 Bacteriophage

To date, two bacteriophages have been described that infect chlamydiae. The first phage to be reported was from a *C. psittaci* duck isolate (Richmond *et al.*, 1982) and was named Chp1. Electron microscopy of cell sections infected with Chp1 showed that the phage was a regular polyhedron with a diameter of 22nm. The second phage, reported by Hsia *et al.* (1996) was also a *C. psittaci* GPIC phage. Recent work by Hsia *et al.* (1998) on the ultrastructure of phage (Φ CPG1)-infected *C. psittaci* RBs have shown that infected RBs have an altered developmental cycle giving rise to enlarged 'maxi' RBs.

1.3.5 Chlamydial antigens

1.3.5.1 Cysteine rich proteins

OMP2 and OMP3 are developmental stage specific cysteine rich proteins that have only been demonstrated in elementary bodies (Hackstadt *et al.*, 1985; Hatch *et al.*, 1986; Moulder, 1993). It is thought that MOMP, OMP2 and OMP3 are extensively di-sulfide cross-linked mediating the structural and osmotic resistance of the chlamydial elementary body.

One biochemical difference between LGV and the trachoma biovars is that OMP2 has different pI values (Batteiger *et al.*, 1985; Allen *et al.*, 1990). OMP2 has been shown to be sensitive to trypsin treatment, and OMP2 in elementary body lysates selectively bind eukaryotic cells suggesting OMP2 may have a role in chlamydiae/host cell interactions (Ting *et al.*, 1995).

1.3.5.2 Heat shock proteins

When cells infected with *C. trachomatis* are subjected to heat shock there is an increased synthesis of at least 20 heat shock proteins (HSP), the most extensively studied being HSP60 (approximate molecular weight 57kDa), and HSP70 (approximate molecular weight 75kDa). A recent study by Raulston (1998b) has shown that both HSP60 and HSP70 remain confined within the inclusion during the course of the chlamydial developmental cycle *in vitro*.

Both chlamydial HSP60 and HSP70 are constitutively expressed, and HSP60 transcription is upregulated under heat stress, in IFN- γ treated cells (Beatty *et al.*, 1994), penicillin exposed cells (Morrison *et al.*, 1989a), and under iron limiting conditions (Raulston *et al.*, 1997). It has been suggested that the chlamydial *dnaK* (encoding HSP70) and *groE* (encoding HSP60) operons may be regulated by a mechanism similar to that described in *Bacillus subtilis* in which, when the transcription of HSP60 is turned

off the *dnaK* operon is activated, and if HSP60 is over produced there is decreased expression of the *dnaK* operon. (Tan *et al.*, 1996; Raulston *et al.*, 1998b).

Chlamydial HSP70 is found in both elementary and reticulate bodies as a dithiothreitol extractable protein, and shares 46% amino acid sequence identity to the mammalian homologue. Schmiel *et al.* (1991) originally suggested the serovar E *dnaK* gene product as a potential chlamydial ligand, with recombinant *E. coli* expressing chlamydial HSP70 on their surface specifically attaching to epithelial cells. Recent studies have suggested that membrane associated HSP70 may influence entry into host cells (Raulston *et al.*, 1998a). It has also been shown that polyclonal antibody to recombinant chlamydial HSP70 can neutralize chlamydial infectivity *in vitro* (Danilition *et al.*, 1990).

Chlamydial HSP60, like HSP70 is found in both developmental forms, and is extractable with sarkosyl or dithiothreitol from elementary bodies. Within the chlamydial genus HSP60 amino acid sequence homology is greater than 80%, but possibly more importantly the chlamydial HSP60 protein shares 48% homology to human HSP60 (Morrison *et al.*, 1990).

Chlamydial heat shock proteins HSP60 and HSP70 have both been shown to be highly immunogenic during the course of natural infection (Brunham *et al.*, 1994). In women the presence of anti-chlamydial HSP60 antibodies has been correlated with pelvic inflammatory disease (PID) (Chernesky *et al.*, 1998), tubal factor infertility (Dieterle and Wollenhaupt, 1996) and ectopic pregnancy (Sziller *et al.*, 1998). In a recent study by Witkin *et al.* (1998) immunity to a specific chlamydial HSP60 epitope was associated with autoimmunity to human HSP60, and with a history of two or more spontaneous abortions. Conversely, anti-chlamydial HSP70 antibodies have been associated with protective immunity in women against ascending infection and tubal disease (Brunham *et al.*, 1987).

1.3.5.3 Lipopolysaccharide

Chlamydial lipopolysaccharide (LPS) is a group specific antigen which has been shown to contain as an immunodominant group a 3-deoxy-2-keto sugar similar but not identical to 3-deoxy-octulosonic acid (Kdo) (Dhir *et al.*, 1972; Brade *et al.*, 1987). A unique 2.8 linkage (coded by the *gseA* gene) joins the two terminal chlamydial Kdo units forming a chlamydial specific epitope. Data has suggested that chlamydiae produce a smooth LPS variant (Lukacova *et al.*, 1994) in addition to a rough form (Nurminen *et al.*, 1985).

Originally it was thought that LPS was poorly exposed on the surface of chlamydial elementary bodies (Kuo *et al.*, 1987; Collett *et al.*, 1989), but chemical cross-linking has demonstrated that LPS and MOMP lie within 10Å of one another on the bacterial cell surface (Birkelund *et al.*, 1988). Studies have demonstrated that LPS can escape the inclusion during the chlamydial development cycle, and can be detected on the surface of infected epithelial cells (Richmond and Stirling, 1981; Karimi *et al.*, 1989; Campbell *et al.*, 1994; Wyrick *et al.*, 1994). The early release of chlamydial LPS likely serves as one of the first signals for the influx of polymorphonuclear leukocytes observed in patients with cervicitis and endometritis (Paavonen *et al.*, 1985). It has also been suggested that chlamydial LPS may alter the fluidity of the plasma membrane of the infected cell thereby helping prevent the destruction of the cell by cytotoxic T cells (Wilde *et al.*, 1986).

Antibody against chlamydial LPS is commonly elicited during natural infection (Brunham *et al.*, 1987), however such antibodies have been shown not to be protective, do not elicit inflammation in animal models, and are not neutralizing *in vitro* (Caldwell *et al.*, 1984; Watkins *et al.*, 1986).

1.3.5.4 Major outer membrane protein

The major outer membrane protein (MOMP) makes up approximately 60% of the chlamydial outer membrane complex of elementary bodies (Caldwell *et al.*, 1981). The gene for MOMP (*omp1*) which is found as a single copy gene (Stephens *et al.*, 1985),

was initially sequenced from LGV2 and consisted of 1,182-bp, encoding 394 amino acids (Stephens *et al.*, 1986).

Comparisons of *C. trachomatis* serovars demonstrates that the *omp1* gene is between 84-97% identical at both the nucleotide and amino acid levels (Fitch *et al.*, 1993; Kaltenboeck *et al.*, 1993). Variation in the *omp1* gene is clustered into four variable domains (VD1, VD2, VD3, and VD4) regularly interspersed with five conserved regions (Stephens *et al.*, 1987; Baehr *et al.*, 1988). The surface exposed variable domains of *C. trachomatis* MOMP gives rise to species-, subspecies-, serovar-, and serogroup-specific epitopes (Zhang *et al.*, 1987; Conlan *et al.*, 1988; Stephens *et al.*, 1988; Zhang *et al.*, 1989; Batteiger *et al.*, 1990; Zhong *et al.*, 1990) which react with human immune sera (Zhang *et al.*, 1987; Zhang *et al.*, 1989a). VD1, VD2 and the amino terminal segment of VD4 specify type-specific epitopes while VD3 and the C-terminal of VD4 specify species-, sub-species, and serogroup epitopes. Monoclonal antibodies directed against MOMP are neutralizing in cell culture and in some animal models (Peeling *et al.*, 1984; Zhang *et al.*, 1987; Baehr *et al.*, 1988; Zhang *et al.* 1989a; Morrison *et al.* 1992). MOMP is a focus of *C. trachomatis* vaccine development even though protective immunity is serovar specific, and MOMP variants can escape *in vitro* neutralization by both monoclonal antibody and human immune sera (Lamp *et al.*, 1997)

Like chlamydial LPS, MOMP has been demonstrated to be surface exposed (Kuo and Chin, 1987; Collett *et al.*, 1989) having trypsin sensitive antigenic epitopes (Baehr *et al.*, 1988); exhibits potential porin function (Bavoil *et al.*, 1984); is post-translationally glycosylated (Baehr *et al.*, 1988; Collett *et al.*, 1989; Swanson and Kuo, 1991; Kuo *et al.* 1996); and is thought to play a role in the structural integrity of the organism (Caldwell *et al.*, 1981; Hatch *et al.*, 1981; Caldwell *et al.*, 1982c).

It has been suggested that MOMP plays an electrostatic role in adherence following a conformational change in VD4, a nonapeptide sequence (previously located in a cryptic, immunoinaccessible cleft) which becomes immunoaccessible and promotes binding (Su *et al.*, 1988; Su *et al.*, 1990; Su *et al.*, 1996). MOMP can contain between 7-10

cysteines which may form homo- or hetero-oligomers with itself and/or other outer membrane proteins (Newhall *et al.*, 1983; Newhall *et al.*, 1986).

1.4 Pathogenicity

1.4.1 Immune mechanisms

1.4.1.1 Innate response

The importance of the host's innate response to chlamydial infection has only recently begun to be examined. Acute chlamydial infection is characterized by an early influx of neutrophils (possibly mediated by CD18 which is involved in lipopolysaccharide binding) which have been shown to play an important role in controlling the early stages of infection (Bartaneva *et al.*, 1996).

An alternative explanation for the influx of neutrophils to the site of infection may be the action of pro-inflammatory cytokines. Chlamydial infection of epithelial cell lines, and of monocytic cells has been shown to elicit the production of pro-inflammatory cytokines including IL-1 α , IL-6, IL-8, TNF- α , growth regulated oncogene (GRO) α and granulocyte-macrophage colony stimulating factor (GM-CSF) independent of nascent LPS (Ault *et al.*, 1996; Heinemann *et al.*, 1996; Bianchi *et al.*, 1997; Rasmussen *et al.*, 1997). The production of the pro-inflammatory cytokines is possibly important in the recruiting of neutrophils and other inflammatory cells to the site of infection before the recruitment of lymphocytes.

After re-infection, or after the initiation of an immune response, the continued stimulation of epithelial cells may exaggerate the local inflammatory response, and IFN- γ has been postulated to have such an effect during repeated infections. In chlamydial infected epithelial cell lines IFN- γ has been shown to increase the production and secretion of IL-8 (Rasmussen *et al.*, 1996). The production of IFN- γ as part of a Th1 response may thus contribute to increased inflammation. Such an enhanced cytokine response has been reported to occur in natural infection. For example, *C. trachomatis* infection stimulates local cytokines which promote a strong cell-mediated and pro-inflammatory response in both the early and late stages of trachoma (Bobo *et al.*, 1996). IFN- γ has also been

detected in sera from women with PID (Grifo *et al.*, 1989), and in endocervical secretions of women infected with *C. trachomatis* (Arno *et al.*, 1990).

Studies using gene knockout mice and neutralization with antibody have been used to study the importance of IFN- γ in the resolution of chlamydial infection (Rank *et al.*, 1992a; Williams *et al.*, 1993; Cotter *et al.*, 1997; Johansson *et al.*, 1997; Perry *et al.*, 1997; Williams *et al.*, 1997). Although these studies have given conflicting results, it appears that IFN- γ is dispensable for the resolution of local infection and protection against challenge but it may play an important role in the prevention of chlamydial dissemination from the mucosal surface. It has been suggested that IFN- γ induces the inducible nitric oxide synthase (iNOS) pathway, although recent studies have shown iNOS is not required for the elimination of *C. trachomatis* from epithelial cells of the female genital tract in mice, but may contribute to the control of dissemination of the infection by infected macrophages (Igietseme *et al.*, 1998; Ramsey *et al.*, 1998).

TNF- α has also been shown to be secreted in the genital tract, as early as 3 days post-infection in the guinea pig model (Darville *et al.*, 1995). While TNF- α most likely plays an important role in the pathogenesis of infection, and the potential role in the recruiting of various cells to the genital tract via its induction of ICAM-1, there is also evidence that it may have direct anti-chlamydial activity (Shemer-Avni *et al.*, 1988; Williams *et al.*, 1990).

1.4.1.2 Humoral response

Studies using *C. trachomatis* MoPn in murine models have confirmed that antibody contributes to, but is not essential for protection (Perry *et al.*, 1997; Su *et al.*, 1997; Williams *et al.*, 1997). However, it has been demonstrated that antibody is able to neutralize *C. trachomatis* infection *in vitro* (Banks *et al.*, 1970; Howard *et al.*, 1975; Byrne and Moulder, 1978; Caldwell *et al.*, 1982b; Peeling *et al.*, 1984; Lucero and Kuo, 1985; Byrne *et al.*, 1993).

Neutralization has been demonstrated with (Lucer and Kuo, 1985; Peterson *et al.*, 1988), and without (Caldwell *et al.*, 1982b; Su *et al.*, 1990; Peterson *et al.*, 1993) the presence of complement, and occurred by either preventing the attachment of chlamydial elementary bodies to the cell (Byrne *et al.*, 1978; Su *et al.*, 1991) or by allowing the internalization of the chlamydiae but preventing/inhibiting replication (Caldwell *et al.*, 1982b). Opsonization of *C. psittaci* has also been demonstrated, resulting in the fusion of phagosomes containing chlamydiae with lysosomes (Wyrick *et al.*, 1978a; Wyrick *et al.*, 1978b).

The predominant molecule in the chlamydial outer membrane is MOMP. The *C. trachomatis* serovars display significant sequence variation in MOMP possibly due to immune pressure (MOMP is a predominant target for antibody). Lampe *et al.* (1997) showed that serovars that vary in MOMP variable regions can escape neutralization with both monoclonal antibodies and immune serum. It has also been shown that the *in vitro* neutralization of *C. trachomatis* serovars isolated from humans was serovar specific and dependent upon conformational epitopes (Fan *et al.*, 1997).

In the human there is a strong antibody response in both genital secretions and serum, but whether they play a protective role is unclear. An inverse relationship between the number of chlamydiae isolated from genital swabs and level of IgA has been documented (Brunham *et al.*, 1983). Jones *et al.* (1994), reported that the presence of neutralizing antibody in patients did not correlate with the immune status of the patient.

Little is known about antibody profiles during acute or chronic chlamydial genital tract infections in humans. Superficial infections (eg. cervicitis) are considered to provide a poor stimulus for antibody formation, whereas infiltrating disease (eg. salpingitis) is associated with seroconversion. IgG can persist for years, and can be used as a marker for a past infiltrating chlamydial disease (Ngeow, 1996).

The specificity of circulating antibodies may change during the course of the immune response. In *C. pneumoniae* infections during the first 3 weeks after the primary

exposure, there is a systemic appearance of IgM, IgA and IgG against less specific epitopes (eg. LPS). Only after 4-8 weeks does more specific IgG against MOMP appear (Ekman *et al.*, 1993).

1.4.1.3 Cell mediated response

Humans have been shown to develop cell mediated immune responses as measured by lymphocyte proliferative assays, against chlamydial genital tract infection. (Hanna *et al.*, 1979; Brunham *et al.*, 1981; Hanna *et al.*, 1982; Witkin *et al.*, 1993a). Like humoral immunity, as yet there is no direct evidence that cell mediated immunity is protective in humans. Studies of both animals and humans have suggested that the protective response to chlamydial infection is predominantly Th1 related (Bobo *et al.*, 1996; Kelly *et al.*, 1996; van Voorhis *et al.*, 1996; Darville *et al.*, 1997; Perry *et al.*, 1997; Williams *et al.*, 1997; van Voorhis *et al.*, 1997).

It has been proposed that in reference to scarring disease, individuals with weak cell mediated immune responses and strong antibody responses are those susceptible to re-infection, are slow to resolve infection, and have high levels of clinical inflammation and consequently disease (Bailey *et al.*, 1993; Brunham and Peeling, 1994). Conversely individuals with a strong cell mediated immune response and a low antibody response are those resistant to infection and are less susceptible to disease.

1.4.1.4 Immunopathology

It is believed that immune pathogenesis is the underlying mechanism of chlamydial disease but it is still not clear if specific antigens coupled with cellular immune responses are directly responsible (eg. delayed-type hypersensitivity [DTH]), or if other inflammatory or immune regulatory processes contribute to disease. Chlamydial infection has been shown to evoke a DTH response, and that re-infection is required to induce disease (Grayston *et al.*, 1985). Evidence suggests that chlamydial heat shock protein 60 (HSP60) may be the mediator of pathogenesis. Many studies have shown an

association between the detection of anti-chlamydial HSP60 antibodies and pelvic inflammatory disease (Eckert *et al.*, 1997; Money *et al.*, 1997; Peeling *et al.*, 1997; Chernesky *et al.*, 1998), tubal factor infertility (Toye *et al.*, 1993; Clanman *et al.*, 1997), ectopic pregnancy (Yi *et al.*, 1993), and trachoma (Taylor *et al.*, 1987; Morrison *et al.*, 1989b; Taylor *et al.*, 1990).

It has been suggested that increased levels of chlamydial HSP60 production may result in the induction of self anti-HSP60 antibodies inducing autoimmunity which ultimately results in tissue damage and scarring.

1.4.2 Genetic susceptibility to chlamydial disease

Studies have shown that some murine strains are more susceptible to chlamydial infection than others (Tuffrey *et al.*, 1992a; Darville *et al.*, 1996; Darville *et al.*, 1997), and in humans not all those infected develop the scarring sequelae. It is believed that host genetic factors may play a role in the tissue damage associated with chlamydial disease.

Conway *et al.* (1996) studied HLA linkage and disease outcome in patients with scarring trachoma and showed that the A*6802 allele of the HLA-A28 (class I) was significantly more common in study patients than controls. No particular HLA type was associated with protection from disease, and no class II alleles were associated with disease.

Conway *et al.* (1997) also demonstrated that scarring trachoma was also associated with a polymorphism in the TNF- α promoter region, independent of HLA type. In a macaque model of PID the relative susceptibility or resistance to the development of tubal adhesions was correlated with specific MHC class I alleles (Lichtchenwalner *et al.*, 1997).

1.4.3 *Chlamydia trachomatis*

1.4.3.1 Trachoma and inclusion conjunctivitis

Trachoma is a chronic keratoconjunctivitis associated mainly with *C. trachomatis* serovars A, B, Ba, and C. Trachoma is hyperendemic in communities with poor hygiene and sanitation and of low economic resources. Active trachoma may range from a mild asymptomatic inflammation with collections of immune cells visible on the tarsal conjunctiva (follicular trachoma) to an intense inflammatory response in which most of the tarsal plate is obscured by capillary congestion (intense trachoma). Repeated ocular infections cause scarring of the conjunctiva (scarring trachoma), inversion of the eyelids and eyelashes (trichiasis), which ultimately can lead to blindness following damage to the cornea by intumed lashes.

Inclusion conjunctivitis is caused by *C. trachomatis* serovars D to K and is mainly seen in the sexually active age groups. Transmission of the disease is usually by self-inoculation with infective genital discharge. Concomitant genital tract infection of patients with inclusion conjunctivitis is seen in 80-90% of females and approximately 50% of males. Clinical manifestations include swollen eyelids, mucopurulent discharge, papillary hypertrophy, and follicular hypertrophy; corneal scarring is rarely found. As in trachoma, repeated episodes lead to the development of more severe disease possibly by the same mechanisms.

1.4.3.2 Female genital tract infections

1.4.3.2.1 Lower genital tract infections

In women *C. trachomatis* is one of the major causes of urethritis and urethral symptoms. Stamm *et al.* (1980) associated dysuria and/or frequency without significant bacteriuria with *C. trachomatis* infection, although Horner *et al.* (1995) in a recent study showed no

such association. There is little evidence of urethral inflammation and *C. trachomatis* infection, although mucopurulent cervicitis is suggestive of urethral syndrome.

Chlamydial mucopurulent cervicitis is often asymptomatic, and if left untreated can ascend to the upper genital tract often leading to the development of PID. It has been reported that 64% of women with chlamydial mucopurulent cervicitis have subclinical PID (Paavonen *et al.*, 1985a). Paavonen *et al.* (1992) introduced a simple set of diagnostic criteria which included the detection of eight or more polymorphonuclear leukocytes per high power field in a cervical smear, the presence of a yellow mucopurulent endocervical discharge, increased erythema, oedema, and induced mucosal bleeding in an area of ectopy and in the cervical transformation zone.

Histological findings associated with chlamydial cervicitis include the presence of dense stromal inflammation (plasma cell infiltrations), intraepithelia and intraluminal inflammation, and well-formed lymphoid follicles comprising transformed lymphocytes (Kiviat *et al.*, 1990).

1.4.3.2.2 Pelvic inflammatory disease

"Pelvic inflammatory disease (PID), or salpingitis, refers to infection of the uterus, Fallopian tubes and adjacent structures not associated with pregnancy or surgery. PID is almost always an ascending infection in which pathogenic microorganisms spread from the cervix and vagina to the upper genital tract" (McCormack, 1994).

PID is one of the most important complications of sexually transmitted disease, and over half the reported cases are caused by *C. trachomatis*, *N. gonorrhoeae* or both. Each repeat episode of PID doubles the risk of tubal disease associated with possible permanent tubal damage and development of ectopic pregnancy (EP) or infertility (Westrom, 1975). As a consequence of tubal scarring approximately 20% of women with laparoscopically confirmed PID will become infertile, 18% will develop prolonged

pelvic pain and 9% will have ectopic pregnancies (Westrom, 1980; Westrom *et al.*, 1992).

Manifestations of acute PID can include endometritis, salpingitis, pelvic peritonitis and ovarian abscesses. The ascending infection can also spread to the appendix (periappendicitis) and the liver capsule (perihepatitis). Diagnosis of PID is to say the least difficult. Minimum criteria for the diagnosis of PID include lower abdominal tenderness, bilateral adnexal tenderness, cervical motion tenderness and no evidence of a competing diagnosis.

In many instances the condition is asymptomatic or silent as demonstrated by Cates and Wasserheit (1991) who showed a strong link between serum anti-chlamydial antibodies and tubal factor infertility or EP in patients with and without a history of PID. Women with PID often have damaged Fallopian tubes at the time of diagnosis, and studies in both primate and murine models suggest that antibiotic treatment with or without anti-inflammatory agents had little effect on tubal inflammation (Patton *et al.*, 1997; Verhoest *et al.*, 1997).

The mechanism by which chlamydial PID mediates tubal damage is thought to be immunopathological. The deleterious sequelae of chlamydial infection appears to be caused by a DTH response to chlamydial heat-shock protein 60 (CHSP60) (Morrison *et al.*, 1989b). Many studies have suggested a correlation between serum antibodies to CHSP60 and PID, tubal factor infertility (TFI) or ectopic pregnancy (EP) (Miettinen *et al.*, 1990; Wager *et al.*, 1990; Brunham *et al.*, 1992; Toye *et al.*, 1993; Paavonen and Lehtinen, 1994; Clanman *et al.*, 1997; Eckert *et al.*, 1997; Money *et al.*, 1997; Peeling *et al.*, 1997; Chernesky *et al.*, 1998; Sziller *et al.*, 1998). In women with chlamydial PID, a prior history of PID, cervicitis or chlamydial infection, laparoscopically verified tubal obstruction, the degree of tubal inflammation and the presence of adhesions have all been associated with the presence of HSP antibodies (Stamm *et al.*, 1994).

The actual mechanism by which CHSP60 mediates damage is unclear, one hypothesis is that antibodies directed against CHSP60 cross react with human HSP60 resulting in autoimmunity and damage. It may also be that the presence of detectable levels of antibodies against CHSP60 may simply be a marker, for example of chlamydial persistence.

1.4.3.3 Male genital tract infections

1.4.3.3.1 Epididymitis

Epididymitis is the most common intrascrotal inflammatory disorder, usually resulting from the spread of microorganisms from the prostatic urethra, prostate or seminal vesicle. Idiopathic epididymitis exhibits an acute or chronic inflammatory infiltration with or without abscess formation and ductal destruction. Chlamydial epididymitis has been characterized as being nondestructive and proliferative compared to epididymitis which is destructive and abscess forming often found associated with other bacteria (Hori and Tsutsumi, 1995).

Studies in both the UK and USA have demonstrated that the microorganisms causing acute epididymitis vary with age and sexually activity. 30-60% of men with acute epididymitis under the age of 35 years show the presence of *C. trachomatis* in epididymal aspirates (Berger *et al.*, 1978; Doble *et al.*, 1989a; Robinson *et al.*, 1990).

1.4.3.3.2 Non-gonococcal urethritis

Non-gonococcal urethritis (NGU) is defined as urethritis where *N. gonorrhoeae* is not the aetiological agent. Many studies have shown that *C. trachomatis* can be demonstrated in urethral specimens of up to 13%-50% of NGU cases (Oriel and Ridgway, 1982; Janier *et al.*, 1995), the remaining cases have been attributed to several other organisms such as *Trichomonas vaginalis*, Herpes simplex virus (Swartz *et al.*, 1978), and *Mycoplasma*

genitalium (Jensen *et al.*, 1993; Taylor-Robinson, 1996). There is no clinical difference between chlamydial or non-chlamydial NGU.

The average incubation period of NGU following sexual contact is between 1 and 3 weeks, with many men complaining of dysuria and urethral irritation with a mucoid or mucopurulent discharge. Up to 25% of all cases of NGU clear spontaneously within three weeks and most will resolve within two months (Mardh *et al.*, 1989), although many symptomatic cases become asymptomatic within three weeks (Shahmanesh, 1994).

More than 10 polymorphonuclear leukocytes (PMNs) in the centrifuged sediment of first-catch urine per high-power field (x400), or more than 5 PMNs per high-power field (x1000) in the urethral smear is usually included in the definition of male urethritis (Swartz *et al.*, 1978; Bowie *et al.*, 1978; Desai *et al.*, 1982). While asymptomatic urethritis in men is recognized (Swartz *et al.*, 1978; Munday *et al.*, 1985), NGU is more frequently associated with discharge and/or penile irritation.

1.4.3.3.3 Post-gonococcal urethritis

Post-gonococcal urethritis (PGU) has been defined as a persistent non-gonococcal urethritis following treatment for gonococcal urethritis. It occurs due to a dual infection with an organism which causes NGU, with up to 80% of cases being attributed to *C. trachomatis* (Stamm *et al.*, 1984). Whether the two infections were acquired simultaneously, or whether *C. trachomatis* was acquired first remaining dormant until triggered by a gonococcal infection (Mardh *et al.*, 1989) is unclear. PGU can be prevented by both treatment for gonorrhoea and an effective anti-chlamydial regimen.

1.4.3.3.4 Proctitis

C. trachomatis serovars D-K have been isolated from rectal samples from both heterosexual and homosexual men (Munday *et al.*, 1981; Bauwens *et al.*, 1995). LGV can cause severe proctitis, with symptoms that include rectal pain and discharge (Bauwens *et al.*,

1995). The other genital serovars of *C. trachomatis* have been recovered from homosexual men, but they have not been associated conclusively with any particular rectal symptoms (Munday *et al.*, 1983).

1.4.3.3.5 Prostatitis

Weidner *et al.* (1991) suggested that nearly one third of cases of 'non-bacterial' prostatitis were of chlamydial origin. Chlamydial urethritis can be diagnosed by culture in approximately 10% of prostatitis cases (Mardh *et al.*, 1978). Bruce *et al.* (1981) examined early morning urine specimens and/or prostatic fluid or semen of fifty patients with chronic prostatitis by tissue culture, 56% proved positive for *C. trachomatis*. However, Doble *et al.* (1989b) using transperineal prostate biopsies from 50 men with 'non-bacterial' chronic prostatovesiculitis failed to identify *C. trachomatis* in prostatic tissue. Recently *C. trachomatis* has been identified by *in situ* hybridization in prostate tissue (Corradi *et al.*, 1996; Krieger *et al.*, 1996) and expressed prostatic secretions (Guo *et al.*, 1997) by PCR. Symptoms of prostatitis can include discomfort, urethral discharge, dysuria, an increased frequency of urination, painful ejaculation and a reduced libido.

1.4.3.4 Lymphogranuloma venereum

Lymphogranuloma venereum (LGV) is a systemic, sexually transmitted disease caused by *C. trachomatis* serovars L1 to L3. The disease is endemic in East and West Africa, India, South-East Asia and South America. It is uncommon in the UK with a combined total of 91 reports of chancroid, granuloma inguinale or LGV from GUM clinics in 1995 (Dept. Health, 1996).

The primary lesion of LGV usually appears 3-12 days post-infection, classically the ulcer is transient commonly effecting the corona; sulcus, frenulum or prepuce in men, and the posterior vaginal wall, fourchette or posterior cervical lip in women. Other symptoms during the primary stage may be urethritis, endometritis or salpingitis. The secondary

stage is characterized by inflammation and swelling of the regional lymph nodes, which may take up to 6 months post-infection. This secondary stage is also associated with the systemic spread of LGV. The tertiary stage is characterized by proctocolitis, perirectal abscesses, fistula formation and rectal stricture/stenosis.

1.4.3.5 Neonatal infections

C. trachomatis can cause conjunctivitis and pneumonitis in neonates born to genitally infected mothers (Claesson *et al.*, 1989; Hammerschlag *et al.*, 1989). Neonatal conjunctivitis is characterized by swelling of the eyelids, erythematous conjunctivae and a mucopurulent discharge. Neonatal pneumonitis symptoms include a pertussis-like cough, and infants with chlamydial pneumonia are at an increased risk for developing pulmonary dysfunction and possibly chronic respiratory disease at a later time (Weiss *et al.*, 1991).

1.4.3.6 Sexually acquired reactive arthritis

Sexually acquired reactive arthritis (SARA) develops in between 1-3% cases of NGU, and approximately one third of patients with SARA also have Reiter's Syndrome (arthritis in association with uveitis and urethritis). Anti-chlamydial IgG titres in both serum and in synovial fluid have been reported to be elevated in patients with SARA when compared to controls (Bas *et al.*, 1996; Wollenhaupt, 1996), although the organism itself has not been isolated. However, chlamydial DNA, rRNA and MOMP have all been detected in the affected joints of SARA patients (Keat *et al.*, 1987; Hammer *et al.*, 1992; Rahman *et al.*, 1992; Taylor-Robinson *et al.*, 1992).

1.4.4 *Chlamydia pneumoniae*

1.4.4.1 Acute respiratory disease

C. pneumoniae was first described as a major cause of mild, community-acquired human respiratory infection (Saikku *et al.*, 1985; Kuo *et al.*, 1986) resembling *Mycoplasma pneumoniae* pneumonia (Cotton *et al.*, 1987) except for the frequent occurrence of pharyngitis, sinusitis (Hashigucci *et al.*, 1992), and otitis media (Ogawa *et al.*, 1990).

Clinical and radiological evidence of consolidation can range from sub-segmental lesions to extensive bilateral pneumonitis. The onset of disease is prolonged with upper respiratory tract symptoms followed by increased coughing and other symptoms indicative of lower respiratory tract disease. Other diseases that have been linked to *C. pneumoniae* infection include encephalitis, the Guillain-Barré syndrome (Haidl *et al.*, 1992), arthritis, and erythema nodosum (Erntell *et al.*, 1989). *C. pneumoniae* has also been associated with asthma in both children and adults (Hahn *et al.*, 1991; Hahn *et al.*, 1992; Hahn *et al.* 1996; Cook *et al.* 1998; Cunningham *et al.*, 1998; Magee, 1998; Miyashita *et al.*, 1998).

1.4.4.2 Coronary heart disease

C. pneumoniae was first demonstrated in autopsy coronary atheroma specimens by electron microscopy (Shor *et al.*, 1992), the results of which were later confirmed by immunohistochemistry and PCR (Kuo *et al.*, 1993). The organism has been demonstrated in several vascular tissue types using a variety of techniques (Campbell *et al.*, 1995; Kuo *et al.*, 1995; Grayston *et al.*, 1995). Using immunohistochemistry *C. pneumoniae* has been localised to macrophages and smooth muscle cells from aortic atherosclerotic lesions (Ong *et al.* 1996), and foam cells of carotid plaques (Grayston *et al.* 1995).

The question of whether *C. pneumoniae* is an 'innocent' bystander or the cause of endothelial damage, hypercoaguability, and macrophage activation still remains to be fully elucidated. It has been suggested that macrophages may act as a 'Trojan horse' in that *C. pneumoniae* is ingested in the lung and taken to atheromatous lesions.

Alternatively, *C. pneumoniae* may actively induce immune activation, cytokine release, endothelial damage, and thrombosis leading to atherogenesis. A recent study has shown that *C. pneumoniae* induces macrophage foam cell formation which may initiate or promote atheroma development (Kalayoglu and Byrne, 1998). Animal models have shown that *C. pneumoniae* does have a tropism for atherosclerotic lesions (Moazed *et al.*, 1997), and is capable of inducing inflammatory atherosclerosis-like changes in the aorta (Laitinen *et al.*, 1997).

1.4.5 *Chlamydia psittaci*

C. psittaci is spread via the inhalation of aerosols derived from animal faeces or other animal products and causes predominantly pneumonia in humans. Disease in humans can vary from a mild like flu to severe generalised illness with symptoms ranging from a fever, to anorexia, and photophobia. In severe cases, nausea, vomiting, and diarrhoea may be seen. Abortion in humans has been associated with *C. psittaci*, the infection being transmitted from infected placental/foetal material. *C. psittaci* has been shown to produce endocarditis in humans (Regan *et al.*, 1979; Lamaury *et al.*, 1993), myocarditis (Jannach, 1958; Dymock *et al.*, 1971), pericarditis (Sutton *et al.*, 1967), and major arterial embolism (Pesanti and Smith, 1979).

1.5 Infertility

1.5.1 Structure and function of the female genital tract

The female genital tract is composed of five main structures, the ovaries, the Fallopian tubes, the uterus, the cervix and the vagina (Figure 2). The morphology of the female genital tract is not static, undergoing repetitive cyclic changes which are a consequence of the re-occurring fluctuations in ovarian hormone production which are an important component of the 28 day ovulatory cycle (Figure 3).

During the ovulatory cycle a single follicle develops into a mature ovum secreting large quantities of oestrogen, under the influence of rising follicle stimulating hormone (FSH). Ovulation occurs at approximately mid-cycle in response to a surge of luteinizing hormone (LH) which is provoked by a critical oestrogen level. The ovum is expelled from the follicle which then undergoes conversion into a corpus luteum. Under the influence of LH the corpus luteum secretes large amounts of both oestrogens and progesterones but in the absence of pregnancy the corpus luteum degenerates accompanied by a decline in oestrogen and progesterone. The tissues of the female genital tract possess steroid hormone receptors and are therefore capable of responding to the cyclic hormonal changes, these changes representing a recurrent 'priming' for potential pregnancy.

1.5.2 Causes of infertility

Data from the USA suggest that less than 50% of infertile couples seek medical advice (Wilcox and Mosher, 1993). A 1982-1985 WHO multi-centred study found that in 20% of infertility cases the problem was predominantly male, in 38% the problem was attributed to the female, 27% had abnormalities in both partners, and the remaining 15% had no clearly defined cause of infertility (WHO, 1987).

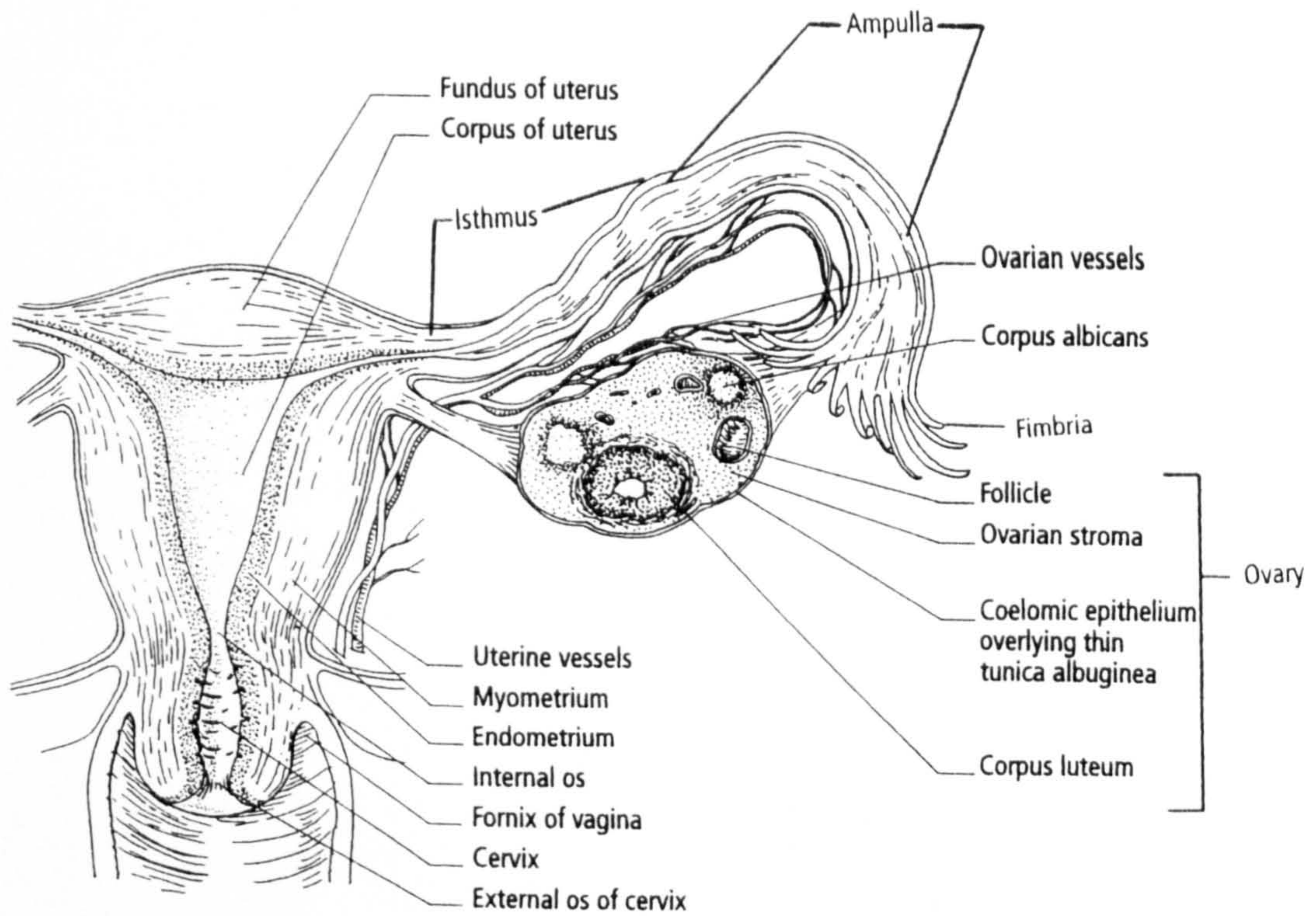


Figure 2. Sectioned view of the female genital tract.

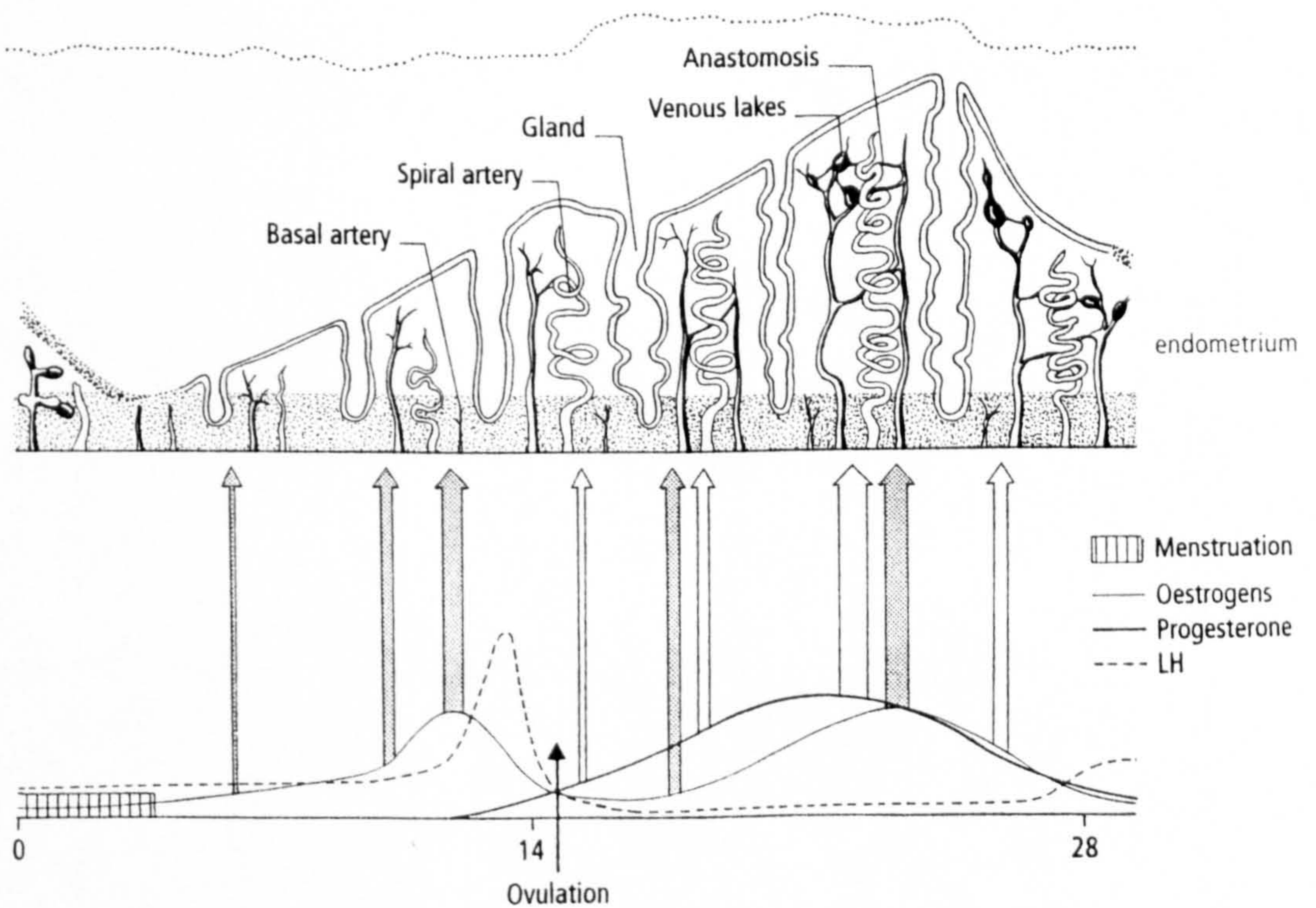


Figure 3. Changes in the human endometrium during the menstrual cycle. Thickness of arrows (oestrogens shaded; progestagens out-lined) indicate the strength of action.

1.5.2.1 Causes in the female

Tubal disease includes an array of disorders including blockage, both distally and proximally, peritubal adhesions, salpingitis, and hydrosalpinx. PID, previous EP, previous sterilization or surgery, and a history of peritonitis which can cause such conditions leading to the development of TFI. Severe tubal disease, specifically distal tubal obstruction leading to hydrosalpinx has been associated with a poor fertility prognosis (Bahamondes *et al.*, 1984; Marana *et al.*, 1995; Nackley *et al.*, 1998).

1.5.2.2 Causes in the male

To be fertile the male requires a normal spermatogenesis, successful epididymal maturation and storage of sperm, normal sperm transport and accessory gland function. Male infertility can be categorized as due to three aetiological factors, pre-testicular (altered endocrine function, coital disorders and ejaculatory failure), testicular (genetic, congenital, infective, effect of anti-spermatogenic agents, vascular, immunological and idiopathic), and post-testicular (obstructive, vasal, accessory gland infection, immunological, and epididymal hostility).

1.5.3 *Chlamydia trachomatis* and female infertility

1.5.3.1 Miscarriage/spontaneous abortion

Serological studies investigating the possible role of *C. trachomatis* in miscarriage have given conflicting results (Witkin *et al.*, 1992; Osser and Persson, 1996; Witkin *et al.*, 1998). When considering women undergoing *in vitro* fertilization (IVF), antibodies against *C. trachomatis* have been associated with spontaneous abortion (Licciardi *et al.*, 1992; Witkin *et al.*, 1994; Neuer *et al.*, 1997).

C. psittaci can cause abortion in women by infection of the placenta, the question remains does this process take place in pregnant women infected with *C. trachomatis*? *C.*

trachomatis DNA has been detected in the placenta of a stillborn foetus by *in situ* hybridization (Gencay *et al.*, 1997) but the importance of *C. trachomatis* in miscarriage/abortion still remains to be elucidated.

1.5.3.2 Ectopic pregnancy

The incidence of ectopic pregnancy (EP) ranges from between 0.25%-1.4% of all pregnancies (ie. the sum of reported live births, legal abortions and EP) (Chow *et al.*, 1987; Coste *et al.*, 1994b) . EP was responsible for 11% of maternal mortality in the UK during 1988-1990.

EP refers to the implantation of the fertilized ovum outside the uterine corpus. The basic cause of EP is the impediment of the passage of the fertilized ovum down the Fallopian tube. Approximately 92% of EPs occur in the ampulla where fertilization takes place and 4% in the isthmus, but they may occur anywhere along the tube from the fimbriae to cornu as well as in extratubal sites such as the cervix, ovary and abdominal cavity. The commonest pathology is damage to the ciliated epithelium and peristaltic activity of the tube by a previous infection with such microorganisms as *C. trachomatis*, and *N. gonorrhoeae*. It has been suggested that chlamydial associated EP may be the result of infectious damage/loss of Fallopian tube function and ongoing infection

1.5.3.3 Tubal factor infertility

The Fallopian tubes act as a conduit for the sperm to reach the ovum, and for the fertilized ovum to reach the uterus. Tubal occlusion resulting from hydrosalpinx and peritubal adhesion formation and tubal scarring are the underlying pathological changes associated with TFI following acute salpingitis.

The mechanism by which *C. trachomatis* induces adhesion formation and scarring may be immune mediated, and similar to that observed in scarring trachoma. Animal model studies have shown that repeated chlamydial infection of the Fallopian tubes resulted in

pathological changes associated with a delayed type hypersensitivity response (DTH) (Patton *et al.*, 1982; Patton *et al.*, 1987; Patton *et al.*, 1989a; Tuffrey *et al.*, 1990; Patton *et al.* 1994a; Rank *et al.*, 1995a), and hydrosalpinx formation (Sweet *et al.*, 1980; Patton *et al.*, 1982; Schachter *et al.*, 1982; Rank *et al.* 1992b; Rank *et al.* 1995a). Campbell *et al.* (1993) and Patton *et al.* (1994) demonstrated that chlamydial DNA could persist in human Fallopian tube tissue from TFI patients, and suggested that such persistence may stimulate immune-mediated tissue damage.

There have been many serological studies that have linked exposure to *C. trachomatis* and the subsequent development of tubal pathology (Brunham *et al.*, 1985; Campbell *et al.*, 1993; Patton *et al.*, 1994b). Data has also associated the presence of anti-chlamydial HSP60 antibodies with TFI (Toye *et al.*, 1993; Sziller *et al.*, 1998).

1.5.4 *Chlamydia trachomatis* and male infertility

Asymptomatic infection of the reproductive tract of infertile men has been acknowledged for over two decades (Ulstein *et al.*, 1976; Nikkanen *et al.*, 1979). The impact of *C. trachomatis* infection on male infertility, and semen parameters is controversial. *C. trachomatis* has been shown to attach (Wolner-Hansen and Mardh, 1984) and enter into spermatozoa (Erbengi, 1993), it can be detected in cytoplasmic droplets of spermatozoa (Villegas *et al.*, 1991), and chlamydial DNA has been demonstrated in prostate tissue specimens (Corradi *et al.*, 1996).

It has been suggested that *C. trachomatis* infection of the genital tract may stimulate the immune system inducing autoimmunity to spermatozoa (Witkin and Toth, 1983; Shamanesh *et al.*, 1986; Soffer *et al.*, 1990; Naz and Menge, 1994; Munoz and Witkin, 1995; Witkin *et al.*, 1995a). Anti-sperm antibodies can be directed against various sites of the spermatozoa (Peters and Coulam, 1992), attachment of antisperm antibodies can effect sperm motility (Zouari *et al.*, 1993); head-directed antibody binding may prevent fertilization due to the occlusion of binding sites for the zone of pellucida (Bronson *et al.*,

1984). The acrosome reaction, one of many crucial steps in sperm function may also be altered by anti-sperm antibodies (Lansford *et al.*, 1990).

1.5.5 Treatment of infertility

1.5.5.1 *In vitro* fertilization

The first baby conceived by *in vitro* fertilization (IVF) was born in 1978 (Steptoe and Edwards, 1978). IVF tends to be used in women with absent, damaged or blocked Fallopian tubes. The technique involves the use of ovulation-enhancing drugs so multiple oocytes mature, the retrieval of the oocytes followed by *in vitro* fertilization, and finally after approximately 48h the re-introduction of the developing embryo into the uterus.

1.5.5.2 Gamete intrafallopian tube transfer

Gamete intrafallopian tube transfer (GIFT) was first described by Asch *et al.* (1986) as an alternative to IVF in situations where IVF had been previously utilized, but where the Fallopian tubes were normal. The basic procedure involves the *in vitro* mixing of oocytes and sperm followed by the re-introduction of the mixture into the distal end of the Fallopian tube where fertilization may occur *in vivo*.

1.6 Diagnosis of chlamydial infections

1.6.1 Specimens

Specimen collection and transport is important in the diagnosis of chlamydial infections, both the sensitivity and specificity of many diagnostic tests have been related to the 'quality' of the specimen (Phillips *et al.*, 1987; Moncada *et al.*, 1990; Schwebke *et al.*, 1990; Howard *et al.*, 1991; Kellogg *et al.*, 1991). Due to the intracellular nature of *Chlamydia* the main objective of sampling should be to collect host cells containing the microorganism.

For culture, specimens should be collected in a medium specifically designed to maintain the host cell and chlamydial viability, for example 0.2M sucrose in phosphate buffered saline (2SP). Antibiotics which chlamydiae are not susceptible to are usually added to the transport medium to prevent the growth of other bacteria and fungi in the specimen. In women the most common site for the isolation of *C. trachomatis* is the endocervix; the type of swab used for collection has been shown to be of some importance due to toxicity to cell cultures, or the direct inhibition of chlamydial growth (Mahony *et al.*, 1985). The pooling of a urethral and an endocervical swab has been reported to increase culture sensitivity by 23% (Jones *et al.*, 1986a). In males, the preferred site of sample collection is the anterior urethra.

Specimen storage can also effect chlamydial isolation, isolation is optimized if specimens are kept and transported at temperatures between 2-8°C immediately after collection. The time between specimen collection and processing should ideally be less than 48h, although freezing at -70°C is acceptable but can lead to a 20% loss of chlamydial viability after three days (Reeve *et al.*, 1975; Mahony *et al.*, 1985).

For non-culture tests, specimens such as urine can be used for antigen tests, polymerase chain reaction (PCR) and ligase chain reaction (LCR). Culture of urine specimens has not always proved to be successful, with the best performance on urine from

symptomatic males (Chernesky *et al.*, 1990; Chernesky *et al.*, 1995). Tests such as PCR and LCR do not require intact chlamydial elementary bodies since only a few copies of target DNA are required for the generation of a positive signal; Kellogg *et al.* (1995) showed that the number of host cells and hence the number of chlamydiae present in a sample can affect the outcome of DNA amplification.

Recently vaginal introitus and vulval specimens have been investigated using DNA amplification techniques. PCR testing of vaginal introitus specimens proved to be as sensitive as culture of cervical specimens (Wiesenfeld *et al.*, 1995), and LCR testing of vulval specimens was as sensitive as cervical specimens (Stary *et al.*, 1995). The importance of these sample types is that they are non-invasive, and in the case of the vaginal introitus sample can be taken by the patient.

1.6.2 Tissue culture

Cell culture for the *in vitro* growth of *C. trachomatis* was first described by Gordon and Quan (1965) using irradiated McCoy cells. Culture is performed by inoculating patient specimens onto cell monolayers. While the technique has a specificity reaching 100%, sensitivity is lower (70-85%) when compared to DNA amplification techniques (Chernesky *et al.*, 1994a; Chernesky *et al.*, 1994b; Lee *et al.*, 1995). Many cell lines have been used for the propagation of *C. trachomatis* including McCoy cells (Yoder *et al.*, 1981; Smith *et al.*, 1982; Stamm *et al.*, 1983), HeLa 229 (Rota *et al.*, 1971; Kuo *et al.*, 1995), and BGMK (Krech *et al.*, 1989; Hosein *et al.*, 1992).

Ideally specimens should be sonicated prior to culture to disrupt cellular material which in turn liberates chlamydial elementary bodies (Watford *et al.*, 1985). Before inoculation the maintenance medium should be removed from the cell monolayer, and replaced by the specimen. Centrifugation of the specimen onto the cell monolayer (2,500-3,000xg at 35°C for 1h) after inoculation has been shown to improve the recovery of viable chlamydiae (Rota *et al.*, 1971). It has also been shown that the addition of polyethylene glycol before centrifugation increased the number of inclusions, improved the sensitivity

of the culture and decreased the possibility of missing weak positive specimens (Mohammed and Hillary, 1985; Gibson *et al.*, 1993)

After centrifugation the remaining sample is aspirated and replaced with fresh culture maintenance medium thus reducing the possibility of a cytotoxic effect. The culture should then be incubated at 37°C in 5% CO₂ for between 48-72h. The blind passage of samples after an initial 48h incubation period has been reported to aid in the recovery of an additional 3-10% of isolates (Barnes, 1989).

The maintenance medium routinely contains antibiotics to which chlamydiae are not susceptible, and a cytostatic agent. Generally cycloheximide (Ripa and Mardh, 1977), a eukaryotic protein synthesis inhibitor is added to the maintenance medium preventing cellular growth and multiplication. It is believed that the inhibition of cellular protein generation results in a pool of amino acids and ATP that the chlamydiae can then utilise for growth.

The identification of chlamydial inclusions in cell culture is routinely accomplished using either genus specific anti-chlamydial lipopolysaccharide fluorescent antibodies, or species specific anti-*C. trachomatis* MOMP fluorescent antibodies. The direct visualization of fluorescent chlamydial inclusions with a distinctive morphology is deemed a positive culture result. Other stains have been used to determine the presence of chlamydial inclusions including Gram, Giemsa, and iodine but due to their lack of sensitivity and specificity, fluorescent antibody staining is the preferred method (Stephens *et al.*, 1982; Stamm *et al.*, 1983; Schoenwald *et al.*, 1988).

1.6.3 Direct sample analysis

1.6.3.1 Enzyme immunoassay

Enzyme immunoassays (EIA) for the detection of chlamydiae were originally developed in the 1980s, and based on the immunochemical detection of chlamydial genus-specific

lipopolysaccharide (LPS). Two basic types of EIA have been described, a direct and an indirect methodology. Direct EIA uses enzyme-labelled antibodies that recognise and bind chlamydial LPS on the surface of elementary bodies present in the clinical sample. Indirect EIA utilises a primary anti-chlamydial LPS antibody as a detector reagent followed by the addition of a secondary enzyme-linked antibody. The enzyme component of both EIAs converts a colourless substrate to a coloured product which can then be detected using a spectrophotometer. Alternatively, the conjugated enzyme may be used to convert a fluorescence-generating substrate to a fluorescent signal.

One of the main disadvantages of EIA is that antibodies to other bacterial LPS may cross-react with the chlamydial LPS, generating a false positive result (Stamm *et al.*, 1988; Barnes, 1989; Centers for Disease Control, 1991; Kellogg *et al.*, 1992). Another problem with EIA is the inability of the system to differentiate between chlamydial species.

There are many commercially available EIAs including Chlamydiazyme (Abbott), IDEIA (Dako), and Clearview (Unipath). Many of the commercially available EIAs include a secondary blocking assay to verify positive EIA results; this uses a monoclonal antibody that competitively inhibits, or blocks the LPS epitope resulting in a reduced signal which is taken as verification of the initial EIA result. The use of blocking antibodies has improved the specificity of EIA (Newhall *et al.*, 1994).

1.6.3.2 Direct fluorescent antibody

Direct fluorescent antibody (DFA) testing is based on the use of a fluorescein-conjugated monoclonal antibody to stain chlamydial elementary bodies in genital, ocular and other smears. DFA using monoclonal antibodies against chlamydial MOMP of *C. trachomatis* (MicroTrak, Syva, USA; Pathfinder, Kallestad, USA; Monofluor, Synbiotics, USA) produces brighter and less non-specific staining than do antibodies against chlamydial LPS (Imagen, Dako).

The reported sensitivity of DFA ranges from 70-100% for men and from 68-100% for women, and a specificity of 87-99% for men and 82%-100% for women (Taylor-Robinson, 1991a; Taylor-Robinson, 1991b). The major disadvantage of DFA is that it requires technical expertise, and discrimination between specific and non-specific staining.

1.6.3.3 Nucleic acid detection

1.6.3.3.1 DNA hybridization probes

At present there is only one commercially available DNA probe for the detection of *C. trachomatis* (PACE 2, Gen-Probe, San Diego, CA). The test employs a chemiluminescent DNA probe that is designed to hybridize to a species specific 16S rRNA sequence. After the formation of the DNA probe/rRNA complex, it is adsorbed onto magnetic beads and the chemiluminescent response is detected and quantified using a luminometer.

The system theoretically should be more sensitive (approximately 10^3 chlamydial elementary bodies) than antigen detection methods due to the fact that actively dividing chlamydiae contain at least 10^4 copies of 16S rRNA. The sensitivity and specificity of PACE 2 compared to culture and an expanded culture standard has been estimated to be between 85% and 98-99% respectively. Like EIA, a probe competition assay has been developed which has been reported to confirm the high number of PACE 2 positive results (Limberger *et al.*, 1992; Stary *et al.*, 1994; Beebe *et al.*, 1997).

1.6.3.3.2 Polymerase chain reaction (PCR)

PCR is a method for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands. PCR involves three main steps; 1) denaturation of the double stranded DNA at high temperature, at temperatures between 90-95°C depending on the G+C content of the sample DNA; 2)

annealing of the primers to the single stranded DNA, 37-65°C; 3) and the primer extension reaction at 72°C which involves the formation of the desired PCR product flanked by both primers.

There are five major reaction components for conducting PCR, a DNA polymerase; deoxynucleoside triphosphates (dNTPs); an appropriate reaction buffer; specifically designed primers that flank the target sequence; and the target DNA.

The first FDA approved PCR for the detection of *C. trachomatis* in the USA was developed by Roche Diagnostics (Amplicor) (Loeffelholz *et al.*, 1992). The Amplicor test targets a 207-bp fragment of the 7.5kb endogenous plasmid of *C. trachomatis*. Plasmid-free *C. trachomatis* isolates have been described (Peterson *et al.*, 1990; An *et al.*, 1992; Farencena *et al.*, 1997) but their abundance in the general population, and clinical significance is unknown.

Detection of amplified products is by an immobilized oligonucleotide capture probe consisting of a sequence complimentary to the amplified target. An avidin-horse radish peroxidase conjugate is then added that binds to the biotinylated PCR product, after washing a peroxidase substrate is added giving rise to a colour change which is read in a spectrophotometer.

Amplicor PCR has been widely evaluated for both urogenital and urine specimens, with an overall sensitivity of 90-99% and 100% specificity and has been approved for use with cervical, male urethral, and male urine specimens. In some studies with Amplicor PCR, a small number of false-negatives that become positive after storage and repeat testing have been reported (Loeffelholz *et al.*, 1992; Bass *et al.*, 1993; Bauwens *et al.*, 1993a; Mahony *et al.*, 1994). It is believed that SDS in the Amplicor transport buffer was the problem, and tests using a 'universal' non-detergent transport buffer (Salmon *et al.*, 1994) or 2SP (Verkooyen *et al.*, 1995) have been shown to improve sensitivity.

MOMP PCR is not available as a diagnostic test commercially, and its sensitivity is below that of PCR based on the *C. trachomatis* plasmid. The reduced sensitivity of MOMP reflects the fact that the MOMP gene is a single copy gene (Stephens *et al.*, 1985), compared to the *C. trachomatis* plasmid which can be present in 5-10 copies (Comanducci *et al.*, 1990). MOMP PCR has been used by Roche Diagnostics for the discrepant analysis of the Amplicor PCR system (Lin *et al.*, 1992; Bawens *et al.*, 1993b).

1.6.3.3.3 Ligase chain reaction

The ligase chain reaction (LCR) was first described by Backman (1987), four oligonucleotide probes attached to two different haptens are used to recognise and bind the target sequence. If the target sequence is present the probes bind adjacently to each other and are enzymatically joined by a ligase to form the amplification product. The amplification product then serves as the target sequence during successive rounds of amplification. Finally the amplified target sequence is detected by hybridization utilising a microparticle enzyme assay.

Abbott have introduced a commercial LCR named LCx. This system has been used to detect *C. trachomatis* in female endocervical swabs, male urethral swabs and in both male and female urine specimens; and has been shown to be highly sensitive in the detection in female chlamydial genital infections (Chernesky *et al.*, 1994a; Chernesky *et al.*, 1994b; Chernesky *et al.*, 1994c; Lee *et al.*, 1995).

1.6.3.4 Other molecular methods

Several new molecular amplification methods have been used to detect *C. trachomatis* DNA in clinical samples. These include the Q-beta replicase-amplified hybridization assay (Gene-Track) (Shah *et al.*, 1994); Gen-Probe AMPLIFIED *Chlamydia trachomatis* Assay (AMP CT) which is based on transcription mediated amplification (TMA) technology (Chambers *et al.*, 1995); and nucleic acid sequence based amplification

(NASBA) for the detection of *C. trachomatis* RNA in cervical and urine specimens (Morre *et al.*, 1996).

The Q-beta replicase-amplified hybridization assay requires an RNA-directed RNA polymerase and two different probes. The reporter probe consists of Q-beta replicase RNA synthetically linked to a *C. trachomatis* rRNA tail, and the capture probe consists of *C. trachomatis* DNA with a polyadenine tail. Both probes hybridize to adjacent regions of *C. trachomatis* 16S rRNA. The hybridization complex is captured by polythymidine coated beads, and the attached products are detected by a fluorimetric system.

The TMA assay at present targets 16s rRNA, and works as an isothermal system that utilizes enzymatic target amplification followed by chemiluminescent detection. AMP CT has been shown to be highly sensitive and specific for the detection of *C. trachomatis* DNA in urine specimens from women and men (Pasternack *et al.*, 1997; Crotchfelt, 1998) and endocervical specimens (Crotchfelt, 1998).

NASBA uses a system similar to that of TMA. One primer contains a T7 RNA polymerase promoter and a sequence homologous to a portion of *C. trachomatis* RNA. A reverse transcriptase extends the primer to make a DNA copy of the target sequence forming an RNA-DNA hybrid. The RNA strand is subsequently degraded by RNase H, and the remaining DNA strand forms the template for a second primer, the extension of which is accomplished by a reverse transcriptase forming a DNA-DNA hybrid. The double stranded DNA then forms a template for the production of further RNA transcripts by T7 RNA polymerase.

1.6.4 Serology

1.6.4.1 Complement fixation test

The complement fixation test (CFT) is based on the detection of genus specific antibodies aimed at chlamydial LPS. The sensitivity of CFT is insufficient to detect uncomplicated

genital infections, but it can be used in the diagnosis LGV (Perine *et al.*, 1990). While the test is still used for the presumptive diagnosis of *C. psittaci* infections based on a four fold rise in titre between acute and convalescent sera, it cannot differentiate between chlamydial species.

1.6.4.2 Enzyme immunoassay/enzyme-linked immunoassay

These tests are designed to detect antibody reactivity to genus-specific antigens such as LPS. As for other serological tests a single measurement of a patient's serum will not give any evidence whether the infection is a current or past infection. Although EIAs for the detection of anti-chlamydial antibodies are less sensitive than MIF, it has been shown that for the detection of IgM in children with chlamydial pneumonitis, EIA is comparable to MIF (Mahony *et al.*, 1986).

1.6.4.3 Micro-immunofluorescence

Wang and Grayston (1970) developed the micro-immunofluorescence (MIF) test which is based on purified chlamydial elementary bodies spotted onto a glass slide. The test serum is diluted and applied to the glass slide spotted with chlamydial elementary bodies, and bound antibody is subsequently detected by the addition of a class specific anti-human fluorescein conjugated antibody. Single antigen types from a specific *C. trachomatis* serovar, or a pooled antigen can be used. *C. psittaci* and *C. pneumoniae* antigen can also be used, allowing species specific antibody to be distinguished.

1.7 Treatment and prevention

1.7.1 Chemotherapy

The current recommended treatment regime for *C. trachomatis* infections entail a 7-10 day course of tetracycline, doxycycline or erythromycin. The major problem associated with multi-dose therapies is the potential for patient non-compliance. The azalide antibiotic azithromycin has emerged as an effective alternative treatment for *C. trachomatis* infections. Azithromycin concentrates within cells, and has a tissue half life of up to 5 days (Wyrick *et al.*, 1993). It been shown to be an effective treatment of uncomplicated chlamydial urethritis, and cervicitis when given as a single 1g dose (Worm and Osterlind, 1995; Ridgway, 1996; Thorpe *et al.*, 1996).

1.7.2 Vaccine development

Antibiotic therapy can effectively eliminate chlamydial infection but can have little if any effect on established pathology, or immune-mediated damage. Prevention of infection rather than treatment could be potentially less costly than a screening program, and computer modelling has suggested that even a partially effective vaccination program may considerably reduce the prevalence of chlamydial genital tract infection (de la Maza *et al.*, 1995).

Early chlamydial vaccine attempts were not totally successful. In the 1960s, trachoma vaccine trials in both humans and other primates demonstrated that neutralizing antibodies could be produced and a degree of protection against conjunctival challenge could be elicited. However, it became apparent that some vaccinated individuals developed more severe disease (Woolridge *et al.*, 1967; reviewed in Sowa *et al.*, 1969; Schachter and Dawson, 1978), and that trachoma was an immune mediated disease. Subsequent challenge with different serovars led to more severe inflammation and it was suggested that a delayed type hypersensitivity response was the cause (Wang *et al.*, 1967). More

recent studies have implicated chlamydial HSP60 in the pathogenesis of ocular inflammation (Morrison *et al.*, 1989b).

In animal models live vaccine have been shown to confer a degree of immunity, they have been used successfully in preventing abortion in sheep (McEwen *et al.*, 1951; Rodolakis *et al.*, 1984; Chalmers *et al.*, 1997), and in murine MoPn induced infertility (Pal *et al.*, 1996). In humans, whole-organism vaccines are unlikely to be used in the future due to the problems encountered with the early trachoma vaccine trials.

Recent research has focused on the development of sub-unit vaccines based on the major outer membrane protein (MOMP) of *C. trachomatis*. Most of the MOMP-based vaccines have been designed to provoke protective antibody responses, and have been shown to elicit both mucosal and serum antibody responses (Tuffrey *et al.*, 1992b; Batteiger *et al.*, 1993; Su *et al.*, 1995), even though immunization with MOMP-derived peptide vaccines have given protection in the absence of antibody production (Campose *et al.*, 1995). One of the problems associated with MOMP vaccines is that variation within the same serovar can be sufficient to evade neutralization by antibody (Lampe *et al.*, 1997).

Chapter 2.0
Enzyme-linked immunoassay (ELISA)

2.1 Aims of chapter

The initial aim of the work in this chapter was to develop and validate a *C. trachomatis* specific enzyme-linked immunoassay (ELISA) which could be used to detect both IgG and IgM in serum samples.

Once established the ELISA system was used to determine the presence of antibodies against *C. trachomatis* (IgG and IgM) in two different studies. One study was designed to look at the prevalence of anti-chlamydial antibodies in women diagnosed with miscarriage, ectopic pregnancy, and tubal factor infertility in both the UK (Sheffield and Bristol) and Trinidad (Mount Hope and Port of Spain) compared to non-pregnant, and antenatal controls.

The second study was used to determine the impact of anti-chlamydial IgG on the success of *in vitro* fertilization (IVF) with respect to the patient's response to gonadotropin stimulation prior to IVF. This study involved two IVF centres (Bristol and Southampton), with the patients from each divided into two groups, those who responded well to gonadotropin stimulation (good responders), and those who responded poorly to gonadotropin stimulation (poor responders). Non-pregnant, and antenatal controls were used to establish a baseline prevalence of anti-chlamydial IgG.

2.2 Introduction

Tubal disease is one of the major causes of female infertility (Westrom *et al.*, 1980) and includes a vast range of conditions including tubal blockage both proximally and distally, peritubal adhesions and hydrosalpinx formation. *C. trachomatis* has been associated with urethritis, endometritis, and more importantly salpingitis and pelvic inflammatory disease (PID) both of which have been shown to be risk factors for ectopic pregnancy (EP) and the development of tubal disease. Other risk factors of tubal disease include a previous EP, endometriosis, previous sterilisation, previous tubal surgery and a history of peritonitis.

Serological studies have supported the role of *C. trachomatis* in the development of PID and its sequelae. In an early study Mardh *et al.* (1977) reported that 80% of sixty women with acute PID had antibodies to *C. trachomatis*. Studies by Punnonen *et al.* (1979), Moore *et al.* (1982), and Conway *et al.* (1984) looked at the detection rate of antibodies to *C. trachomatis* in women with infertility, all concluded that tubal disease was associated with the presence of anti-chlamydial antibodies. Many studies have also related EP to the detection of antibodies against *C. trachomatis*, Chrysostomou *et al.* (1992) found a significant rate of detection of anti-chlamydial IgG in women with EP compared to pregnant controls.

Severe tubal disease, specifically distal tubal obstruction leading to the formation of hydrosalpinx has been reported to be associated with a poor fertility prognosis (Edwards *et al.*, 1984, Marana *et al.*, 1995). Englert *et al.* (1987) found that patients with infertility of tubal origin had lower *in vitro* fertilization (IVF) success rates than patients with partners diagnosed with male infertility, or idiopathic causes.

Lewis *et al.* (1977) was the first to develop an enzyme-linked immunoassay (ELISA) technique for the detection of antibodies to chlamydiae using antigens derived from *C. psittaci* which involved the fixation of chlamydial elementary bodies to an EIA plate. Evans and Taylor-Robinson in 1982 described an ELISA system based on sodium

deoxycholate extracted *C. trachomatis* lipopolysaccharide (LPS) as antigen, and although this method was found to be more sensitive than both micro-immunofluorescence (MIF) and the complement fixation test (CFT), it was unable to distinguish between chlamydial species.

In a study of a UK STD population it was reported that antibodies to *C. pneumoniae* and *C. psittaci* accounted for up to half of all the chlamydial specific IgG (Moss *et al.* 1993). In a recent paper by Chernesky *et al.* (1998) five serological assays were compared in the detection of antibodies to *C. trachomatis* in women with pelvic pain, with and without evidence of chlamydial infection. The best sensitivity and specificity was found with the whole inclusion fluorescence test (WIF), and even though a recombinant anti-LPS ELISA measured antibodies in the majority of the test patients the specificity was low. Due to the presence of genus cross reactive epitopes on the chlamydial surface it could be argued that for an ELISA with the highest specificity for antibodies against a particular chlamydial species, a purified species specific antigen should be used (Puolakkainen *et al.*, 1984); or alternatively there should be the removal of genus cross reactive epitopes (eg. chlamydial LPS) from antigen preparations.

In 1989 Ladney *et al.* described an ELISA method based on the pre-treatment of chlamydial elementary bodies to extract the LPS. This system showed a decreased cross reactivity which was able to distinguish antibodies against *C. trachomatis* and *C. pneumoniae*. In the same year Ossewaarde *et al.* (1989) also published an ELISA method that used either a periodate or Triton X-100 pre-treatment, that could measure antibodies to *C. trachomatis* proteins or LPS. This method was validated against a standard ELISA and WIF (Ossewaarde *et al.*, 1994b) the results of which concluded that oxidation of chlamydial LPS reduced the binding of genus cross reactive antibodies to *C. trachomatis* elementary bodies pre-treated with sodium periodate, enhancing ELISA specificity.

2.3 ELISA Materials

2.3.1 Reagents

Reagents were purchased from Sigma (Dorset, UK) unless otherwise stated.

2.3.2 Cell culture

McCoy cells (Cat. No. 90010305) were purchased from ECACC (Salisbury, UK), and were *Mycoplasma* free. The cell line was originally derived from the synovial fluid of a patient suffering from degenerative arthritis, but subsequent sublines have been shown to be of mouse origin.

Glutamine (Gibco, Paisley, UK) was purchased as a 200mM solution and stored in 5ml aliquots at -20°C.

McCoy cell growth medium

RPMI 1640	445ml
Foetal calf serum (Bioclear Ltd., Wilts, UK)	50ml
200mM glutamine	5ml

The pH growth medium was adjusted to 7.4 and stored at 4°C.

Amphotericin B (Fungizone) was obtained from Gibco as a 250µg/ml sterile solution, it was aliquoted into 2ml volumes and stored at -20°C.

Cycloheximide was dissolved in sterile water to give a final concentration of 1mg/ml, aliquoted in 500µl volumes and stored at -20°C.

Gentamicin (Royal Hallamshire Hospital Pharmacy) was purchased as a 40mg/ml sterile solution and was stored in 250µl amounts at -20°C.

Glucose was dissolved in sterile water to give a 20% (w/v) solution and was then sterilised through a 0.2µm Millipore filter and stored at 4°C.

Streptomycin was obtained from Flow Laboratories, and dissolved in water to give a concentration of 40mg/ml, aliquoted in 1ml amounts and stored at -20°C.

x10 trypsin (Gibco) was purchased as a sterile 2.5% solution and diluted to a 0.25% solution in PBS. 5ml aliquots were stored at -20°C.

Vancomycin hydrochloride was dissolved in sterile water to give a concentration of 20mg/ml, aliquoted in 1ml amounts and stored at -20°C.

Versine ($C_{10}H_{14}N_2O_5Na_2 \cdot 2H_2O$) was purchased from BDH Chemicals (Poole, UK), dissolved in PBS to give a final concentration of 5mM and the pH adjusted to 7.2-7.4. The solution was then aliquoted into 5ml volumes, autoclaved and stored at -20°C.

McCoy cell maintenance medium

RPMI 1640	416.0ml
Heat inactivated foetal calf serum (Bioclear Ltd.)	50ml
glutamine	5ml
1% glucose	25ml of stock
1µg/ml amphotericin B	2ml of stock
20µg/ml gentamicin	250µl of stock
20µg/ml vancomycin	500µl of stock
20µg/ml streptomycin	250µl of stock
2µg/ml cycloheximide	1ml stock

The pH of the maintenance medium was adjusted to 7.4 and stored at 4°C.

Sterility checking of medium

Before use, both types of medium were subjected to sterility checking. Briefly, 1ml of medium was inoculated in two bottles of thioglycolate broth and incubated at both 33°C or

37°C for 1 week. The medium was deemed sterile if no growth was visualised.

2.3.3 Antigen preparation

Urografin 150 was purchased from Schering Health Care Ltd (West Sussex, UK).

The solution contained sodium diatrizoate (0.04g/ml) and meglumine diatrizoate (0.26g/ml), and was stored at 4°C.

0.02% (v/v) formalin-PBS for the preservation of chlamydial antigen preparations was prepared by adding 20µl of formalin to 100ml of PBS.

Bradford dye solution

Coomassie brilliant blue G-250 (BDH)	100mg
95% ethanol	50ml
85% orthophosphoric acid	100ml
purified water	50ml

The Coomassie brilliant blue was initially dissolved in the 95% ethanol and left for 15 min. Finally the 100ml of 85% orthophosphoric acid was added and the total volume made up to 200ml with purified water. The dye was stored at 4°C, and used within 6 months.

2.3.4 ELISA solutions

EIA/RIA plates were purchased from CoStar (Cambridge, USA). The plates were non-sterile, flat bottomed and high affinity binding.

Sodium bicarbonate buffer (coating buffer)

Na ₂ CO ₃	0.795g
NaHCO ₃	1.465g
NaN ₃	0.1g
purified water	500ml

The pH of the coating buffer was adjusted to 9.6 with 1M NaOH, and stored at 4°C.

PBS-Tween

Tween 20 (BDH)	0.5ml
PBS	1L

The solution was stored at room temperature.

Serum diluent

PBS	500ml
0.3M NaCl	8.766g
0.001M MgCl ₂	0.0476g
0.5% (w/v) BSA fraction V (Winlab, Middlesex, UK)	2.5g
0.5% (w/v) gelatine	2.5g

The diluent was aliquoted in 20ml amounts and stored at -20°C.

Citrate phosphate buffer

0.1M citric acid	2.1g
purified water	100ml
0.2M Na ₂ H _p O ₄ 12H ₂ O	7.3g
purified water	100ml

24.3ml of the citrate solution and 25.7ml of the phosphate solution were added to a final volume of 100ml. The pH was adjusted to 5.0 with either solution, and the buffer stored at 4°C.

Substrate buffer

10mg of σ -phenylenediamine dihydrochloride	1 tablet
citrate phosphate buffer	25ml

This was prepared fresh, followed by the addition of 10 μ l of 30% hydrogen peroxide (H₂O₂) immediately before use.

Antibody conjugates

Goat anti-human IgG (whole molecule), conjugated with horseradish peroxidase and IgM conjugated with horseradish peroxidase were obtained from Sigma. The antibodies were aliquoted in 10 μ l amounts and stored at -70°C.

2M sulphuric acid

98% H ₂ SO ₄	19.6ml
water	90.2ml

The acid was stored at room temperature.

1% (w/v) sodium periodate-PBS

sodium periodate	10g
PBS	500ml

The solution was stored at room temperature.

1% Triton X-100 was prepared in PBS containing 5mM EDTA and stored at 4°C.

***Chlamydia* MIF Kits** for the detection of anti-chlamydial IgG and IgM were obtained from MRL Diagnostics (Cypress, CA, USA) and stored at 4°C. Before use the MIF slides and reagents were allowed to reach room temperature.

2.3.5 Specimens

Specimen preparation

Patient samples were either delivered as non-heparinised whole blood or had been pre-separated to leave serum. Whole blood samples were centrifuged briefly to sediment out the red blood cells, and the serum removed. Finally each sample was coded blind and stored at -20°C.

Serum samples from the West Indies were sent frozen in dry ice, and were immediately stored at -20°C on arrival. Samples from Bristol, and Southampton (IVF samples only)

were transported overnight in liquid nitrogen containers, and placed at -20°C on arrival in Sheffield.

Control sera

Human control sera with known MIF titres (IgG) for *C. trachomatis*, *C. pneumoniae* and *C. psittaci* were donated by Dr. Treharne (Institute of Ophthalmology, London). Each serum sample was aliquoted in 50µl volumes and stored at -20°C. In addition a selection of known IgG MIF titre samples from the Northern General Hospital (Sheffield) serum sample bank were collected and stored at -20°C.

Also from the UK, serum samples from twenty eight pregnant women and from fifty three non-pregnant females (serum obtained during routine medical management) were collected and stored at -20°C. From Trinidad sixty four antenatal serum samples were collected, and stored at -20°C. These samples were used to determine a baseline prevalence of anti-chlamydial IgG and IgM for both geographic locations.

2.4 Methods

2.4.1 Cell culture

Growth of McCoy cells

The McCoy cells were originally purchased from ECACC and were confirmed as being *Mycoplasma* free. Initially a frozen 1ml vial of trypsinised McCoy cells suspended in growth medium containing 10% DMSO was revived by washing once in fresh growth medium. The cells were then seeded into a 25cm² culture flask (Bibby Sterilin Ltd., Staffordshire, UK) and incubated at 37°C in 5% CO₂ for 4 days, or until confluent.

Once a confluent cell monolayer was achieved the cells were passaged. The growth medium was removed and the cell monolayer was gently washed with PBS. The PBS was replaced with 2ml of trypsin and 2ml of versine pre-warmed to 37°C, and the cell culture flask was incubated for 5 min at 37°C or until the monolayer had become detached from the flask surface. 10ml of growth medium was then added and the cellular suspension was divided equally between four 25cm² culture flasks, each flask was topped up with growth medium to give a final volume of 25ml. The flasks were then incubated for up to 4 days at 37°C in 5% CO₂ until confluent and either passaged into 25cm² or 75cm² flasks, or infected with *Chlamydia*.

Shell vial culture of McCoy cells

A confluent 25cm² flask of McCoy cells was gently washed with PBS after the removal of growth medium. The PBS was replaced with 2ml of trypsin and 2ml of versine pre-warmed to 37°C, and the cell culture flask incubated for 5 min at 37°C or until the cells had become separated from the flask surface. Growth medium was then added to give a final cell concentration of approximately 1-2 x 10⁵ cells/ml of growth medium. 1ml of this suspension was then used to seed 5ml Trac bottles containing a glass coverslip (Sterilin). The Trac bottles were then incubated for up to 4 days in 5% CO₂ at 37°C until confluent and were then infected with *Chlamydia*.

Growth of *Chlamydia trachomatis* LGV1

C. trachomatis serovar LGV1 was obtained from Dr. Treharne (Institute of Ophthalmology, London) and after revival was confirmed as LGV1 by MOMP PCR (Lan *et al.*, 1994) and DNA restriction pattern analysis.

Initially McCoy cells grown in Trac bottles were used to revive the LGV1 serovar. The growth medium was removed and 5ml of maintenance medium containing 1µg/ml cycloheximide was added. The original egg culture was diluted 1:200 in PBS and 100µl was used to inoculate each Trac bottle. The cells were then incubated at 37°C in 5% CO₂ for 3 days. 3ml of the maintenance medium was removed from each Trac bottle and the monolayer was scraped off the glass coverslip using the end of a sterile 1ml syringe. 100µl of the resulting cellular suspension was then used to inoculate a new Trac bottle containing maintenance medium. The chlamydial culture was passaged three times before the presence of *Chlamydia* was confirmed using either iodine staining, or immunofluorescence (Syva MicroTrak *Chlamydia trachomatis* Culture Confirmation Test, Behring Diagnostics, France).

Once the culture had been established in Trac bottles and there was an approximately 100% infection of the McCoy cells, the culture was transferred firstly into 25cm² flasks and finally 75cm² flasks. Before the passage of a flask the cell monolayer was iodine stained to determine the percentage of infection. When the percentage of infection reached approximately 80%, the cells were removed by agitation with sterile glass beads and the resulting cellular suspension was stored at 4°C until chlamydial elementary body purification.

2.4.2 Chlamydial staining

Iodine staining

The maintenance medium was removed from either the Trac bottle or flask, and the cell monolayer was washed briefly in PBS. The McCoy cells were then fixed in 1ml of methanol for 10min, which was then replaced with 1ml of iodine for 10min. For Trac

bottles the glass coverslip was removed, mounted in glycerol on a glass microscope slide and examined under light microscopy at x100 and x400 magnifications (Figure 4). The number of chlamydial inclusions (dark brown spheres within cells) were counted per coverslip.

Immunofluorescent detection of *C. trachomatis*

The immunofluorescent detection of *C. trachomatis* was achieved using the Syva MicroTrak *Chlamydia trachomatis* Culture Confirmation Test (Behring Diagnostics, France) as per the manufacturer's instructions. Briefly, the maintenance medium was aspirated from the Trac bottle, 1ml of ethanol was added and the monolayer fixed for 10min. The ethanol was poured away, the coverslip was removed with forceps and placed cell side up on a clean microscope slide. 30µl of reagent (supplied) was added to the monolayer and the cells incubated at 37°C in a moist chamber for 30min. The coverslip was then rinsed gently in PBS, mounted cells side down in the medium supplied and viewed under a fluorescence microscope (fitted with a FITC filter system) at x100 and x400 magnification (Figure 5). Again, the number of elementary bodies (fluorescent apple green spheres within red counterstained cells) was determined.

2.4.3 Antigen preparation

Preparation of LGV1 elementary bodies

The McCoy cell/*Chlamydia* suspension was disrupted by sonication at a 12 micron amplitude for 30sec, followed by a 1min interval to allow the dissipation of the heat generated during sonication into a surrounding ice cushion. The process was repeated three times before the suspension was centrifuged at 500xg for 15min at 4°C to remove cellular debris. The supernatant was kept for further purification.

Purification of LGV1 elementary bodies

The crude elementary body preparation was centrifuged at 30,000xg for 1h at 4°C to pellet the elementary bodies, each pellet was resuspended in 8ml of PBS and sonicated as previously described to give a homogeneous suspension. 15ml of the suspension was

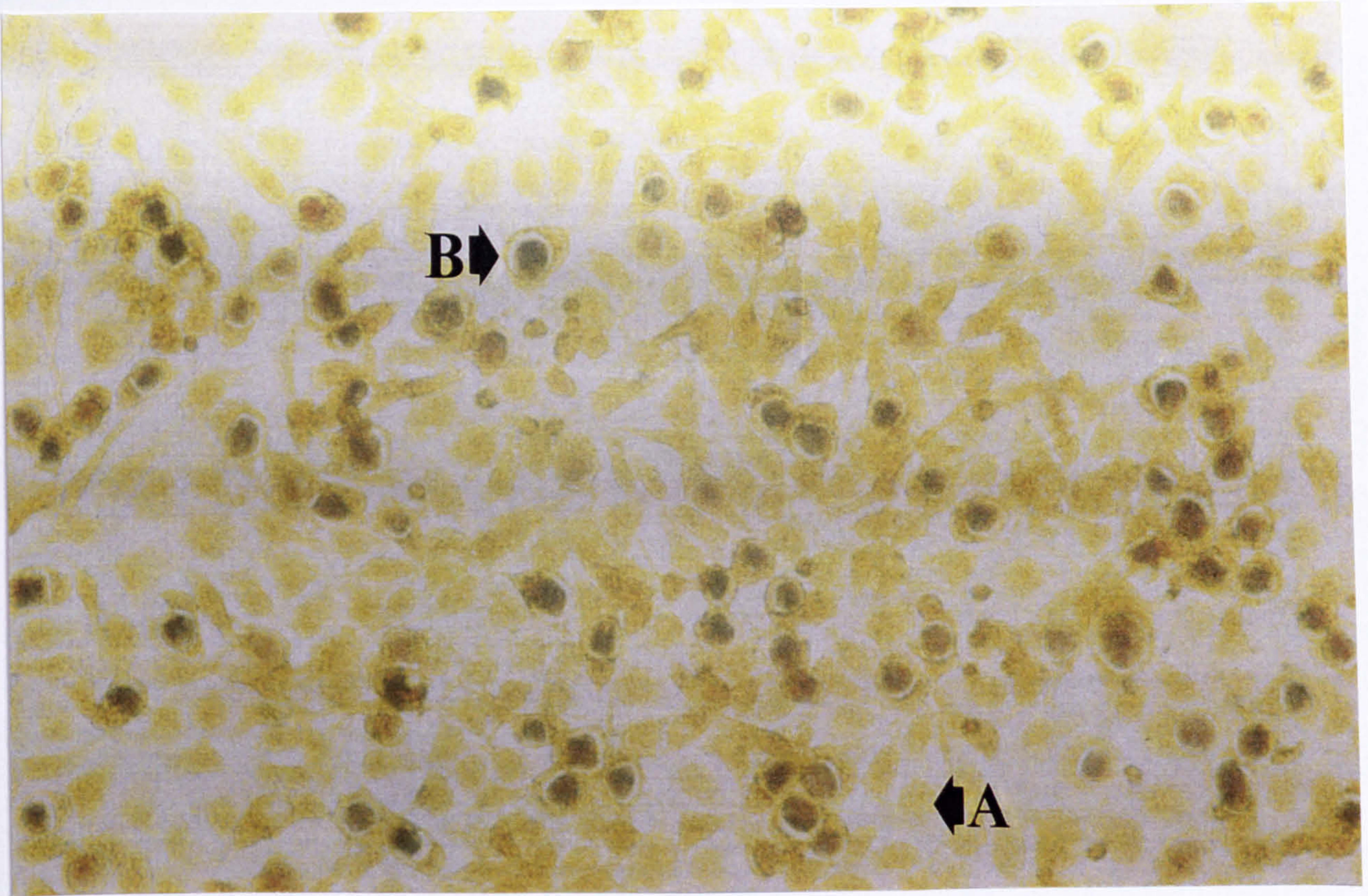


Figure 4. McCoy cell monolayer infected with *C. trachomatis* LGV1 (48h) after iodine staining (mag.x400). A- uninfected McCoy cell; B- LGV1 inclusion.

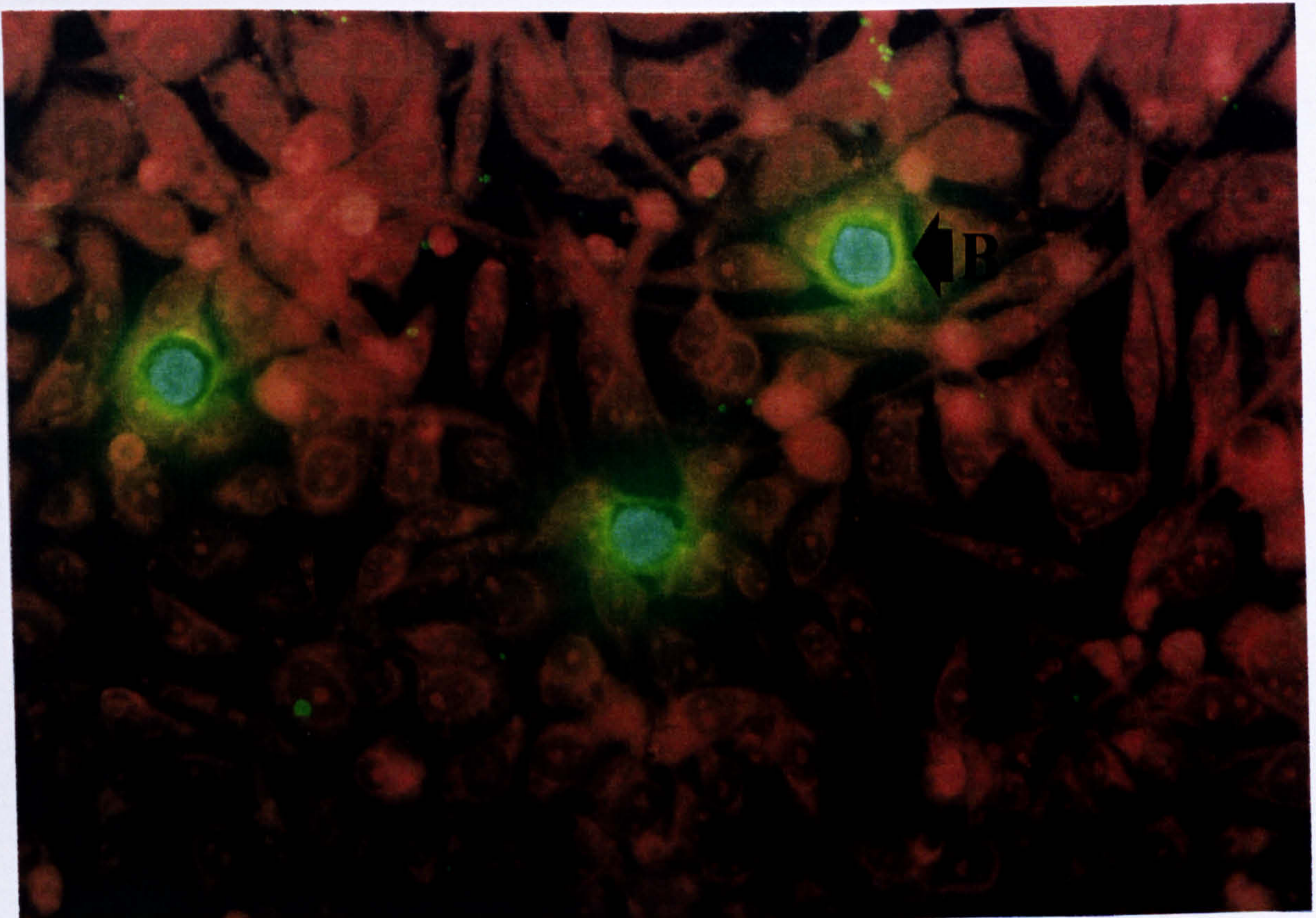


Figure 5. McCoy cell monolayer infected with *C. trachomatis* LGV1 (48h) after immunofluorescent staining (mag.x1000). A- uninfected McCoy cell; B- LGV1 inclusion.

then layered onto 15ml of Urografin and centrifuged at 4°C for 1h at 30,000xg. The pellet was washed in PBS, sonicated and centrifuged as above. The final pellet containing the elementary bodies was resuspended in a small volume of 0.02% formalin-PBS, sonicated and the protein concentration measured. The antigen preparation was ultimately aliquoted into 100µl amounts and stored at -70°C.

McCoy cell antigen preparation

A negative control McCoy cell antigen preparation was prepared in the same manner from uninfected McCoy cells.

***Chlamydia pneumoniae* and HL cell antigen preparations**

C. pneumoniae strain IOL-207 and HL cell antigen preparations of known protein concentrations were kindly donated by Dr. M. Khan (formally of the Department of Medical Microbiology, University of Sheffield). The method of elementary body preparation of *C. pneumoniae* was the same as that for *C. trachomatis*.

Measurement of protein concentration (Bradford method)

The protein concentration of both the LGV1 and McCoy cell antigen preparations was measured using the Bradford dye binding assay (Bradford, 1976).

A series of BSA solutions containing 6.25µg/ml to 1000µg/ml of protein were examined by the Bradford method to establish a standard protein curve (Table 1, Graph 1.). The Bradford dye was diluted 1:5 with purified water in a plastic disposable cuvette and 100µl of each standard protein solution was added. The protein standards were left for 15min for colour to develop, and then the absorbance of each was measured twice at 600nm. A standard curve was constructed relating protein concentration to OD, the standard curve being linear between 20-150µg of protein in 100µl.

Each antigen preparation was diluted to 1:7 and the absorbance measured as previously described. The absorbance value was compared to the standard protein curve and the protein concentration of each antigen preparation was determined (BSA gives an OD two

fold higher than its weight). The LGV1 antigen preparation was found to give an approximate protein concentration of 357µg/ml, and the McCoy antigen 110µg/ml.

Table 1. Absorbance values (OD) at different protein concentrations using the Bradford method.

protein conc. µg/ml	mean OD at 600nm	standard deviation
1000	1.245	0.008
400	1.1065	0.0025
200	0.706	0.004
100	0.4365	0.0035
50	0.235	0.002
25	0.1035	0.0005
12.5	0.043	0
6.25	0.0095	0.0005

antigen prep. (1:7 dilution)	mean OD at 600nm	standard deviation
LGV1	0.45	0.002
McCoy cell	0.16	0.01

2.4.4 ELISA development

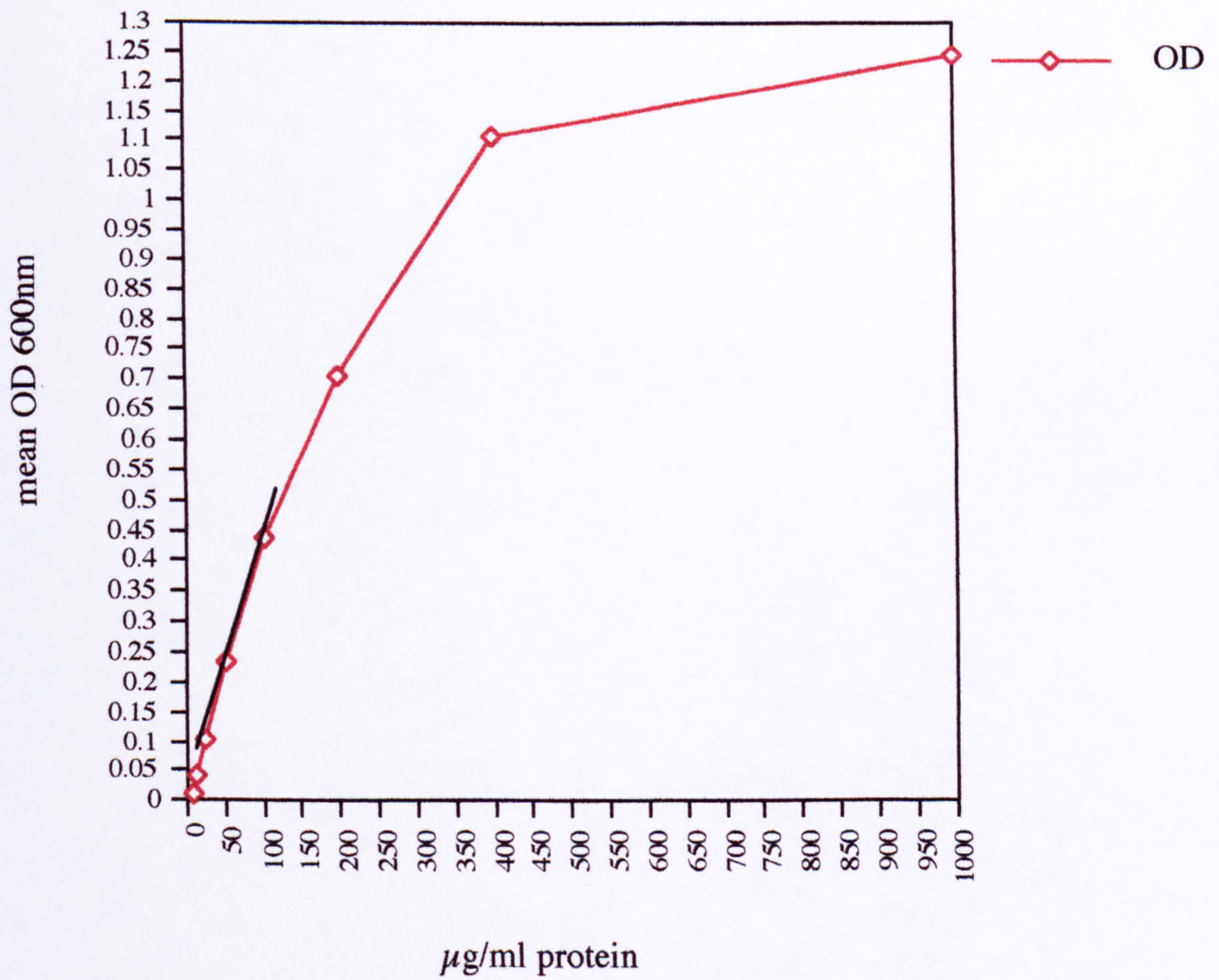
The development of both the IgG and IgM ELISA methodologies can be found in Appendix 1.

2.4.5 ELISA of study samples

IgG ELISA specific for *C. trachomatis*

The *C. trachomatis* LGV1 antigen preparation was diluted to 20µg/ml in coating buffer, and 50µl was dispensed into each well of an EIA plate. The plate was left overnight at 4°C in a moist chamber, was then washed once in PBS-Tween and 50µl of 1% sodium periodate was added to each well and the plate incubated at 37°C for 10min. The plate

Graph 1. Protein (BSA) standard curve using the Bradford method for the determination of antigen protein concentration



was then washed twice in PBS-Tween before the addition of the diluted serum.

Each study sample was diluted 1:200 in serum diluent, and 50µl was placed into three wells; the plate was then incubated for 1h at 37°C in a moist chamber. The plate was then washed three times in PBS-Tween, 50µl of HRP-conjugated anti-human IgG pre-diluted 1:4000 in serum diluent was then added to each well and the plate incubated again at 37°C for 1h. Following incubation the plate was washed three times to remove unbound antibody that could contribute to the generation of a background signal, and excess liquid removed by inverting the plate on tissue paper. 50µl of substrate buffer was placed in each well and the plate left to develop at room temperature for 15min. The enzyme reaction was stopped by the addition of 50µl of 2M H₂SO₄ to each well, and the absorbance was read immediately at 492nm in an ELISA reader.

Control samples (IgG)

Control sera of known MIF titres for *C. trachomatis* and *C. pneumoniae* were used on each EIA plate to ascertain the success of the ELISA and to determine the presence of *C. trachomatis* IgG. Due to the possibility of plate to plate variation, samples were compared only to the controls from the plate on which they were tested. An EIA plate was repeated if negative control sera and positive control sera did not give the appropriate results.

For the IgG ELISA, five control sera were included on each plate; an MIF 1:512 *C. trachomatis* positive serum; two MIF 1:16 *C. trachomatis* positive serum samples; two serum samples negative for *C. trachomatis* by MIF; and a dual species *Chlamydia* serum containing chlamydial IgG against both *C. trachomatis* and *C. pneumoniae* (MIF 1:16 against *C. trachomatis*, and MIF 1:256 against *C. pneumoniae*). Samples were considered negative for *C. trachomatis* IgG if the mean OD value was below that of the mean negative control OD value plus three standard deviations (+3SD). A sample was considered positive if the mean OD was greater than the mean OD of the MIF 1:16 samples +3SD. Samples with a mean OD between that of the mean negative control OD value +3SD, and the mean OD of the MIF 1:16 samples +3SD were tested by MIF (MRL

Diagnostics, USA) as per the manufacturer's instructions.

IgM ELISA specific for *C. trachomatis*

Study sera were tested in triplicate at a serum dilution of 1:200, against 20µg/ml of the LGV1 antigen preparations using a sodium periodate pre-treatment, and an IgM conjugate dilution of 1:2000. The method of EIA plate coating of the antigens and the actual ELISA methodology were as previously described for the IgG ELISA (p.65) with one exception; the substrate buffer was left to develop at room temperature for 35min.

Control samples (IgM)

Again, due to the possibility of plate to plate experimental variation, test samples were only compared to the controls found on the same plate.

For the IgM ELISA, four control sera were included on each plate; an MIF >1:40 *C. trachomatis* positive serum; an MIF 1:10 *C. trachomatis* positive serum; and two serum samples negative for *C. trachomatis* by MIF. Samples were considered negative for *C. trachomatis* IgM if the mean OD value was below that of the mean negative control OD value +3SD. A sample was considered positive if the mean OD was greater than the mean OD of the MIF 1:10 sample +3SD. Samples with a mean OD between that of the mean negative control OD value +3SD, and the mean OD of the MIF 1:10 sample +3SD were considered 'query' positive. Due to problems with circulating rheumatoid factor giving rise to false positive results, all samples deemed positive for anti-chlamydial IgM were tested by an IgM MIF (MRL Diagnostics, USA) as per the manufacturer's instructions.

2.4.6 IgG and IgM micro-immunofluorescence

Both micro-immunofluorescence (MIF) systems used were based on the fluorescent detection of anti-chlamydial antibodies against purified elementary bodies which allowed the semi-quantification and speciation of human serum IgG and IgM antibodies. The test utilised purified elementary body preparations of *C. psittaci*, *C. trachomatis* and *C. pneumoniae* diluted in yolk sac. All three antigen preparations were spotted within a

single well on a slide, each slide contained twelve such wells. Yolk sac was used as a negative antigen control. Patient samples were screened at a single dilution (1:16 for IgG and 1:10 for IgM) to determine the presence/absence of anti-chlamydial antibodies.

For the IgG MIF, slides were allowed to reach room temperature before being opened to prevent condensation developing on the slides. The serum samples were pre-diluted in PBS at a factor of 1:16 and 25 μ l was added to the appropriate well. Each slide also contained a positive and negative control serum (supplied). The test slide was then incubated at 37°C for 30 min in a moist container, rinsed gently in PBS for 10 min, dipped in distilled water and left to air dry. 25 μ l of the fluorescein-labelled goat anti-human anti-mouse IgG (supplied) was added to each slide well and the slide incubated for 30 min at 37°C in a moist container. The slide was washed again in PBS for 10 min, left to air dry and finally mounted with a coverslip. The slide was then viewed using a fluorescence microscope at a magnification of x400.

Each serum sample was scored according to fluorescent intensity of the elementary bodies for each chlamydial species. A score of ++ to ++++ was given if moderate to intense apple-green fluorescence was seen, + if a definite but dim fluorescence was observed and a negative score if no fluorescence or fluorescence equal to that of the corresponding yolk sac control or negative control was seen. The positive control serum was expected to exhibit ++ to ++++ fluorescence for all three chlamydial antigen preparations, and the negative no fluorescence. A score of + for a study sample was considered as positive evidence of antibodies against the appropriate chlamydial species.

The IgM MIF was similar to the IgG test with one exception. Each serum sample was initially diluted (1:10) in an IgM pre-treatment diluent (supplied), which contained monospecific goat antiserum to human IgG in PBS. The IgG was removed since IgG may compete with IgM giving rise to false negative results, and complexed IgG (rheumatoid factor) may give rise to the generation of a false positive result.

2.4.7 Statistical analysis

The results for each patient group were compared to the appropriate study control group using the Fisher Exact Probability Test (2 tailed). The Bonferroni correction was used for multiple comparisons. A probability (P) value of <0.05 was considered to be statistically significant.

2.5 ELISA results

2.5.1 Serology results for Sheffield/Bristol

The serological results from all the Sheffield/Bristol groups are shown in Table 2, and Graph 2. The results for individual patients can be found in Appendix 3 .

Table 2. Serology results (IgG and IgM) for Sheffield/Bristol.

Patient Group	Prevalence of anti-chlamydial IgG	Prevalence of anti-chlamydial IgM
Antenatal controls mean age= 27yr SD= 5.3 age range= 18-42yr	14% (4/28)	7% (2/28)
Study controls mean age= 40yr SD= 6.2 age range= 33-57yr	15% (3/20)	10% (2/20)
Ectopic pregnancy (EP) mean age= 30.5yr SD= 5.7 age range= 24-40yr	67% (6/9)	22% (2/9)
Tubal factor infertility (TFI) mean age= 30yr SD= 3.7 age range= 24-36yr	75% (9/12)	8% (1/12)

Study control group vs. EP IgG	P<0.05	significant difference
Study control group vs. EP IgM	P>0.1	no significant difference
Study control group vs. TFI IgG	P<0.05	significant difference
Study control group vs. TFI IgM	P>0.1	no significant difference

Of the twenty study controls anti-chlamydial IgG was detected in three patients, and IgM in two. For the twenty eight antenatal controls, anti-chlamydial IgG was found in four patients, and IgM in two. There was no significant difference found between the two control groups in the detection of antibodies against *C. trachomatis* (IgG P>0.5; IgM P>0.5).

2.5.2 Serology results for Trinidad

The serological results from all the Trinidad groups are shown in Table 3, and Graph 3.

The results for individual patients can be found in Appendix 4.

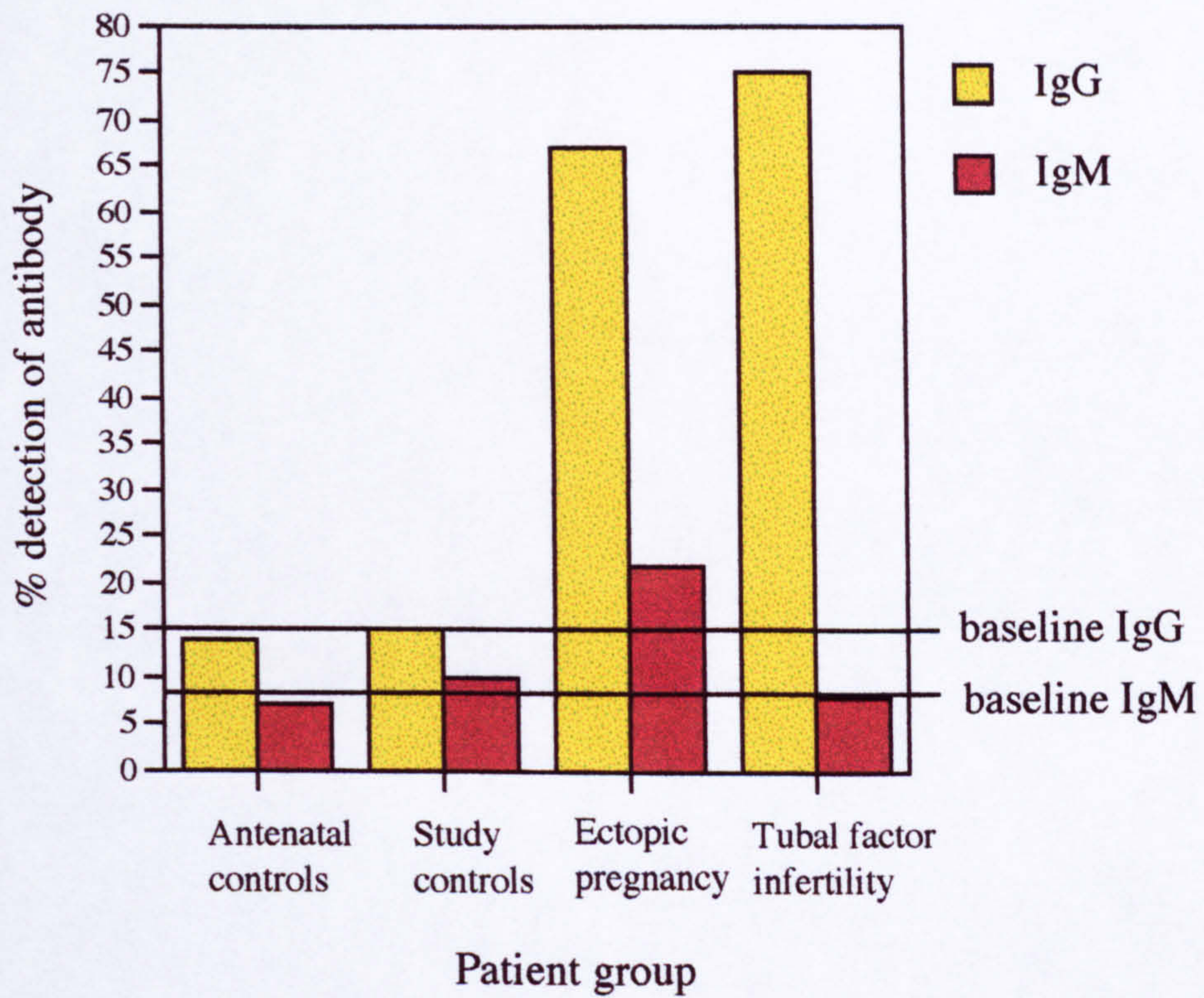
Table 3. Serology results (IgG and IgM) for Trinidad (Mount Hope and Port of Spain).

Patient Group	Prevalence of anti-chlamydial IgG	Prevalence of anti-chlamydial IgM
Antenatal controls mean age= 25.3yr SD= 6.5 age range= 15-43yr	23% (15/64)	8% (5/64)
Study controls mean age= 31.7yr SD= 5.3 age range= 22-44yr	36% (10/28)	11% (3/28)
Ectopic pregnancy (EP) mean age= 30yr SD= 6.3 age range= 16-40yr	62% (13/21)	38% (8/21)
Miscarriage (Msc) mean age= 33yr SD= 1.0 age range= 24-41yr	39% (7/18)	11% (2/18)
Tubal factor infertility (TFI)	100% (2/2)	0% (0/2)

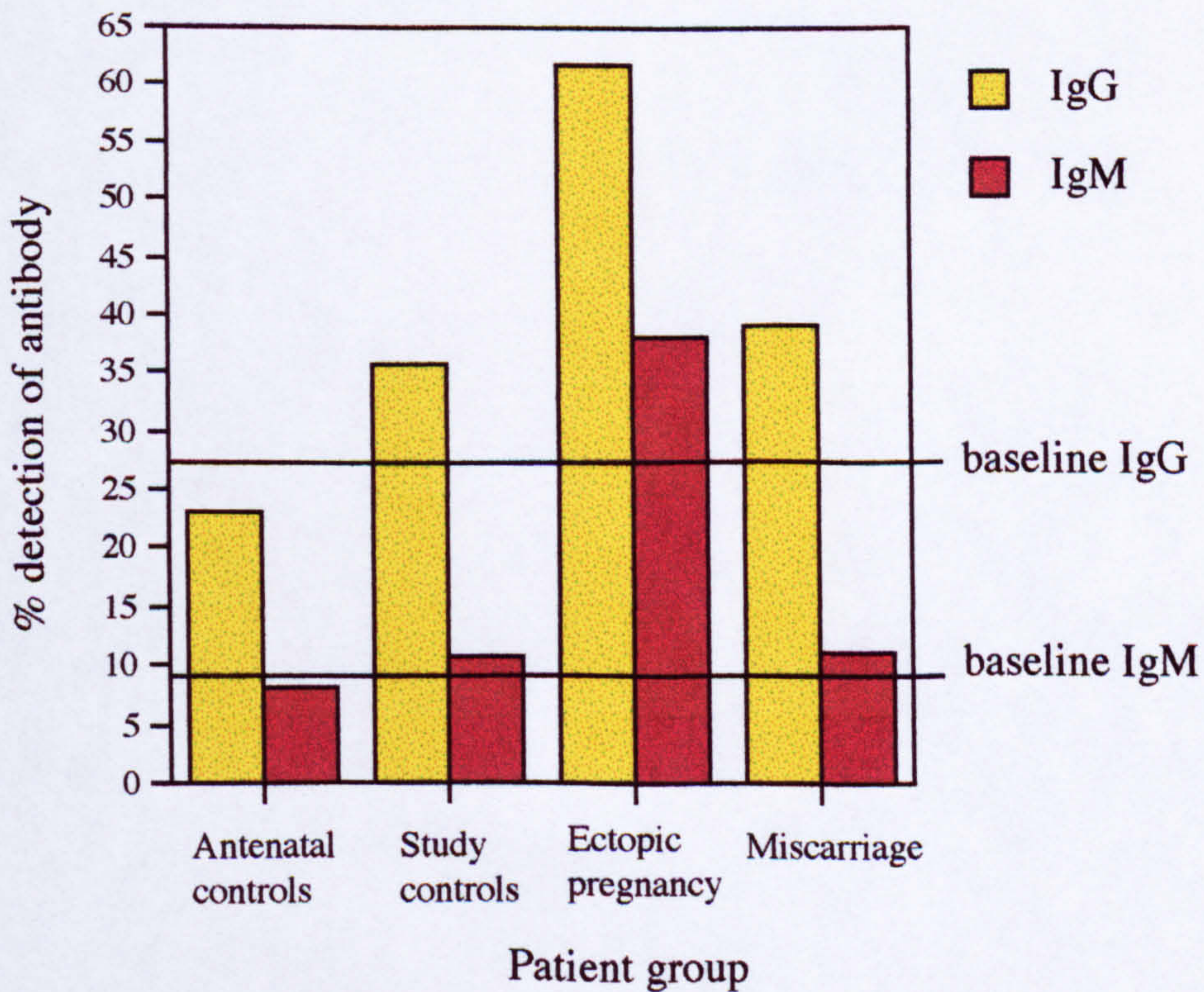
Study controls vs. EP IgG	P>0.1	no difference
Study controls vs. EP IgM	P>0.05	no difference
Study controls vs. Msc IgG	P>0.5	no significant difference
Study controls vs. Msc IgM	P>0.5	no significant difference

Of the twenty eight study controls anti-chlamydial IgG was detected in ten patients, and IgM in three. Of the sixty four antenatal controls anti-*C. trachomatis* IgG was found in fifteen patients, and IgM in five. No significant difference in the detection of antibodies against *C. trachomatis* was found in the two control groups (IgG P>0.1; IgM P>0.5).

Graph 2. Percentage detection of IgG and IgM in Sheffield/Bristol by enzyme-linked immunoassay (with sodium periodate pre-treatment).



Graph 3. Percentage detection of IgG and IgM in Trinidad by enzyme-linked immunoassay (with sodium periodate pre-treatment).



2.5.3 Validation of ELISA results

All of the Sheffield/Bristol serum samples were sent to the lab of Dr. Owen Caul (Bristol PHLS Laboratory, UK) for an independent assessment of the IgG ELISA results. The samples were re-coded, and tested by both inclusion immunofluorescence (IF) and the complement fixation test (CFT) to determine the presence of anti-chlamydial IgG.

Sixty serum samples were sent to be tested (Appendix 5), with some samples represented more than once. Two serum samples were found to be negative by ELISA (IgG) and positive by IF/CFT; four samples were positive by ELISA (IgG) and negative by IF/CFT (two of these samples were from the same patient); and one sample which was tested in duplicate was both positive and negative by IF/CFT.

All the samples with discrepant serological results were re-tested by ELISA for the presence of anti-chlamydial IgG, all but one of the samples gave the same results as when previously tested by ELISA. Of the re-tested samples, two samples previously ELISA positive were only weakly positive, but IF/CFT negative. It may be that these samples were in fact weak positives missed by IF/CFT, or that defrosting/freezing had facilitated the 'integrity' of the serum ultimately giving discrepant IF/CFT serological results. The ELISA negative, but IF/CFT positive samples may be a reflection of the non-specific nature of IF/CFT; and the ELISA positive, IF/CFT negative samples may be due to 'sticky sera' giving a false positive ELISA reading. The percentage correlation between ELISA and IF/CFT results was calculated to be 85%.

2.6 *In vitro* fertilization and anti-chlamydial IgG

2.6.1 Definition of IVF groups

The purpose of this retrospective study was to investigate the relationship between IgG antibodies against *C. trachomatis* and poor ovarian response to gonadotropin stimulation prior to *in vitro* fertilization (IVF) (Keay *et al.*, 1998). Although there is no standard definition of poor response to gonadotropin stimulation, it may be defined as the failure to develop sufficient mature follicles to proceed to oocyte retrieval after ovarian stimulation (Keay *et al.*, 1997).

Two IVF groups were studied, Group A consisted of serum samples previously collected from patients (n=97) on the University of Southampton IVF programme between March 1992 and June 1993; and Group B comprised of serum samples (n=145) from the IVF programme at the University of Bristol, collected between January 1993 and December 1994.

For Group A, pituitary down-regulation was achieved using nasal buserelin (200µg) three times daily, commenced in the luteal phase of the menstrual cycle prior to the start of IVF. From day four of the ensuing menses four ampoules of human gonadotropin (hMG) were administered daily (each ampoule containing 75IU of follicle stimulating hormone [FSH] and 75IU of luteinizing hormone [LH] [Pergonal, Serono, UK]). Blood samples were taken on days 4, 7 and 9 of the IVF cycle, the serum was separated for oestradiol assay and the remainder was stored at -20°C.

For Group B, the stimulation protocol for IVF has been previously described by Hull *et al.* (1992). Briefly, suppression of pituitary gonadotropin with buserelin was started mid-luteal phase of the preceding ovarian cycle. After 10-14 days of buserelin treatment, ovarian stimulation was initiated by subcutaneous injection of 150IU purified FSH (Metrodin HP Serono, UK). Ovarian follicular development was monitored by vaginal ultrasonography and the measurement of serum oestradiol. Serum was obtained

during the stimulation attempt and was separated for oestradiol assay, the remaining serum was stored at -20°C.

Both groups were further sub-divided into poor, and good responders of gonadotropin stimulation. In Group A, patients were classified as poor responders if fewer than four follicles developed after six days of hMG stimulation (Jenkins *et al.*, 1991). By this definition 49 patients were identified as poor responders. Each poor responder was age matched (within two years) to 48 patients receiving IVF treatment exhibiting a good response to the standard regime (ie. sufficient to reach oocyte retrieval). For Group B, poor responders were classified as patients who failed to reach oocyte retrieval despite 300IU FSH daily (n=45), and good responders as those who reached oocyte retrieval (three or more follicles) on no more than 150IU FSH daily (n=100).

Control sera from non-IVF patients were also tested, these included sera from 28 pregnant patients, and from 53 non-pregnant females taken in the course of routine medical management.

2.6.2 IgG ELISA method

The ELISA used has been previously described (p.65), in summary each serum sample was tested in triplicate at a dilution of 1:200 in PBS against 20µg/ml LGV1 antigen and 20µg/ml of negative McCoy cell antigen. The presence of anti-chlamydial IgG was determined using a goat anti-human IgG HRP conjugate diluted 1:4000 in serum diluent. The determination of the serological status of each test sample was achieved using the appropriate controls as previously described by IgG ELISA and IgG MIF (p.68).

2.6.3 Statistical analysis

The Chi squared (χ^2) test and Student t test were used for comparisons between groups where appropriate, using the statistical package ARCUS Protstat (Medical Computers, Aughton, UK). A P value of <0.05 was considered to be statistically significant.

2.7 IVF ELISA results

There were no significant differences in age between the good and poor responders in both Group A and Group B (Table 4). The incidence of detectable levels of anti-chlamydial IgG was significantly higher in all the IVF patients (Group A plus Group B) when compared to the control patients ($P < 0.0001$). When the response to gonadotropin stimulation was considered, both poor and good responders in both groups had significantly higher detection rates of IgG antibodies against *C. trachomatis* than the combined control group (Table 4).

A significantly higher incidence of serum IgG against *C. trachomatis* was observed in the poor responders in comparison to the age matched good responders in Group A ($P < 0.05$). A similar association was seen in Group B between poor and good responders (Table 5, Graph 4), but it was found not to be statistically significant. Combining the results for both groups of poor responders and good responders, the incidence of anti-chlamydial IgG was significantly higher in those patients that responded poorly to gonadotropin stimulation than those who responded well ($P < 0.05$).

The patients in Group A were subdivided on the basis of the infertility diagnosis (Table 6) to determine whether those patients with tubal infertility had a higher incidence of anti-chlamydial IgG than those with infertility of a non-tubal nature. Some patients had more than one infertility diagnosis and were included in all the appropriate infertility diagnosis groups. The corresponding information for Group B was unavailable.

Table 4. Comparison of the incidence of serum IgG against *C. trachomatis* between *in vitro* fertilization (IVF) patients compared to controls.

	Percentage of patients seropositive for anti-chlamydial IgG
Poor responders	45% (42/94)
Good responders	30% (45/148)
All IVF patients	36% (87/242)
Pregnant controls	14% (4/28)
Non-STD female controls	11% (6/53)
All controls	13% (10/81)

All IVF poor responders vs. all controls
 All IVF good responders vs. all controls
 All IVF vs. all controls

P<0.0001
 P<0.0005
 P<0.0001

significant difference
 significant difference
 significant difference

Group A	poor responders	mean age= 34yr	SD 3.9
	good responder	mean age= 33yr	SD 3.8
Group B	poor responders	mean age= 39yr	SD 4.8
	good responder	mean age= 37yr	SD 4.1

Graph 4. The detection of IgG against *C. trachomatis* by enzyme-linked immunoassay (sodium periodate antigen pre-treatment) in poor and good responders in two *in vitro* fertilization (IVF) populations (Groups A and B).

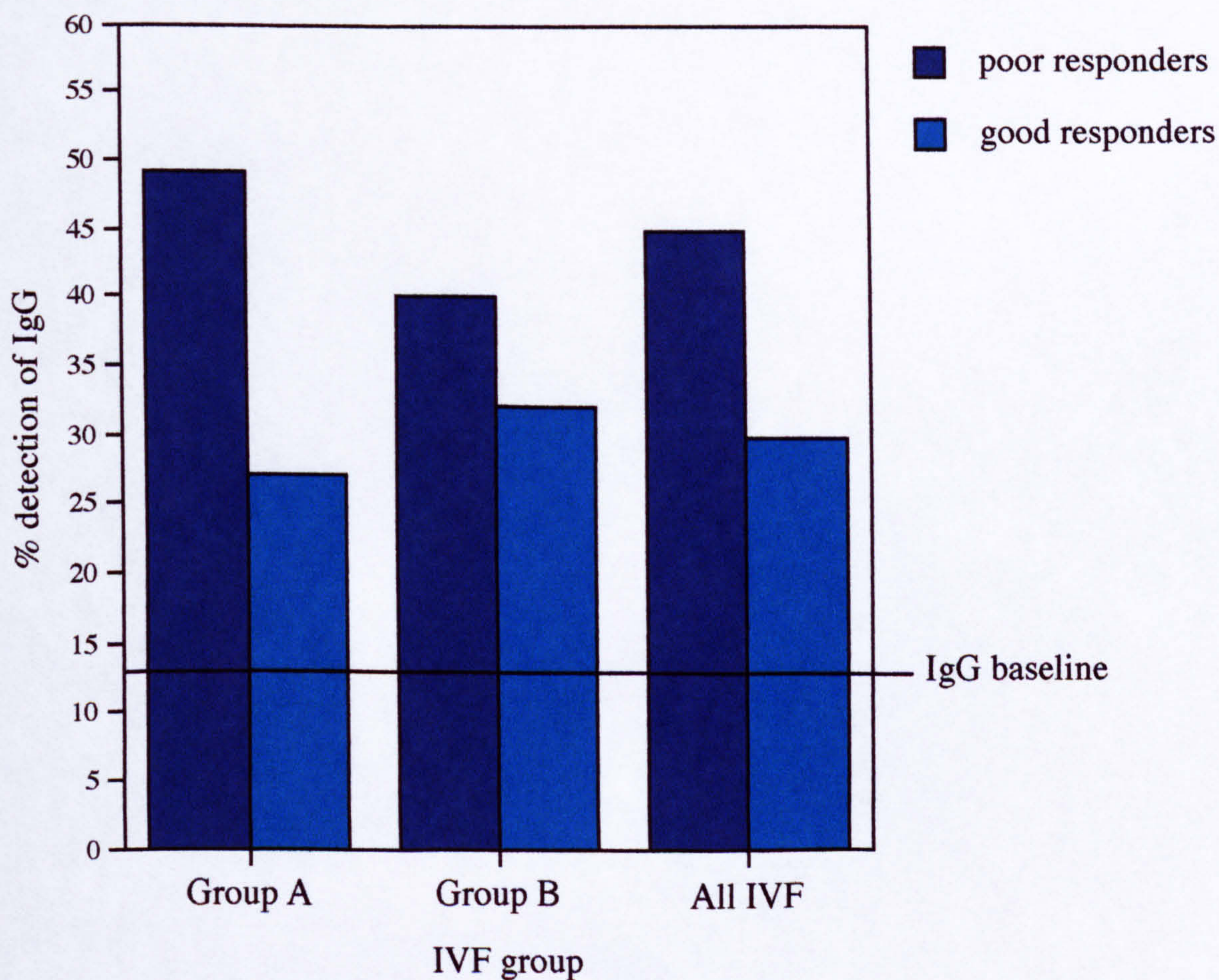


Table 5. A comparison of the incidence of significant levels of anti-*C. trachomatis* IgG in *in vitro* fertilization (IVF) patients with good and poor response to ovarian stimulation.

Seropositive for anti-chlamydial IgG			
	Group A (n=97)	Group B (n=145)	Groups A+B (n=242)
Poor responders	49% (24/49)	40% (18/45)	45% (42/94)
Good responders	27% (13/48)	32% (32/100)	30% (45/148)

Group A

poor responders vs. good responders

P<0.05

significant difference

Group A+B

poor responders vs. good responders

P<0.05

significant difference

Table 6. Comparison of infertility diagnosis between good and poor responders to ovarian stimulation, and prevalence of anti-chlamydial IgG in Group A.

	Group A diagnosis of infertility		Group A prevalence of anti- chlamydial IgG	
	poor response (n=49)	good response (n=48)	poor response (n=49)	good response (n=48)
Tubal	47% (23/49)	33% (16/48)	52% (12/23)	44% (7/16)
Endometriosis	14% (7/49)	8% (4/48)	14% (1/7)	50% (2/4)
Male factor	22% (11/49)	35% (17/48)	55% (6/11)	18% (3/17)
Unexplained	16% (8/49)	25% (12/48)	38% (3/8)	8% (1/12)
Other	8% (4/49)	12% (6/48)	50% (2/4)	33% (2/6)

Tubal infertility diagnosis

poor responders vs. good responders

P>0.5

no significant difference

Male factor infertility diagnosis

poor responders vs. good responders

P<0.05

significant difference

2.8 Conclusion

2.8.1 Sheffield/Bristol and Trinidad study

The reported prevalence of *C. trachomatis* in women from Bristol in 1980 in a GUM clinical setting was 19% by culture (Richmond *et al.*, 1980). In other areas of the UK figures ranged from 3.6-9.1 % of women attending gynaecology/antenatal or family planning clinics (Fish *et al.*, 1989; Hopwood *et al.*, 1990). When considering only IgG the prevalence of antibodies against *C. trachomatis* in the Sheffield/Bristol control groups is comparable to those previously reported in the UK.

In Trinidad the prevalence of anti-chlamydial IgG was higher in the study control group, when compared to the antenatal group ($P>0.1$). Although this was not statistically significant it may be accounted for by differences in the actual patient populations sampled. The IgM results ($P>0.5$) for the two groups were found to be similar to each other, and the UK control groups.

The results for the Trinidad control groups raise some important questions about the prevalence of chlamydial disease in the West Indies. The apparent difference in the detection of IgG may simply be age related (Chout *et al.*, 1995), the mean age of the study controls being approximately six years greater than the antenatal patients. A recent study by Dowe *et al.* (1998) showed that the incidence of *C. trachomatis* infection of the endocervix of pregnant Jamaican women by DIF was 16%. This value is lower than that seen in the Trinidad antenatal group, but the Dowe *et al.* results reflect the actual detection of *C. trachomatis*, and not the prevalence of past/present or persistent chlamydial infection as determined by the detection of circulating IgG or IgM. In Martinique the reported prevalence of *C. trachomatis* by culture of the cervix and urethra in pregnant women was reported to be 27% (Chout *et al.*, 1995). This value is comparable to the IgG serology data for the Trinidad control groups.

Comparisons of the Trinidad and Sheffield/Bristol study controls groups and antenatal

controls reveal a very different picture for the prevalence of *C. trachomatis*. It is an important consideration when comparing the UK control groups with the Trinidad control groups to take into account factors such as ethnicity and its impact on differing risk factors for EP (eg. vaginal douching [Chow *et al.*, 1990; Kendrick *et al.*, 1997]) and STD (Coste *et al.*, 1994a). In a recent study by Scholes *et al.* (1998) a relationship between vaginal douching and the risk of cervical chlamydial infection was found. The results of this study support the hypothesis that douching predisposes to the acquisition of cervical infection by *C. trachomatis*, which could further lead to the sequelae of upper genital tract chlamydial infection.

Few studies have looked at the possible role of *C. trachomatis* and miscarriage, and those that have been conducted have reported conflicting results. Witkin *et al.* (1992) was the first to report a link between *C. trachomatis* and spontaneous abortion, showing a high titre of anti-chlamydial IgG in those women who miscarried.

A recent study by Witkin *et al.* (1998) evaluated the relationship between immunity to specific regions of *C. trachomatis* HSP60 (CHSP60) and infertility. The results showed that humoral immunity to a particular CHSP60 epitope was associated with sensitization to human HSP60 and with a history of two or more spontaneous abortions, and may be an immunological marker for spontaneous abortion.

In the Trinidad miscarriage group anti-chlamydial IgG was detected in 39% of the patients, and IgM in 11%. Although not statistically significant ($P>0.5$) when compared to the Trinidad study control group, there was still a 12% difference in the detection of IgG. IgM levels in the miscarriage group were approximately the same as the combined control group. The results of the Trinidad miscarriage group are similar to a recent study in Sweden (Osser *et al.*, 1996) where the frequency of IgG antibodies against *C. trachomatis* was 39% in women surgically treated for spontaneous pregnancy loss, compared to 33% of pregnant controls. This study, like the Trinidad miscarriage data, found no association serologically between evidence of chlamydial infection and miscarriage.

There have been many studies over the last two decades which have looked at the association between antibodies against *C. trachomatis*, and ectopic pregnancy (EP) or tubal factor infertility (TFI). The significance of *C. trachomatis* in tubal infertility was originally supported by the work of Jones *et al.* (1982) and Moore *et al.* (1982), who both showed a correlation between TFI and the presence of serum anti-chlamydial antibodies. Gump *et al.* (1983) linked chlamydial salpingitis to EP through serological evidence, patients with elevated anti-chlamydial IgG titres found during an initial infertility evaluation had an increased incidence of EP.

Within the Sheffield/Bristol ectopic pregnancy (EP) group, 67% of the patients had detectable IgG, and 22% had detectable IgM. It is interesting to note that when individual EP patients were considered, all patients with IgM also had anti-chlamydial IgG. The prevalence of anti-chlamydial IgG was statistically significant ($P < 0.05$) when compared to the UK control group, but the detection of IgM although double that of the study control group was not statistically significant ($P > 0.1$).

In the Trinidad EP group 62% of the patients had detectable anti-chlamydial IgG, and 38% had detectable IgM. The prevalence of neither IgG and IgM were statistically significant when compared to the Trinidad study control group (IgG $P > 0.05$; IgM $P > 0.1$). The increased detection of IgM in the Trinidad EP group, compared to the UK EP group, may be explained by the comparatively larger number of patients tested from Trinidad. Interestingly at a significance level of $P < 0.1$ the Trinidad IgM results would become significant warranting further investigation.

Chrysostomou *et al.* (1992) reported similar findings in a study conducted in Greece, where a statistically significant detection of anti-chlamydial IgG by IF was found in women with EP, when compared to pregnant controls (75% vs. 46%, $P < 0.001$). In this Greek study none of the women in the EP group had detectable IgM, it was suggested that this was due to the conventional IgM to IgG switch in response to chlamydial infection. This study concluded that there was a positive relationship between a prior *C. trachomatis* infection and the risk of tubal pregnancy.

Sziller *et al.* (1998) evaluated the serological responses to 13 synthetic peptides corresponding to major epitopes of the chlamydial HSP60, and against an elementary body preparation of LGV2 in 67 women with EP and 45 pregnant controls. This study found that EP patients were more likely to have anti-chlamydial IgG than the pregnant controls. In the EP group, when antibodies against both *C. trachomatis* and the HSP60 epitopes were detected there was an increased incidence of salpingitis, pelvic adhesions and/or a history of PID compared to EP patients negative for antibodies against HSP60 and LGV2. Sziller *et al.* suggested that a prior or prolonged *C. trachomatis* infection may be necessary for the induction of HSP60-related immunopathology that could result in tubal damage predisposing to EP.

In the Sheffield/Bristol TFI group the detection of IgG was higher than that of the UK study control group ($P < 0.05$), and the Sheffield/Bristol EP group. What is interesting is that the IgM levels (8%) were the same as the UK study control group (8%), unlike the UK EP group (22%) and the Trinidad EP group (38%). It may be that the increased prevalence of anti-chlamydial IgG is an indicator of damage/loss of tubal function resulting in TFI from a previous/persistent chlamydial infection; whereas chlamydial associated EP may be the result of damage and ongoing infection suggested by detectable levels of IgM. Brade *et al.* (1994) suggested that IgM may be an indicator of acute infection, this was further supported by the high frequency of anti-chlamydial IgM in female patients who were found to be *C. trachomatis* culture positive in the genital tract.

Claman *et al.* (1997) using MIF for IgG and IgM found no significant difference between the prevalence of anti-chlamydial antibodies in women with infertility of tubal, and non-tubal origin (63% vs. 46%), whereas Brunham *et al.* (1985) did find a significant difference (72% vs. 9%, $P < 0.0001$). Patton *et al.* (1994b) also detected anti-chlamydial IgG in 71% of TFI patients studied. In our study we also saw a significant difference in the detection of anti-chlamydial IgG in the TFI group when compared to the control group. We did not find a significant difference in the detection of anti-chlamydial IgM, neither did Campbell *et al.* (1993) in a study of sixteen women with distal tubal occlusion.

A possible explanation of our results may be since TFI is supposedly the result of damage caused by a past infection, circulating IgM levels may have declined below the positive threshold value for our ELISA. It is also possible that in patients with evidence of having/or having had a *C. trachomatis* infection, and who have no detectable anti-chlamydial antibodies, an inadequate antibody response was originally mounted during infection resulting in tubal damage due to non-clearance/persistence of the organism. Or it may simply infer that the tubal pathology associated with TFI was the result of repeated, past chlamydial infection.

From the results of both the UK, and Trinidad studies it can be seen that *C. trachomatis* is an important aetiological agent in the development of both EP and TFI. The question remains whether chlamydial serology can be used as a screening method for conditions such as asymptomatic PID (Chernesky *et al.*, 1998) and salpingitis, that could predispose to EP or TFI? Many studies have supported the use of chlamydial serology in the diagnosis of tubal pathology, suggesting that chlamydial antibody titres are comparable to the use of hysterosalpingography in the diagnosis of tubal occlusion (Dabekausen *et al.*, 1994; Henry-Suchet *et al.*, 1994; Tanikaw *et al.*, 1996; Mol *et al.*, 1997). However although serology is a 'non-invasive' technique the limitations of the procedure have to be recognized, for example circulating levels of antibody may have declined over time generating false negative results.

The serology data from Sheffield/Bristol and Trinidad suggest that the presence of IgG against *C. trachomatis* appears to be a good indicator of tubal pathology with respect to EP, and TFI. As for the Trinidad miscarriage patients, neither IgG nor IgM was suggestive of chlamydial involvement in the aetiology of miscarriage.

2.8.2 IVF study

There are many factors that affect embryo implantation rates and therefore the success of IVF, one important factor that is not fully understood is the intrauterine environment and

the mechanism by which hydrosalpinx alters the uterine receptive capacity. It has been proposed that connections between the hydrosalpinx and the uterine cavity allow the direct flow of hydrosalpingeal fluid into the uterus exposing the endometrium and embryo to a potentially 'toxic' fluid which has been postulated to contain microorganisms, debris, lymphocytes, cytokines, prostaglandins and leukotrienes which could all interfere with normal endometrial function or be embryotoxic (Strandell *et al.*, 1994; Anderson *et al.*, 1994; Schenk *et al.*, 1996). It has also been suggested that a reflux of hydrosalpingeal fluid into the uterus may also alter integrin expression potentially affecting endometrial receptivity (Lessey *et al.*, 1994). It has been shown that the surgical removal of hydrosalpinx can improve the IVF success rate (Nackley *et al.*, 1998), suggesting that the presence of hydrosalpinx may be a major factor in embryo rejection.

Hydrosalpinx can form as a result of *C. trachomatis* infection. Previous infection with *C. trachomatis* as determined by the presence of anti-chlamydial IgG is more prevalent in patients with tubal disease than in women with infertility of non-tubal origin (Rowland *et al.*, 1985; Sharara *et al.*, 1996). Rowland *et al.* (1985) reported that a past *C. trachomatis* infection halved the success rate of IVF (30% vs. 65.5%), although Driscoll *et al.* (1991) showed that a prior chlamydial infection had no effect on IVF outcome.

The question of whether elevated *C. trachomatis* antibodies adversely effect IVF outcome has been the subject of many studies. Rowland *et al.* (1985) found a significantly lower pregnancy rate in patients with antibodies to *C. trachomatis* whereas Torode *et al.* (1987) found no significant difference.

Licciardi *et al.* (1992) found an association between previous exposure to *C. trachomatis* and spontaneous abortion after IVF. The study looked at chlamydial endocervical culture and the presence of antibodies to *C. trachomatis* in 145 women undergoing IVF. None of the women were found to have a positive endocervical culture, however 48% of those with tubal disease had detectable anti-chlamydial IgG compared to 18% with infertility of a non-chlamydial aetiology. The rate of spontaneous abortion within the IVF population was 20%, of those 69% had IgG antibodies against *C. trachomatis*, compared to 24% of

the women who achieved a successful pregnancy after IVF.

A similar study by Neuer *et al.* (1997) looked at the prevalence of antibodies against *C. trachomatis* (IgG and IgA) with respect to chlamydial MOMP, and recombinant chlamydial LPS (rLPS) in paired follicular fluid and sera of 149 women undergoing IVF. The study also investigated the expression of human HSP60 in follicular fluid. IgA antibodies to both MOMP, and rLPS in follicular fluid were associated with failure to become pregnant after embryo transfer. Anti-chlamydial IgG in sera and follicular fluid, and anti-chlamydial IgA in sera were found to be unrelated to IVF outcome. The expression of human HSP60 in follicular fluid correlated with the presence of both anti-MOMP and anti-rLPS in follicular fluid. The association between anti-chlamydial IgA against two chlamydial antigens in follicular fluid, and the expression of human HSP60 may support the possibility that a persistent upper genital tract chlamydial infection could contribute to IVF failure.

Witkin *et al.* (1994) studied women with previous *C. trachomatis* infection diagnosed by the presence of IgA in cervical mucus by ELISA, and compared their IVF outcomes. Increased levels of chlamydial HSP60 (CHSP60) IgA were observed. CHSP60 is thought to induce a local immune response that may lead to an inflammatory reaction impairing implantation and facilitating immune rejection after embryo transfer, but the actual mechanism remains unknown. Conversely, Claman *et al.* (1996) showed that IVF patients with tubal infertility and with anti-CHSP60 antibodies had a significantly higher pregnancy rate when compared to TFI patients who were seronegative for CHSP60 (42% vs. 7%).

An impaired ovarian response to gonadotropin stimulation signifies a reduced likelihood of pregnancy following IVF, possibly through a reduction in the quality and quantity of oocyte production (Keay *et al.*, 1997). The capacity of the ovary to respond to such stimulation is an indirect measure of ovarian reserve. The results of our study with the combined results from two IVF centres (Group A and Group B) showed a significant number of poor responders to IVF gonadotropin stimulation have had previous exposure

to *C. trachomatis*. This suggests that prior genital tract infection with *Chlamydia* may affect the ovary in an as yet undefined way.

When infertility diagnosis was considered, a greater proportion of the poor responders classified as having male factor infertility also had detectable anti-chlamydial IgG ($P < 0.05$). It would have been interesting to have tested the male partners of these patients for the presence of anti-chlamydial IgG and/or anti-sperm antibodies. There is evidence that *C. trachomatis* infection can cause morphological alterations in the epididymis decreasing sperm maturation and sperm motility (Villegas *et al.*, 1991). Many studies have also shown an association between the presence of *C. trachomatis* (as demonstrated by either the detection of chlamydial DNA, or the presence of anti-chlamydial antibodies) and the production in the male of anti-sperm antibodies (Witkin *et al.*, 1993; Munoz *et al.*, 1995; Witkin *et al.*, 1995; Weidner *et al.*, 1996; Munoz *et al.*; 1996).

Witkin *et al.* (1993b) also showed an association between the detection of *C. trachomatis* in semen, and the presence of circulating anti-sperm antibodies in female partners of couples diagnosed with unexplained infertility. It was suggested that *C. trachomatis* may provoke an immune response to sperm in women, and that the infertility in these couples may have resulted from a direct inflammatory response in the cervix or endometrium to repeated chlamydial infection from their partner, or the ability of *C. trachomatis* to evoke an immune response to sperm.

It is possible that the ELISA system developed in this study simply detected more patients with severe adnexal adhesions and post-infective pelvic damage due to *C. trachomatis* (Tanikawa *et al.*, 1996). Patients with tubal infertility produce fewer oocytes, have a lower peak oestradiol and require more gonadotropin when compared to patients with prolonged unexplained infertility. This demonstrates a subtle but significant impaired ovarian response in women with tubal infertility (FIVNAT, 1992). If the results of our study simply indicate that the ELISA system detected more intense pelvic damage in patients with severe adnexal damage, the underlying mechanism by which the ovary has

been compromised, its relationship to gonadotropin response and hence the poor prognosis for IVF is unknown. It may be that ovarian reserve is influenced by ovarian infection with a microorganism such as *C. trachomatis*, or it may be possible that ovarian function is indirectly affected by the formation of post-infection adhesions.

There is indirect evidence that has linked pelvic infection to adhesion formation and reduced ovarian response (Madehevan *et al.*, 1985; Molloy *et al.*, 1987; Bowman *et al.*, 1993; Czemichy, 1996), which has been supported by experiments in rabbits (McComb and Delbeke, 1984). Hamilton *et al.* (1986) found apparent altered ovarian function in spontaneous cycles in a small study of patients with pelvic adhesions following PID. These studies suggest a link to the sequelae of PID and a reduction in the capacity of the ovaries to respond to gonadotropin stimulation.

In their study of the effect of hydrosalpinx and IVF, Sharara *et al.* (1996) tested each patient for *C. trachomatis* cervical infection, and determined the levels of anti-chlamydial IgG. The study was designed to evaluate the role of a previous chlamydial infection and hydrosalpinx in the success of IVF. Patients with elevated levels of IgG antibodies to *C. trachomatis* were treated with antibiotics prior to IVF. Of the thirty-four women who were seropositive for *C. trachomatis*, and received treatment, 21% became pregnant after IVF compared to 12% of the women who did not receive treatment (found not to be statistically significant). This study also found that the pre-treatment with antibiotics of those patients with hydrosalpinx and positive chlamydial serology had no effect on IVF outcome, suggesting that a current chlamydial infection was not directly linked to IVF success.

The detection of IgG against *C. trachomatis* in the Southampton and Bristol IVF populations suggests that a chlamydial infection can, in some as yet defined way, influence the success of IVF. Witkin *et al.* (1995) using PCR detected *C. trachomatis* DNA in the cervixes of culture negative women undergoing IVF which correlated with adverse IVF outcome. This study suggested that an undetected chlamydial infection (silent/persistent) may be responsible for IVF implantation failure or spontaneous

abortion. It may be useful in the future to include chlamydial serology in the initial routine infertility work-up prior to IVF in order to help identify those patients who may respond 'poorly' to gonadotropin treatment. In this way, different treatment regimes, such as increased gonadotropin stimulation or hydrosalpinx investigation/removal, could be included in the IVF treatment.

Chapter 3.0
Polymerase chain reaction (PCR)

3.1 Aims of chapter

The work described in this chapter had two distinct aims. The first aim was to utilise the polymerase chain reaction (PCR) to determine the prevalence of *C. trachomatis* DNA in two study populations (Sheffield/Bristol and Trinidad). Patients from both study populations included women presenting with ectopic pregnancy (EP), miscarriage, tubal factor infertility (TFI), and controls undergoing either hysterectomy, or tubal sterilization. Three tissue samples from each patient (endometrial, ovarian and Fallopian tube) were examined for the presence of *C. trachomatis* DNA.

The second aim of this chapter was to investigate the distribution of polymorphisms in the human TNF gene in a pilot. Tumor necrosis factor alpha (TNF- α) has been implicated in the pathogenesis of chlamydial disease. TNF- α , and TNF- β gene polymorphisms have been previously linked with functional differences of TNF- α levels, so the possible link between regulatory polymorphisms effecting TNF- α production and the risk of the inflammatory sequelae of *C. trachomatis* infection were investigated. TNF- α -308A, and TNF- β TNFB*1 *Nco*I polymorphisms were studied by PCR and restriction enzyme digestion of the appropriate PCR product in all patient groups, from both Sheffield/Bristol, and Trinidad.

3.2 Introduction

Chlamydia trachomatis is a major cause of infection of the female genital tract often resulting in pelvic inflammatory disease (PID), tubal occlusion which can predispose to ectopic pregnancy, and infertility. One of the major problems associated with genital chlamydial infection, especially PID, is that in many cases the infection is asymptomatic or silent. Cates *et al.* (1993) showed that of 283 infertile women with tubal occlusion, 84% had never had any notable symptoms indicative of upper genital tract infection. Therefore detection of chlamydial infection is of paramount importance in the prevention of the sequelae of chlamydial infection.

Griffais and Thibon (1989) were the first to describe a PCR for the detection of *C. trachomatis* DNA in clinical samples using primers aimed at the endogenous 7.5kb plasmid. In the same year Pollard *et al.* (1989), and Duthil *et al.* (1989) also published primers for the detection of *C. trachomatis*, the former described primers aimed at the chlamydial 16S rRNA gene and the latter targeted the MOMP gene. Claas *et al.* (1990) utilised PCR with primers aimed at both the *C. trachomatis* plasmid, and 16S rRNA gene, to compare culture and PCR with product confirmation by oligonucleotide hybridization. The plasmid primers were found to be specific for *C. trachomatis*, and more sensitive than culture.

The *C. trachomatis* 7.5kb plasmid has been estimated to be present in upto 10 copies per elementary body (Palmer and Falkow, 1986), and as such provides a multiple copy template for PCR increasing sensitivity, compared to for example chlamydial MOMP (single copy gene [Stephens *et al.*, 1985]). Mahony *et al.* (1992) described a *C. trachomatis* plasmid based PCR using a second set of confirmatory primers which were shown to improve specificity and increased sensitivity. This nested PCR, which used a second set of primers aimed at an internal region of the primary PCR product produced by the first set of primers. Although nested PCR can improve sensitivity, contamination problems may arise with the generation of false-positive results.

TNF- α may play a central role in the disease pathogenesis associated with *C. trachomatis*

infection. Conway *et al.* (1997) showed that scarring trachoma was associated with polymorphism in the TNF- α gene promoter, and with elevated TNF- α levels in tear fluid. Persistent or repeated infection of the epithelia with *C. trachomatis* can trigger a chronic inflammatory response causing tissue damage (Taylor *et al.*, 1987), leading to fibrosis and scar formation. In trachoma scarring of the upper eyelid causes inversion and trichiasis which can lead to blindness, and in genital chlamydial infection scarring of the Fallopian tube can cause tubal occlusion and predispose to tubal factor infertility (TFI) or ectopic pregnancy (EP).

The family of tumour necrosis factors comprises three members; TNF- α ; TNF- β (also known as lymphotoxin a, LT- α); and LT- β . The genes encoding TNF- α and TNF- β are tandemly arranged within a 7kb region of the major histocompatibility complex (MHC) (Nedwin *et al.*, 1985; Browning *et al.*, 1993). Both cytokines encoded by the TNF genes are potent immunomodulators, and are essential mediators of the inflammatory response (Beutler *et al.*, 1988; Paul *et al.*, 1988; Fiers *et al.*, 1991; Vilcek *et al.*, 1991). The location of TNF within the MHC region has prompted much speculation about the role of TNF genes in the aetiology of MHC-linked disease, in particular those diseases with an inflammatory or autoimmune component.

Two polymorphisms in the TNF genes have been described, one at position -308 in the promoter region of the TNF- α gene (Wilson *et al.*, 1993) for which allele 2 is associated with higher constitutive and inducible levels of TNF- α (Wilson *et al.*, 1994); the second polymorphism was located in the TNF- β gene at intron 2/exon 3 (Messer *et al.*, 1991).

Both TNF polymorphisms have been associated with both increased, and decreased TNF- α secretion by mononuclear cells, depending on the population studied (Spengler *et al.*, 1992; Abraham *et al.*, 1993; Pociot *et al.*, 1993). The mechanism(s) by which these polymorphisms influence TNF- α secretion is not known, and the effect of these polymorphisms with respect to *C. trachomatis* infection of the genital tract and the development of tubal disease remains to be elucidated.

3.3 Materials

3.3.1 Reagents

Reagents were purchased from Sigma (Dorset, UK) unless otherwise stated.

3.3.2 DNA extraction

Pestle and mortars were soaked in 0.1M HCl overnight before being autoclaved.

Lysis buffer

x 10 KCl PCR buffer (Bioline, London, UK)	10ml
1% Triton X-100 (v/v) (BioRad, Herts, UK)	1ml
500µg/ml proteinase K	0.05g
sterile purified water	89ml

The lysis buffer was stored at -20°C in 1ml aliquots in a DNA-free freezer.

Phenol:chloroform:isoamyl alcohol was purchased as a pre-mixed solution at a ratio of 25:24:1.

Glycogen was obtained from Boehringer Mannheim (Diagnostics and Biochemicals Ltd., East Sussex, UK) at a concentration of 20mg/ml.

Sodium acetate

3M sodium acetate	24.6g
purified water	100ml

The solution was adjusted to pH 4.8, autoclaved and stored at 4°C.

3.3.3 Measurement of DNA concentration

Fluorescent measurement of DNA concentration

Hoechst 33258 dye, for use with the TKO 100 mini-fluorometer (Hoefer Scientific Instruments, San Francisco, USA), was prepared as a stock solution of 1mg/ml in water, and stored in a dark container at 4°C for 6 months.

TNE buffer

100mM Tris-OH	12.1g
10mM EDTA	3.7g
1M NaCl	58.4g
water	1000ml

The TNE buffer was adjusted to pH 7.4, autoclaved and stored at 4°C.

Working dye solution

0.1µg/ml Hoechst 33258 dye	10µl of stock
10xTNE	10ml
water	90ml

The dye solution was pre-filtered through a sterile 0.2µm Millipore filter before use, and was prepared fresh daily.

3.3.4 PCR

OmniGene PCR machine (Hybaid Ltd., Middlesex, UK) with heated lid, was used for all PCR applications.

Sterile aeroguard tips and positive displacement tips were purchased from Alpha Laboratories Ltd. (Hampshire, UK).

0.5 ml PCR tubes were obtained from GeneMate (Kaysville, UT, USA) and were DNase and RNase free. Before use the tubes were autoclaved.

Stock nucleotides containing dATP, dCTP, dGTP, and dTTP were purchased as a polymerisation mix (Bioline). The DNA polymerisation mix contained 10mM of each nucleotide in solution and was stored at -20°C in a designated DNA-free freezer.

Stock nucleotides containing dUTP, dATP, dCTP, and dGTP were purchased as separate solutions from Perkin Elmer (Langen, Germany). Each nucleotide was supplied as a stock solution of 10mM, except dUTP which was supplied as 20mM. A working dNTP solution (1.25mM of each dATP, dCTP, dGTP and 2.5mM of dUTP) was made by adding 125µl of each nucleotide to 500µl of sterile purified water, this was stored at -20°C in a designated DNA-free freezer. In a 50µl PCR reaction mix, 8µl of the stock nucleotides containing dUTP will yield 200µM of dATP, dCTP, dGTP and 400µM of dUTP.

AmpErase™ Uracil N-glycosylase (UNG) was purchased as a 1U/µl solution from Perkin Elmer and kept at -20°C in a designated DNA-free freezer.

Magnesium chloride (MgCl₂) was purchased from Bioline as a 50mM stock solution and stored at -20°C in a designated DNA-free freezer.

x10 PCR reaction buffers NH₄ and KCl were purchased from Bioline. The NH₄ buffer contained 160mM (NH₄)₂SO₄, 670mM Tris-HCl and 0.1% (v/v) Tween-20; the KCl buffer contained 500mM KCl, 100mM Tris-HCl, 15mM MgCl₂ and 1% (v/v) Triton X-100.

BioTaq DNA polymerase was obtained from Bioline at a concentration of 5U/µl. The polymerase was stored -20°C in a designated DNA-free freezer.

3.3.5 PCR primers

PCR primers were purchased from R & D Systems Ltd. at 0.2mM with no further purification, and diluted to 100pmol/µl. Aliquots of 100 µl were stored at -20°C, one

aliquot was stored at 4°C for routine day to day use.

***Chlamydia trachomatis* single plasmid primers (Claas *et al.*, 1990)**

					binding position (nucleotide [nt])
T1	Forward (F)	5'	ggA CAA ATC gTA TCT Cgg	3'	2464-2481nt
T2	Reverse (R)	5'	gAA ACC AAC TCT ACg CTg	3'	2980-2963nt

Oligonucleotide probe for Southern blot analysis (Claas *et al.*, 1990)

					binding position
5'			CgC AgC gCT AgA ggC Cgg TCT ATT TAT gAT	3'	2705-2734nt

The binding positions of primers T1 and T2, and the oligonucleotide probe were based on the published sequence of the *Chlamydia trachomatis* cryptic plasmid pLGV440 (Hatt *et al.*, 1988. Accession number X06707).

***Chlamydia trachomatis* MOMP primers (Lan *et al.*, 1994)**

					binding position
NRO	F	5'	CTC AAC TgT AAC TgC gTA TTT	3'	5632-5653nt
NLO	R	5'	ATg AAA AAA CTC TTg AAA TCg	3'	6759-6739nt
SERO2A	F	5'	TTT CTA gA(T/C) TTC AT(C/T) TTg TT	3'	5696-5715nt
PCTM3	R	5'	TCC TTg CAA gCT CTg CCT gTg ggg AAT CCT	3'	6705-6676nt

The relative binding positions of the MOMP primers were based on the published sequence of the *C. trachomatis* complete genome, section 65 (Stephens *et al.*, 1998. Accession number AE001338).

Human β -globin primers (Saiki *et al.*, 1985; Lan *et al.*, 1995)

					binding position
PC03	F	5'	ACA CAA CTg TgT TCA CTA gC	3'	62150-62169nt

PCO6 R 5' CAT CAg gag Tgg ACA gAT CC 3' 62476-62457nt

The binding positions of the two human β -globin primers were based on the published sequence of the human β -globin region on chromosome 11 (Marotta *et al.*, 1974.

Accession number U01317).

Tumour necrosis factor alpha (TNF α) primers (Cabrera *et al.*, 1995)

						binding position
TNF α 1	F	5'	Agg CAA TAg gTT TTg Agg gCC AT	3'		284-302nt
TNF α 2	R	5'	TCC TCC CTg CTC CgA TTC Cg	3'		390-371nt

The binding position of the TNF α primers were based on the sequence of the human gene for tumor necrosis factor alpha (Nedwin *et al.*, 1985. Accession number X02910).

Tumour necrosis factor beta (TNF β) primers (Messer *et al.*, 1991)

						binding position
TNF β 1	F	5'	CCg TgC TTC gTg CTT Tgg ACT A	3'		129-150nt
TNF β 2	R	5'	AgA gCT ggT ggg ACA TgT CTg	3'		869-848nt

The binding position of the TNF β primers were based on the human tumor necrosis factor beta sequence (accession number M55913) published by Abraham *et al.* (1991).

3.3.6 Gel electrophoresis

Agarose gel electrophoresis

Electrophoresis grade agarose was obtained from Gibco Life Technologies Ltd. (Paisley, UK). Gel solutions were prepared in 0.5xTBE and heated in a microwave.

Ethidium bromide was prepared as a working stock solution of 10mg/ml in water and stored in a dark container at room temperature.

Gel loading dye

0.25% bromophenol blue (BDH Chemicals, Poole,UK)	0.025g
0.25% xylene cyanol (BDH)	0.025g
30% glycerol	3ml
water	10ml

5xTBE buffer

Tris-OH	54g
boric acid	27.5g
EDTA	4.65g
water	1000ml

The buffer was adjusted to pH 8.3, autoclaved and stored at room temperature. A 0.5x TBE solution was prepared for electrophoresis.

Polyacrylamide gel electrophoresis

10% ammonium persulphate

ammonium persulphate	0.5g
purified water	5ml

The solution was prepared fresh each time a polyacrylamide gel was prepared.

Resolving gel

x1 TBE	2ml of of stock
acrylamide/bis acrylamide 30% (w/v) [29:1] soln.	2.34ml
glycerol	90 μ l
10% ammonium persulphate	50 μ l
purified water	5.51ml

10 μ l of TEMED was added to the resolving gel solution immediately before the gel was poured.

Stacking gel

x1 TBE	2ml of of stock
acrylamide/bis acrylamide 30% (w/v) [29:1] soln.	1.3ml
glycerol	90µl
10% ammonium persulphate	50µl
purified water	6.55ml

10µl of TEMED was added to the stacking gel solution immediately before the gel was poured.

Silver staining of polyacrylamide gels was achieved using the BioRad silver staining kit. All reagents were made up as per the manufacturers instructions with purified water.

3.3.7 Southern blotting

Hybond™-N+ positively charged membrane was purchased from Amersham Life Sciences Ltd. (Buckinghamshire, UK)

Denaturation solution

1.5M NaCl	87.66g
0.5M NaOH	20.0g
purified water	1000ml

Neutralization solution

1.5M NaCl	87.66g
0.5M Tris-HCl	78.8g
purified water	1000ml

20xSSC stock solution

0.3M Na ₃ C ₆ H ₅ O ₇ ·2H ₂ O	88.2g
3M NaCl	175.3g
purified water	1000ml

The pH was adjusted to 7.0 and the solution autoclaved.

3.3.8 ECL 3' hybridization solutions

The ECL 3'-oligolabelling and detection kit was purchased from Amersham (Bucks, UK), the anti-fluorescein-horseradish peroxidase (HRP) conjugate, blocking solution and the ECL detection reagents were stored at 4°C. The remaining kit components were stored at -20°C.

Hybridization buffer

5xSSC	95ml
0.1% (w/v) hybridization buffer component (supplied)	0.1g
0.02% (w/v) SDS	0.02g
Blocking solution (as supplied) (1:20 dilution)	5ml

The hybridization buffer was warmed to dissolve the hybridization buffer component, aliquoted in 20ml amounts and stored at -20°C for up to 3 months.

Primary wash solution

20xSSC (1 x SSC)	250ml
0.1% (w/v) SDS	1g
water	750ml

The wash buffer was stored at room temperature.

Secondary wash solution

20xSSC (0.5xSSC)	50ml
0.1% (w/v) SDS	1g
water	950ml

The wash buffer was stored at room temperature.

Buffer 1

0.15M NaCl	8.77g
0.1M Tris-OH	12.1g
water	1000ml

The pH of the buffer was adjusted to pH 7.5 with concentrated HCl, autoclaved and stored at room temperature.

Buffer 2

0.4M NaCl	23.4g
0.1M Tris-OH	12.1g
water	1000ml

The pH of the buffer was adjusted to pH 7.5 with concentrated HCl, autoclaved and stored at room temperature.

Block solution

buffer 2	475ml
liquid block (supplied)	25ml

The block solution was aliquoted in 20ml volumes and stored at -20°C.

Antibody solution

buffer 2	500ml
0.5% (w/v) BSA fraction V (Winlab, Middlesex, UK)	2.5g

The buffer was aliquoted in 15ml amounts and stored at -20°C. Prior to use, 15µl of anti-fluorescein HRP conjugate (supplied) was added.

ECL detection reagents (supplied) were mixed in equal volumes under safe light conditions and used immediately.

Hyperfilm ECL autoradiography paper was obtained from Amersham and stored in the

dark at room temperature.

Ilford PQ universal developer was purchased from Ilford Ltd. (Cheshire, UK) and diluted 1:10 with water before use.

Ilford Ilforspeed fixer was purchased from Ilford Ltd. and diluted 1:5 with water before use.

3.3.9 Patient specimens

Specimens were collected from Sheffield (Jessop Hospital for Women), Bristol (St. Michael's Hospital) and Trinidad (Mount Hope Women's Hospital, St. Augustine; and the General Hospital, Port of Spain). From Sheffield/Bristol, three study groups were examined, women presenting with ectopic pregnancy (EP), women undergoing surgery for tubal factor infertility (TFI), and control patients. From Trinidad, four study groups were considered, women presenting with EP, women with a history of miscarriage, women undergoing surgery for tubal factor infertility, and control patients. The specimens taken from the miscarriage group were taken retrospectively. The study controls from Sheffield/Bristol were hysterectomy patients and controls from Trinidad were patients undergoing sterilization.

Three tissue samples were collected from each patient in the study; an endometrial sample; a sample of the Fallopian tube (ampulla, isthmus or fimbriae); and an ovarian core biopsy. For the patients belonging to the EP groups, tissue samples were taken at the time of surgery, and Fallopian tube samples were taken from the site of the EP. Each set of tissue samples were collected in duplicate, one set was frozen and used for DNA extraction, and the other fixed immediately in buffered formalin for histological examination and *in situ* hybridization. Samples were coded with a patient reference number and the sample type. The study had Ethics Committee approval at all geographical sites.

Specimens for PCR from Trinidad were sent frozen in dry ice, and upon arrival in Sheffield were stored at -20°C. Samples from Bristol were delivered after overnight transport in liquid nitrogen and stored at -20°C. Tissue samples from Sheffield were collected fresh and stored immediately at -20°C.

For the TNF study, the DNA extracted from a single tissue specimen from all patients (Sheffield/Bristol and Trinidad) was tested by PCR.

3.4 Methods

3.4.1 DNA extraction

DNA extraction development

A small amount of tissue was removed from each defrosted tissue sample, and ground to a fine powder in liquid nitrogen. 1ml of lysis buffer (containing 200µg/ml proteinase K) was then added to resuspend the cells, and the suspension transferred to a sterile tube and incubated at 37° for 1h. Each tube was then boiled for 10min to denature the proteinase K, centrifuged at 10,000xg for 5min, and the resulting supernatant divided equally into three tubes. One tube was purified with 200µl of Insta Gene Purification Matrix; one tube was subjected to routine phenol/chloroform DNA extraction followed by ethanol precipitation; and the final tube was not purified further.

The concentration of DNA liberated by each method varied. The lysis buffer extraction procedure yielded the highest yield of DNA and the Instagene Purification the least. The phenol/chloroform extraction method gave marginally less DNA than the lysis buffer method (as determined using a TKO fluorometer), probably due to loss of DNA during the phenol/chloroform extraction procedure.

It became apparent that a problem with contamination may arise from using liquid nitrogen. Once the samples had been covered in liquid nitrogen, small pieces of tissue were observed to become airborne when the tissue was ground. DNA extraction by grinding the sample in the absence of liquid nitrogen and extraction in a lysis buffer containing 500µg/ml proteinase K, was found to be as efficient as DNA extraction with liquid nitrogen and lysis buffer containing 200µg/ml proteinase K.

The effect of varying the incubation time on the recovery of DNA with a 500µg/ml proteinase K lysis buffer was also investigated using a TKO fluorometer. A small amount of tissue (either endometrial, Fallopian tube, or ovarian) was crushed in a pestle and mortar with 1ml of 500µg/ml proteinase K lysis buffer. 250µl of the resulting

cellular suspension was aliquoted into three tubes, and the tubes incubated at 37°C for 1h, 2h, 3h respectively, centrifuged at 10,000xg for 5min and then subjected to conventional phenol/chloroform DNA extraction followed by ethanol precipitation. A steady increase in the DNA concentration recovered was observed over time (Graph 5), with the 3h incubation giving the highest DNA yield. Overnight incubation of samples, at 37°C, was found not to significantly increase the final concentration of extracted DNA.

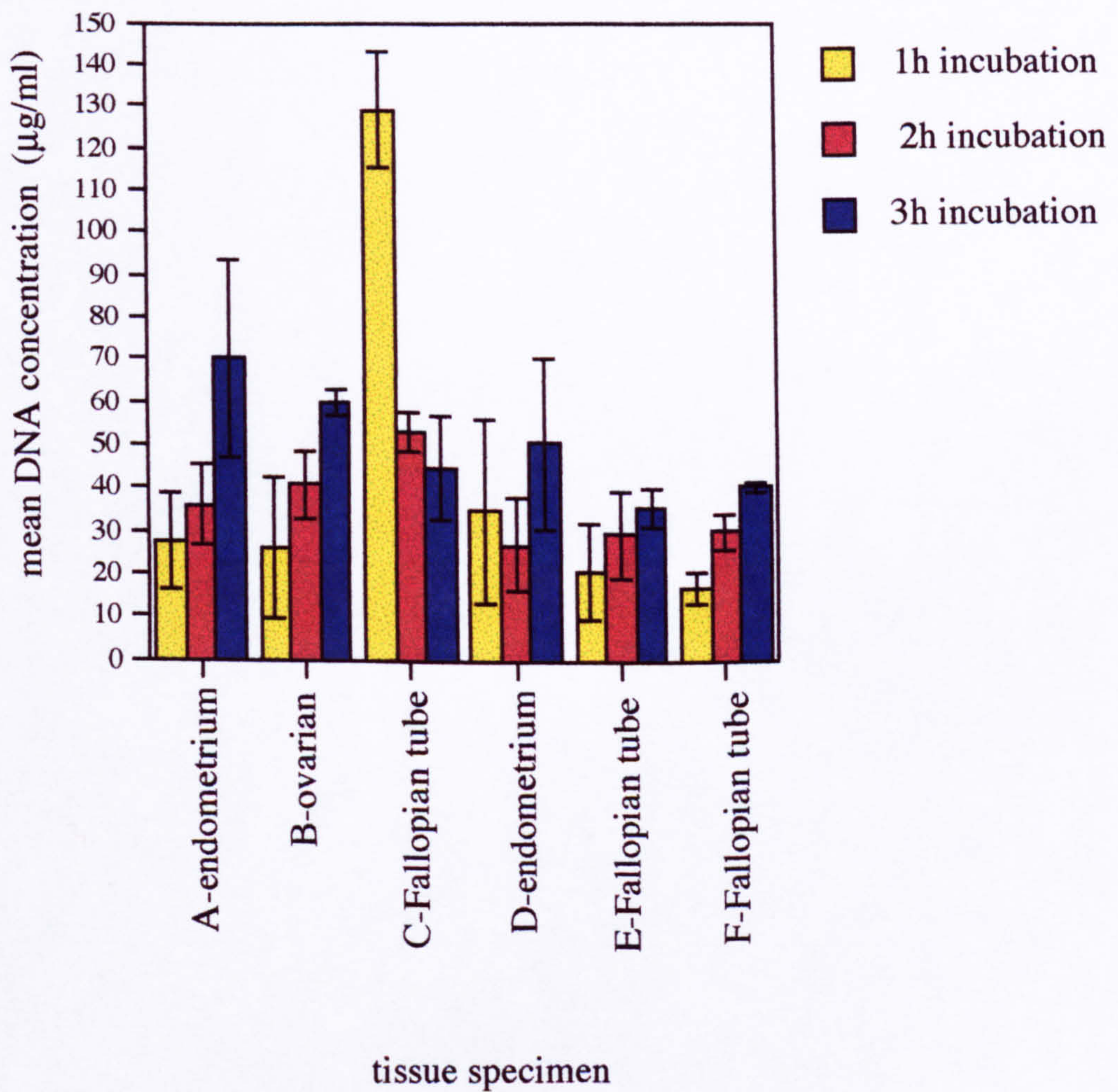
DNA extraction of study samples

Tissue samples were thawed and a measured amount (depending on sample size) was homogenized with a sterile pestle and mortar in 1ml of lysis buffer (containing 500µg/ml proteinase K). The cellular suspension was incubated at 37°C overnight, boiled for 10min, and then centrifuged at x10,000g for 2min to sediment cellular debris. The resulting supernatant was subjected to phenol:chloroform:isoamyl alcohol DNA extraction followed by ethanol precipitation with 50µl 3M sodium acetate and 5µl glycogen at -70°C overnight. The DNA was pelleted by centrifugation at x10,000g for 30min at 4°C, washed twice with 70% ice cold ethanol, air dried and finally resuspended in 100µl of sterile water. Appropriate extraction controls were set up throughout the procedure to detect contamination between samples and from equipment/material and the environment.

Extraction of LGV1 DNA

C. trachomatis serovar LGV1 DNA was extracted from cell culture. LGV1 was grown in Trac bottles containing a McCoy cell monolayer, as previously described (p.61). After two days of growth, the cell monolayer was removed with the end of a sterile 1ml syringe. The resulting cellular suspension was pelleted at x10,000g for 5min, 1ml of lysis buffer (containing 500µg/ml proteinase K) was added to the pellet and sample incubated at 37°C for 1h. The sample was then boiled for 10min, and centrifuged at x10,000g for 2min. The resulting supernatant was subjected to phenol:chloroform:isoamyl alcohol DNA extraction followed by ethanol precipitation with 50µl 3M sodium acetate and 5µl glycogen at -80°C overnight. The DNA was pelleted by centrifugation at x10,000g for 30min at 4°C, washed twice with 70% ice cold ethanol, air dried and finally resuspended in 100µl of sterile water. The extracted LGV1 DNA was used as a positive

Graph 5. The effect of varying incubation time on the concentration of DNA recovered ($\mu\text{g/ml}$) from female genital tract specimens using a $500\mu\text{g/ml}$ proteinase K lysis buffer.



control for *C. trachomatis* plasmid PCR.

3.4.2 Determination of DNA concentration

TKO 100 fluorometer

The TKO fluorometer was used to determine the presence of human genomic DNA after sample extraction. The system depends on the preferential binding of a bis benzimidazole dye (Hoechst 33258) to A-T rich regions of DNA. In the absence of DNA, the emission spectrum of Hoechst 33258 peaks at 492nm, when DNA is present the emission spectrum of the dye peaks at 458nm. The fluorescent characteristics of the dye bound to DNA closely match the emission and detection peaks of the TK100 fluorometer.

Initially the machine was allowed to stabilize for at least 15min before use and was first calibrated with calf thymus DNA (Hoefer) standard of known DNA concentration in 1xTNE buffer. 2ml of the freshly prepared working dye solution was placed in a TKO 105 glass cuvette (supplied) and 2 μ l of standard was added. After gentle mixing, the cuvette was placed in the fluorometer and the instrument calibrated to the appropriate concentration of DNA (taking into account the 1:1000 dilution of the standard in the working dye solution). The DNA concentration of each sample was measured in the same way, in units of ng/ml. The cuvette was washed with working dye solution between samples, and the fluorometer was blanked between each sample.

Spectrophotometer determination of DNA concentration

The DNA concentration of a random selection of extracted study samples was assessed to determine the average DNA content. Briefly, 2 μ l of each study sample was diluted in 998 μ l of sterile water and the optical density (OD) measured in a spectrophotometer at 260nm and 280nm. 1ml of sterile water was used to zero the spectrophotometer, and the microcuvette (quartz) was washed in sterile water between samples.

The OD at 260nm allowed the calculation of the DNA content of the sample, an OD of 1 is equivalent to 50 μ g/ml of double stranded DNA. The average recovery of DNA

(assuming a 100% extraction, and recovery rate) was calculated to be 13 μ g DNA/ μ g (SD 7.4) of wet weight tissue initially extracted in 1ml of lysis buffer. For PCR, 10 μ l of sample was used routinely which equated to an average of 4.68 μ g of DNA in a 50 μ l PCR reaction mix.

Pure preparations of DNA should have a 260nm/280nm ratio of 2.0 (double stranded DNA), protein contamination of the DNA sample would have the effect of lowering this value. None of the study samples tested showed evidence of protein contamination.

3.4.3 PCR detection of human genomic DNA

Titration of human β -globin PCR

The detection of the human β -globin gene by PCR was subjected to magnesium (Mg^{2+}) titration to determine the optimal Mg^{2+} concentration. $MgCl_2$ concentrations tested ranged from 0.5mM to 2.5mM, in 0.5mM increments.

The PCR was performed on 1 μ g of human placental DNA in a final reaction mix of 50 μ l, using primers PCO3 (Saiki *et al.* 1985), and PCO6 (Lan *et al.* 1995). The final reaction mix contained the appropriate concentration of $MgCl_2$; x1 NH_4 PCR buffer; 200 μ M of dNTP; 50pmol of each primer (PCO3, PCO6); and 1U Taq DNA polymerase. The PCR consisted of 40 cycles of amplification, comprising denaturation at 94°C for 1min, primer annealing at 55°C for 40sec and primer extension at 72°C for 40sec. Pre-PCR the tubes were incubated at 94°C for 4min, and post-PCR at 72°C for 8min.

The PCR product (326-bp) was visualised on a 0.8% agarose gel after ethidium bromide staining (Figure 6). The optimal $MgCl_2$ concentration was determined to be 1.0mM, and was therefore used in all subsequent human β -globin PCR experiments.

Human β -globin PCR sensitivity

The average amount of extracted DNA used in each PCR was approximately 4.68 μ g in a 50 μ l reaction mix. The sensitivity of the human β -globin PCR was determined to ensure

that the PCR detection threshold was greater than the average DNA concentration extracted per sample.

Human placental DNA (Sigma) was serially diluted from 1µg/µl to 0.06ng/µl in purified sterile water. 2µl of each DNA concentration was added to 48µl of reaction mix. The final reaction mix contained 1.0mM MgCl₂; x1 NH₄ PCR buffer; 200µM of dNTP; 50pmol of each primer; and 1U Taq DNA polymerase. The PCR consisted of 40 cycles of amplification, comprising denaturation at 94°C for 1min, primer annealing at 55°C for 40sec and primer extension at 72°C for 40sec. Pre-PCR the tubes were incubated at 94°C for 4min, and post-PCR at 72°C for 8min. The expected PCR product of 326-bp was visualised on a 0.8% agarose gel after ethidium bromide staining. The human β-globin PCR was able to detect below 1ng of human DNA in a final reaction volume of 50µl.

Human β-globin PCR of study samples

Detection of human genomic DNA was performed by PCR using primers PCO3 (Saiki *et al.* 1985), and PCO6 (Lan *et al.* 1995) aimed at the human β-globin gene. This PCR was not only used to show the presence of human DNA and the success of DNA extraction, but was also used to show the absence of PCR inhibitors. Only samples positive for the presence of human DNA were subsequently tested for the presence of *C. trachomatis* DNA.

The PCR was performed on 10µl of the extracted DNA sample in a final reaction mix of 50µl. The final 50µl PCR reaction mix contained 1.0mM MgCl₂; x1 NH₄ PCR buffer; 200µM of dNTP; 50pmol of each primer; and 1U Taq DNA polymerase. Prior to PCR, the reaction tubes minus template DNA were subjected to UV irradiation. The PCR consisted of a total of 40 cycles of amplification, comprising denaturation at 94°C for 1min, primer annealing at 55°C for 40sec and primer extension at 72°C for 40sec. Prior to the commencement of PCR cycling the tubes were incubated at 94°C for 4min, and post-PCR at 72°C for 8min. The expected PCR product of 326-bp was visualised on a 0.8% agarose gel after ethidium bromide staining (Figure 6). Human placental DNA was used as a positive control, and purified water as a negative control.

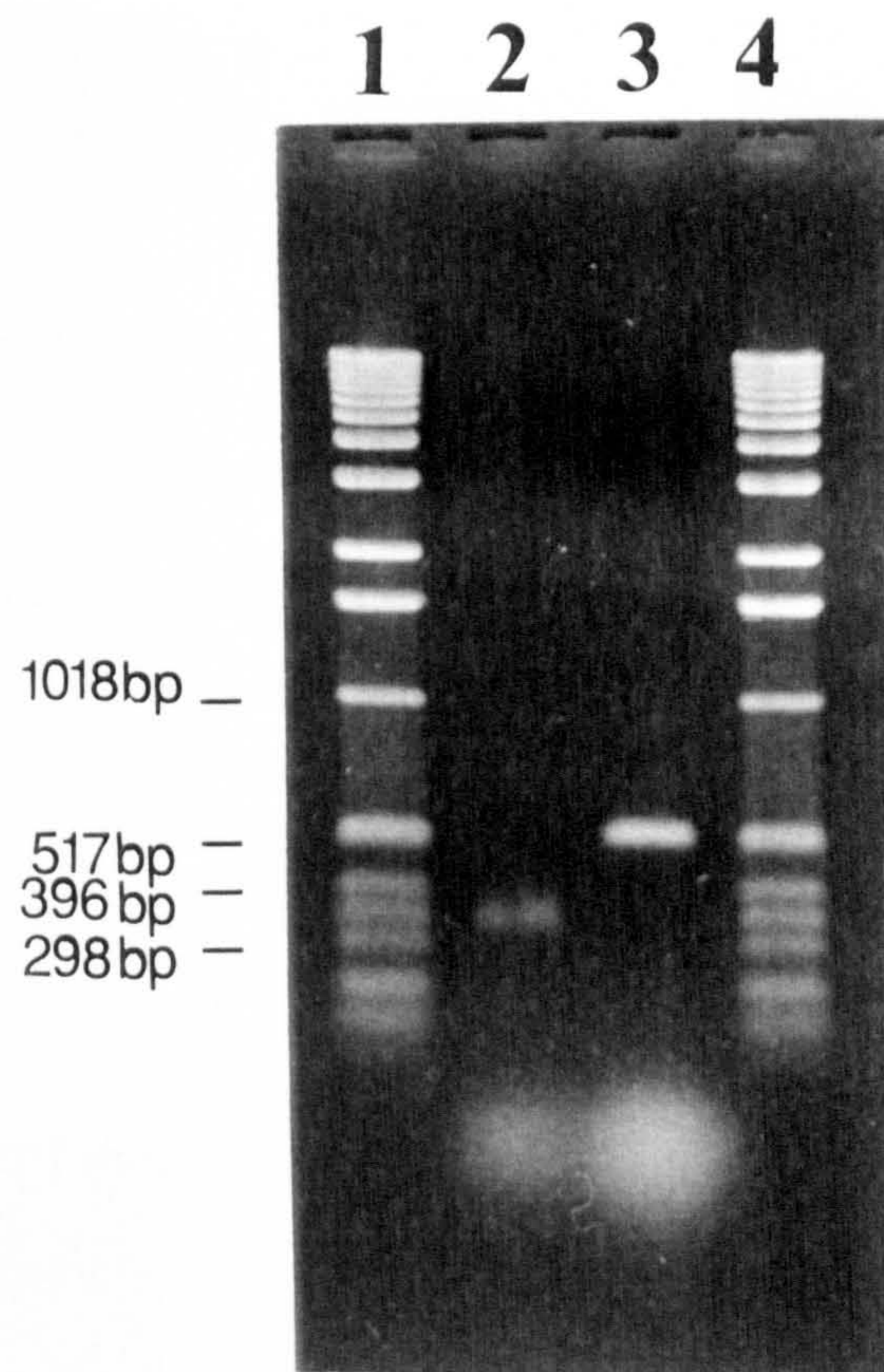


Figure 6. A representative 0.8% agarose gel showing lane 1, 1kb ladder; lane 2, 326-bp human β -globin polymerase chain reaction product; lane 3, 517-bp single plasmid *C. trachomatis* polymerase chain reaction product; and lane 4, 1kb ladder.

3.4.4 PCR detection of *C. trachomatis* DNA

The production of a false positive PCR result due to the amplification of exogenous template from a previous PCR is an important consideration when interpreting the results of PCR. Methods such as the physical separation of pre- and post-amplification steps (Kwok and Higuchi, 1989), the use of designated pipettes, the use of positive displacement tips and filter barrier tips, and UV irradiation of reagents and reaction tubes (Sarker and Summer, 1990) have been employed to reduce the risk of amplicon contamination.

Another method for reducing the possibility of a false positive PCR result is the GeneAmp Carry Over Prevention System (Perkin Elmer). This system uses the enzymatic, and chemical reactions analogous to the restriction-modification and excision-repair systems of cells to specifically degrade PCR products from a previous PCR and mis-matched or mis-primed products produced prior to PCR, but not native DNA. dUTP is substituted for dTTP in the PCR reaction mix, so amplicons containing dUTP are produced. In subsequent PCR reactions, uracil N-glycosylase (UNG), in the presence of alkaline pH and heat, is used to degrade contaminating dU-containing DNA. 0.5U of UNG in a final volume of 50µl and a 10min incubation at room temperature has been shown to prevent the amplification of 1×10^6 copies of dU-containing PCR product. One of the limitations of the UNG system is that it is incompatible with a nested PCR. If UNG were present in the secondary PCR reaction mixture it would facilitate the degradation of the template (primary PCR product containing dU) leading to a false negative result.

Samples found positive for human genomic DNA were tested for the presence of *C. trachomatis* DNA using primers T1 and T2 homologous to specific regions of the chlamydial 7.5kb plasmid (Claas *et al.*, 1990). The PCR (using the GeneAmp Carry Over Prevention System), including the other anti-contamination methods previously described was performed on 10µl of the extracted DNA sample in a final reaction mix of 50µl.

The final reaction mix contained 2.5mM MgCl₂; x1 KCl PCR buffer; 200µM of dATP, dCTP, and dGTP; 400mM dUTP; 100pmol of each primer; 0.5U UNG; and 1U Taq DNA polymerase. Prior to PCR, the reaction tubes minus template DNA were subjected to UV irradiation (9.9x10⁵µJ/cm²). The PCR consisted of 40 cycles of amplification, comprising denaturation at 95°C for 30sec, primer annealing at 55°C for 30sec and primer extension at 72°C for 30sec. Pre-PCR the tubes were incubated at 28°C for 10min to allow the digestion of any contaminating dUTP PCR product, followed by 10min at 95°C to denature the UNG. LGV1 DNA was used as a positive PCR control and sterile water was used as a negative control.

Post-PCR the tubes were held at 72°C and chloroform was added to denature any residual UNG. The expected product, a 517-bp amplicon, was analysed through a 0.8% agarose gel by standard protocols and visualised under UV light after ethidium bromide staining (Figure 6, and Figure 7).

3.4.5 Confirmation of *C. trachomatis* PCR products

Southern blot

The DNA from the agarose gel was transferred to a nylon membrane by Southern blotting. Briefly, after ethidium bromide staining the gel was washed for 10min in purified water, followed by 15min in denaturation solution and 15min in neutralisation solution. A electrophoresis tank was rinsed, and a wick made from filter paper soaked in 20xSSC was laid over the platform and the tank partially filled with 20xSSC. The gel was laid on the wick and a piece of Hybond-N⁺ membrane (pre-soaked in purified water) was placed on top. Air bubbles were carefully removed. Three sheets of filter paper, cut to size and wetted with 20xSSC were placed on top of the membrane followed by absorbent towels and a weight.

Capillary blotting was allowed to proceed overnight. Finally, the blotting apparatus was dismantled, the membrane marked for orientation and left to air dry before being UV cross-linked at 12x10⁴µJ/cm². The agarose gel was re-stained with ethidium bromide to

determine whether DNA transfer had been successful.

Oligonucleotide labelling

The 3' labelling of the oligonucleotide probe with fluorescein was achieved using the Amersham 3'-oligolabelling and detection system.

The required labelling components were allowed to defrost on ice. 10µl of fluorescein-11-dUTP was added to 1µl of the oligonucleotide probe (100×10^{-2} pm/ml) followed by 16µl of cacodylate buffer, 117µl of water and 16µl of terminal transferase. The mixture was then incubated at 37°C for 1h and stored at -20°C in the dark.

Hybridization

The membrane was wetted in 2xSSC, placed in a clean sterile hybridisation tube with 10ml of hybridisation buffer and left to pre-hybridize for 1h at 55°C in a hybridization oven. 2ml of hybridization buffer was then removed from the tube and the probe added to give a final concentration of 10ng/ml. The hybridization buffer containing the probe was returned and the membrane was then left for 2h at 55°C in the hybridization oven.

The blot was then removed from the hybridization solution, placed in a clean container and covered with an excess of wash buffer 1 for 5min with constant agitation. This was repeated. The blot was then placed in a clean hybridization tube and covered with an excess of wash 2 which had been pre-warmed to 55°C, and returned to the hybridization oven for 15min at 55°C. This again was repeated.

The blot was then placed in a clean container and rinsed with buffer 1 for 1min, which was then discarded and replaced with an excess of blocking solution for 1h. The membrane was then washed briefly with buffer 1, incubated with the antibody solution for 30min and then rinsed in an excess of buffer 2 for 5min. This was repeated a further three times to ensure the complete removal of non-specifically bound antibody.

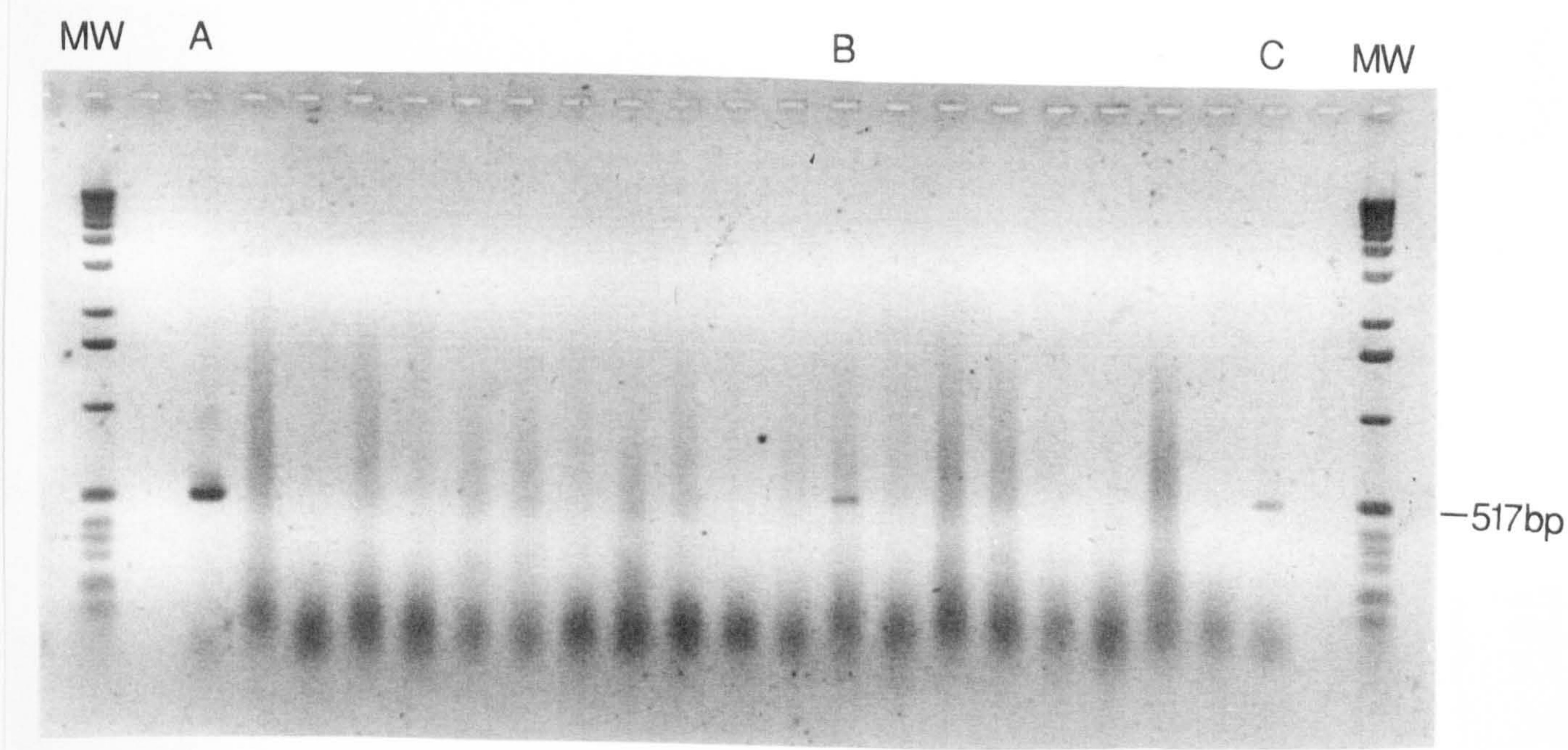


Figure 7. A 0.8% agarose gel of *C. trachomatis* single plasmid polymerase chain reaction products. MW - 1kb ladder; A - LGV1 positive control; B - positive endometrial sample; C - positive endometrial sample; MW - 1kb ladder.

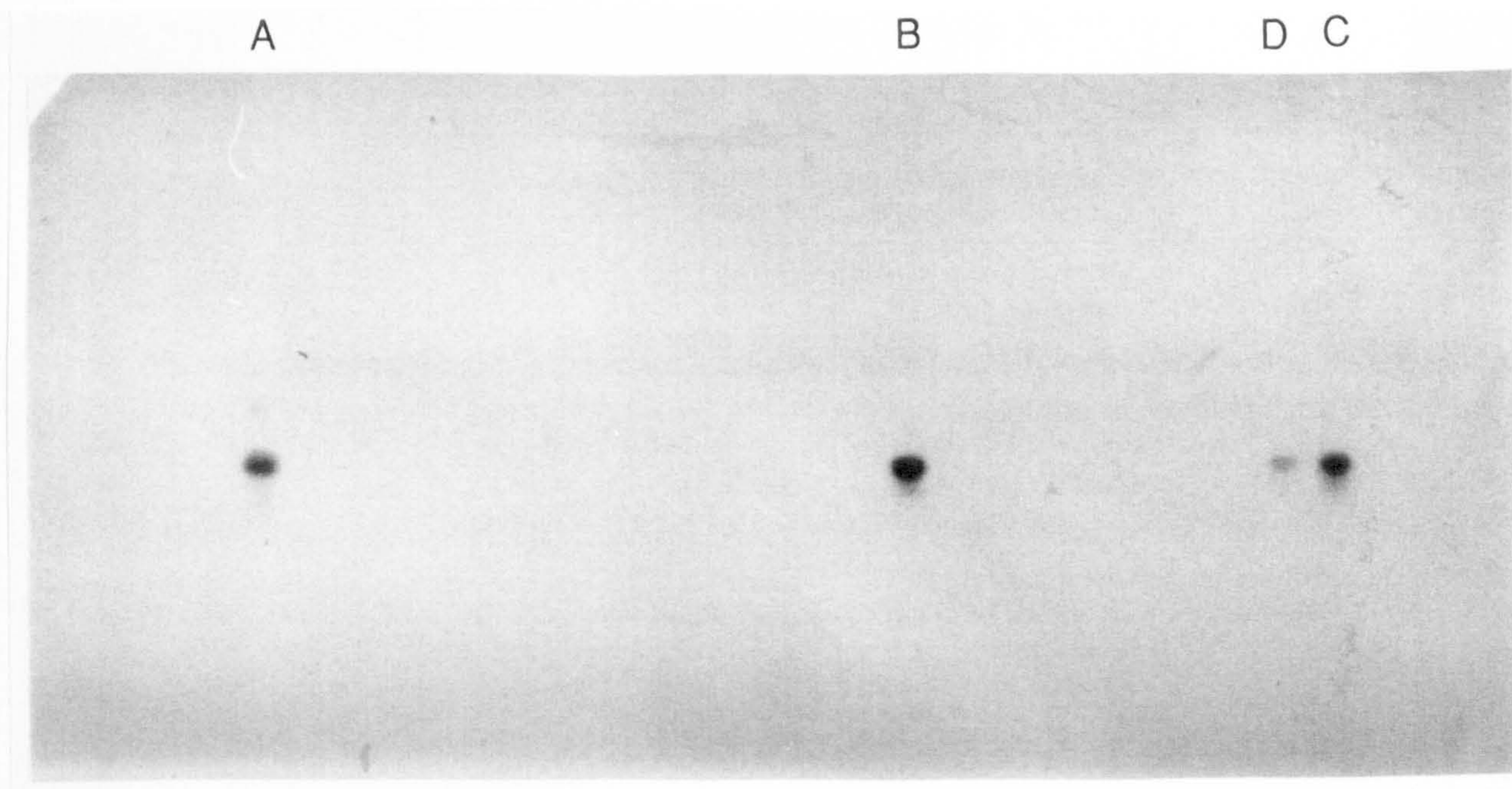


Figure 8. Southern blot of gel shown in Figure 7. A - LGV1 positive control; B - positive endometrial sample; C- positive endometrial sample; D - positive ovarian sample not seen on gel.

Signal generation and detection

Under safe-light conditions in a darkroom, the two ECL detection reagents were mixed in equal volumes, the membrane was drained and placed on an acetate in a developing cassette and covered with another acetate. Hyper film, cut to the appropriate size was placed over the acetate covered membrane and the cassette locked and left for 10 min. The film was then developed, fixed and washed and finally left to air dry. Depending on the strength of the signal, another piece of film was placed over the membrane and left for a maximum of 12h (Figure 8).

3.4.6 Effect of human DNA on *C. trachomatis* PCR

The effect of high concentrations of human genomic DNA on the single plasmid PCR was investigated to ensure that *C. trachomatis* DNA could be detected in samples with a high 'background' of human genomic DNA.

Human placental DNA (Sigma) was diluted in sterile purified water, and 5µl of each dilution was added to a 50µl reaction mix to give final concentrations of 0.1µg/µl, 0.02µg/µl, 0.01µg/µl, 5ng/µl, 2.5ng/µl, and 1.25ng/µl. A duplicate set of samples was made to which 100ng of extracted *C. trachomatis* DNA was added.

The final reaction mix contained 2.5mM MgCl₂; x1 KCl PCR buffer; 200µM of dATP, dCTP, and dGTP; 400mM dUTP; 100pmol of each primer; 0.5U UNG; and 1U Taq DNA polymerase. Prior to PCR, the reaction tubes minus template DNA were subjected to UV irradiation (9.9x10⁵µJ/cm²). The PCR consisted of 40 cycles of amplification, comprising denaturation at 95°C for 30sec, primer annealing at 55°C for 30sec, and primer extension at 72°C for 30sec. Pre-PCR the tubes were incubated at 28°C for 10min to allow the digestion of any contaminating dU PCR product, followed by 10min at 95°C to denature the UNG. Post-PCR 50µl of chloroform was added to each sample to prevent degradation of dU PCR product by any residual UNG. LGV1 DNA was used as a positive PCR control, and sterile water was used as a negative control.

The presence of high concentrations (0.1µg/µl) of human genomic DNA was shown to interfere with the amplification of chlamydial plasmid DNA (Figure 9).

3.4.7 Major outer membrane protein PCR

MOMP PCR of study samples

Lan *et al.* (1994) published a series of primers, for single and nested PCR, targeted at the four variable domains of the chlamydial OMP1 gene. The nested set of primers with the highest reported sensitivity were NLO and NRO, followed by PCTM3 and SERO2A. Both the primary and secondary nested product were suitable for RFLP genotyping. Study samples that were found to be *C. trachomatis* DNA by plasmid PCR were tested by MOMP PCR.

For the primary MOMP PCR the final reaction mix contained 1.5mM MgCl₂; x1 KCl PCR buffer; 200µM of dNTP; 50pmol of each primer (NLO and NRO); 1U Taq DNA polymerase; and 10µl of extracted study DNA. The PCR consisted of 49 cycles of amplification, comprising denaturation at 94°C for 1min, primer annealing at 45°C for 3min and primer extension at 72°C for 3min. Pre-PCR the tubes were incubated at 94°C for 6min, and post-PCR at 72°C for 8min. LGV1 DNA was used as a positive PCR control, and sterile water was used as a negative control. The primary PCR product of 1149-bp was visualised on a 0.8% agarose gel after ethidium bromide staining and UV transillumination.

The secondary MOMP PCR was only used on samples that tested negative by the primary PCR. The secondary PCR final reaction mix contained 1.5mM MgCl₂; x1 KCl PCR buffer; 200µM of dNTP; 50pmol of each primer (PCTM3 and SERO2A); 1U Taq DNA polymerase; and 3µl of the primary MOMP PCR product. The PCR cycling parameters were the same as in primary MOMP PCR. 3µl of the primary product generated from the LGV1 DNA control was used as a positive control for the second round of amplification. Sterile water was used as a negative control. The primary PCR product of 1013-bp was visualised on a 0.8% agarose gel after ethidium bromide staining and UV

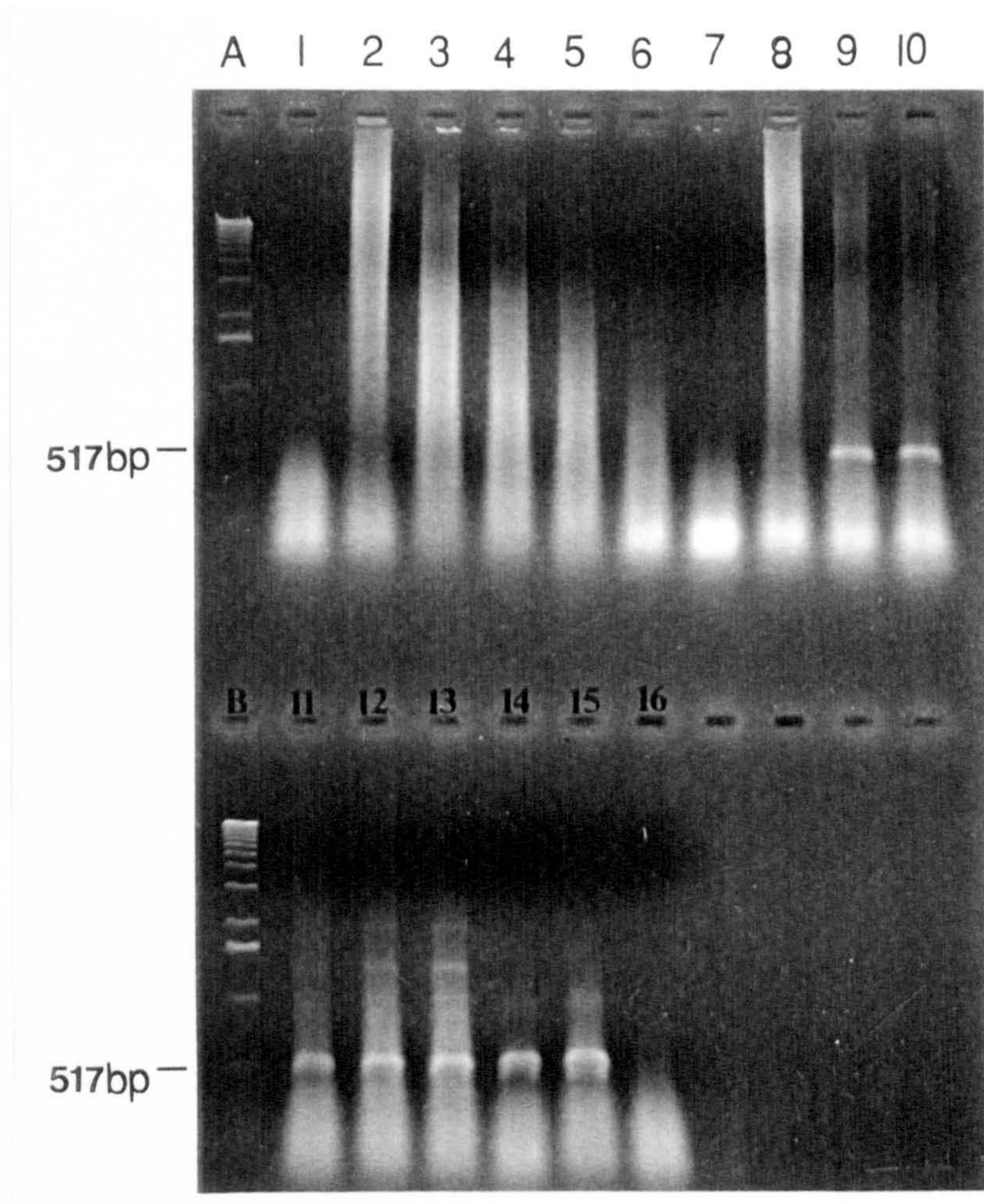


Figure 9. 0.8% agarose gel of *C. trachomatis* single plasmid polymerase chain reaction products with varying concentrations of human DNA. Lane 1, negative control; lane 2, 0.1 $\mu\text{g}/\mu\text{l}$ of human DNA; lane 3, 0.02 $\mu\text{g}/\mu\text{l}$ of human DNA; lane 4, 0.01 $\mu\text{g}/\mu\text{l}$ of human DNA; lane 5, 5ng/ μl of human DNA; lane 6, 2.5ng/ μl of human DNA; lane 7, 1.25ng/ μl of human DNA. Lanes 8-13 are duplicates of lanes 2-7, with the addition of 100ng of LGV1 DNA. Lanes 14, and 15 contained 100ng, and 300ng of LGV1 DNA. Lane 16, negative control. Lanes A and B-1kb ladder.

transillumination.

RFLP genotyping

RFLP genotyping was performed on either the primary or secondary MOMP PCR product. 10µl of PCR product was initially digested with 2.5U of *Alu1* (Gibco) and 2µl of the appropriate enzyme buffer in a total reaction volume of 20µl in a waterbath overnight at 37°C. The samples were then analysed through a 7% polyacrylamide gel, and the gel was then silver stained. The resulting fragment patterns were compared against the RFLP patterns of fifteen reference strains (serovars A to LGV3). Samples that were thought to be of genotypes A, C, H, I, J, and LGV3 were further analysed by a second round of enzyme digestions. For genotypes C and J, 10µl of MOMP PCR product was digested with 2.5U of *Hinf1*; genotypes H, I, and LGV3 were digested with 2.5U of both *EcoR1* and *Dde1*. All enzyme digestions were carried out overnight at 37°C in a waterbath.

Polyacrylamide gel electrophoresis

Polyacrylamide gels were prepared and run using a Mini-Protean II electrophoresis system (Bio-Rad). Before use the glass plates were washed, dried and wiped with 40% methanol. The resolving gel was poured first, and water saturated butanol was pipetted over the top to give a perfectly flat top surface to the gel. After the gel had set, the butanol was removed using 3MM blotting paper, and the stacking gel was laid over the resolving gel and the comb added. The gel was left to polymerise at room temperature for 30min before the comb was removed, and excess liquid removed from the wells using fine strips of filter paper.

The prepared gel was assembled in the Mini Protean gel tank, and the tank was filled with 1xTBE in both chambers. 10µl of digested MOMP PCR product was added to 2µl of loading buffer, and the mixture loaded into one of the gel wells. A 1kb ladder (Gibco) diluted 1:10 was also pre-mixed with loading buffer and loaded onto the gel. Finally the gel was run at 200V for approximately 45min, or until the leading dye front had reached the bottom of the gel.

Silver staining of polyacrylamide gels

The polyacrylamide gel was carefully removed from the glass plates by immersion in 200ml of 40% methanol, once detached the gel was left for 30min. The methanol was replaced with 200ml of 10% ethanol, and the gel gently shaken for 15min, the ethanol was replaced and 200ml of fresh 10% ethanol was added for a further 15min.

The ethanol was decanted and replaced with 50ml of oxidiser (supplied), and the gel shaken gently for 5min. The gel was then washed up to four times in purified water until the orange colour of the oxidizer was removed. 50ml of silver reagent (supplied) was then added to the gel, the gel shaken for 20min and then finally the gel was rinsed in purified water for 1min. The DNA bands were eventually visualized upon the addition of 50ml developing solution (supplied) pre-warmed to 37°C. The developing solution was replaced after 15-20sec with 50ml of fresh developer. 5% acetic acid was used to stop the developing reaction.

3.4.8 Determination of TNF gene polymorphisms

TNF- α PCR

Detection of the human TNF- α gene was achieved using the primers TNF- α 1 and TNF- α 2 (Cabrera *et al.*, 1995). These primers were originally designed to incorporate a polymorphic site at position -308 of the TNF- α promoter region. The optimal Mg²⁺ concentration was determined by an Mg²⁺ titration PCR assay.

The PCR was performed on 5 μ l of the extracted DNA sample in a final reaction mix of 50 μ l. The final reaction mix contained 1.5 mM MgCl₂; x1 NH₄ PCR buffer; 200 μ M of each dNTP; 100pmol of each primer; and 1U Taq DNA polymerase. The PCR consisted of 35 cycles of amplification, comprising denaturation at 94°C for 1min, primer annealing at 60°C for 1min, and primer extension at 72°C for 15sec. Pre-PCR the tubes were incubated at 95°C for 3min, 60°C for 35sec and 72°C for 1min. Post-PCR the tubes were held at 72°C for 5min. The expected PCR product of 107-bp was visualised under UV light on a 2% Nu Seive (FMC Bioproducts) gel under UV after ethidium bromide

staining.

TNF- β PCR

Detection of the human TNF- β gene was achieved using the primers TNF- β 1 and TNF- β 2 (Messer *et al.*, 1991), which amplify a 740bp fragment from exon 1 to intron 3 of the TNF- β gene, and also incorporates a polymorphic *Nco*1 site. The optimal Mg²⁺ concentration for PCR was determined by an Mg²⁺ titration assay.

The PCR was performed on 5 μ l of the extracted DNA sample in a final reaction mix of 50 μ l. The final reaction mix contained 1.0 mM MgCl₂; x1 NH₄ PCR buffer; 200 μ M of each dNTP; 50pmol of each primer; and 1U Taq DNA polymerase. The PCR consisted of 35 cycles of amplification, comprising denaturation at 95°C for 1min, primer annealing at 65°C for 45sec, and primer extension at 72°C for 30sec. Pre-PCR the tubes were incubated at 95°C for 6min, and post-PCR the tubes were held at 72°C for 5min. The expected PCR product of 740-bp was visualised under UV light on a 0.6% agarose gel after ethidium bromide staining.

TNF- α and TNF- β PCR digests

For both *Nco*1 digestions, 10 μ l of the appropriate PCR product was digested overnight at 37°C, with 6U of *Nco*1 (Gibco) and 3 μ l of the appropriate buffer in a final volume of 30 μ l. The TNF- α digests were separated on a 2% Nu Seive gel, and the TNF- β digests on a 0.8% agarose gel before being ethidium bromide stained. The allele distribution for both the TNF- α , and TNF- β genes was determined under UV light.

TNF- α wild type homozygotes (TNF- α -308G/TNF- α -308G) gave two bands after *Nco*1 digestion and gel electrophoresis; one band at 87-bp and the other at 20-bp. TNF- α heterozygotes (TNF- α -308G/TNF- α -308A) gave three bands, one band at 107-bp, one at 87-bp and the final band at 20-bp. TNF- α -308A homozygotes (TNF- α -308A/TNF- α -308A) gave only a single band at 107-bp due to the loss of the *Nco*1 restriction site on both alleles. See Figures 10 and 11.

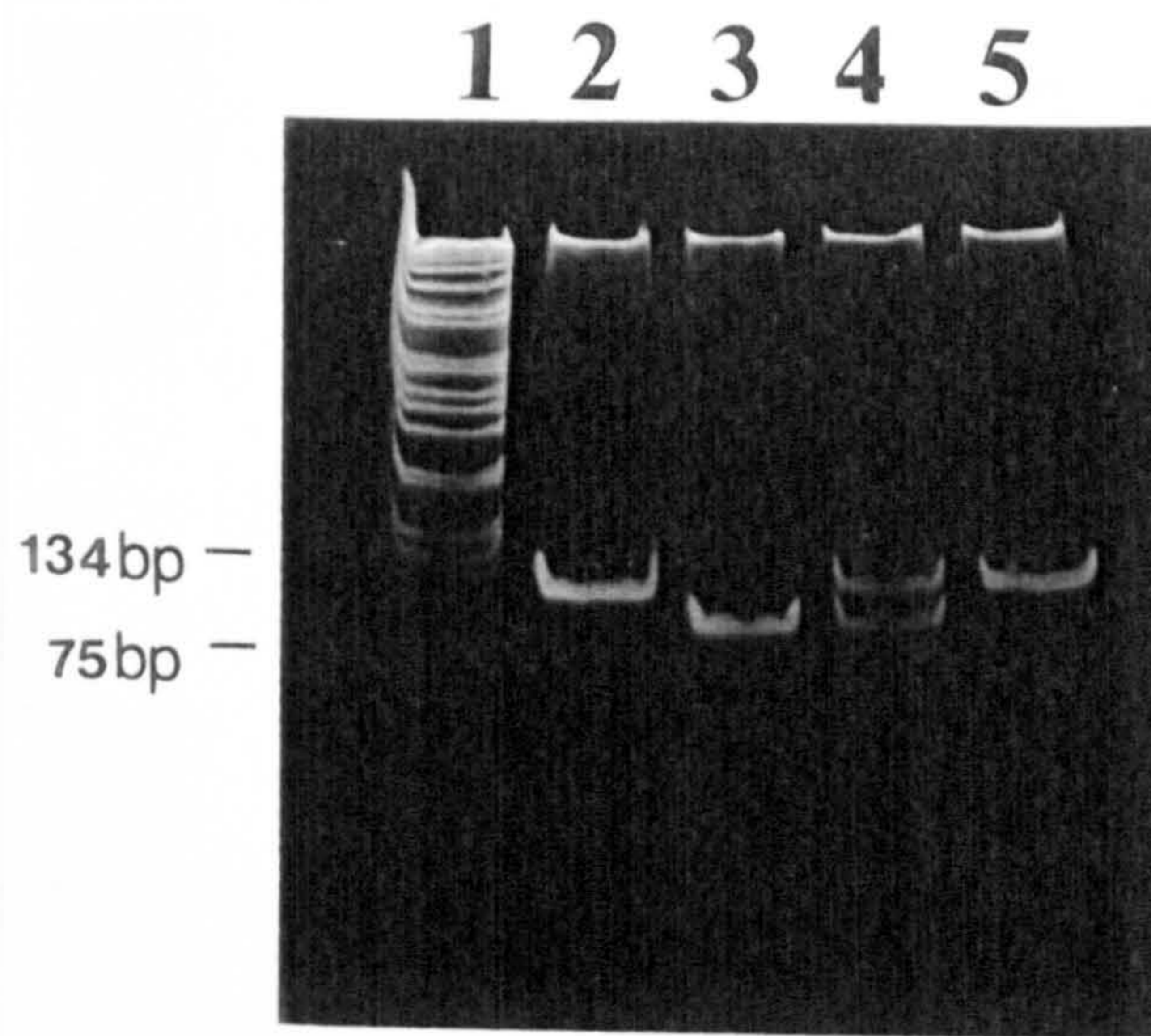


Figure 10. A representative 7.5% polyacrylamide gel of tumor necrosis factor- α polymerase chain reaction products after *Nco*I digestion. Lane 1 1kb ladder; lane 2, uncut tumor necrosis factor- α polymerase chain reaction product; lane 3, 308G/308G; lane 4, 308G/308A; lane 5, 308A/308A. The 20-bp product was observed faintly on actual gel.

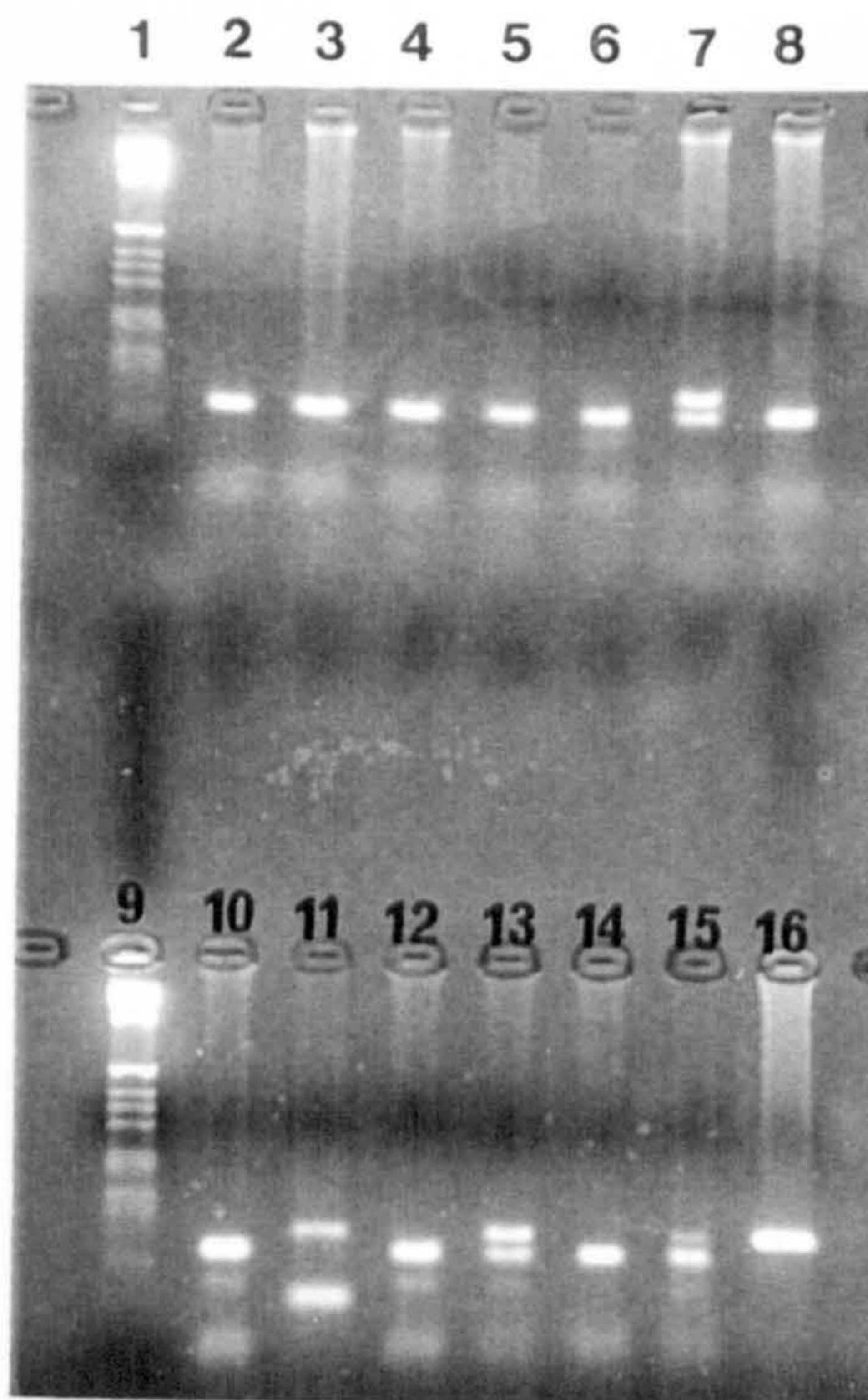


Figure 11. 2% Nu Seive gel of tumor necrosis factor- α polymerase chain reaction product *Nco*I digests of actual patient samples. Lane 1, 1kb ladder; lanes 2-6, 308G/308G; lane 7, 308G/308A; lanes 8 and 10, 308G/308G; lane 9, 1kb ladder; lane 11, unusual tumor necrosis factor- α *Nco*I polymorphism; lane 12, 308G/308G; lane 13, 308G/308A; lane 12, 308G/308G, lane 13, 308G/308A; lane 14, 308G/308G; lane 15, 308G/308A; lane 16, uncut tumor necrosis factor- α polymerase chain reaction product.

TNF- β wild type homozygotes (TNFB*2/TNFB*2) gave a single band of 740-bp after *Nco*I digestion, and gel electrophoresis. TNF- β heterozygotes (TNFB*2/TNFB*1) gave three bands, one band at 740-bp, one at 555-bp and the final band at 185-bp. TNF- β homozygotes for the *Nco*I polymorphism (TNFB*1/TNFB*1) gave two bands, one at 555-bp and the other at 185-bp. See Figures 12 and 13.

3.4.9 Statistical analysis

The prevalence rate of *C. trachomatis* DNA for each study group was compared to the appropriate control group using the Fisher Exact Probability Test (2 tailed). A probability (P) value of <0.05 was considered to be statistically significant.

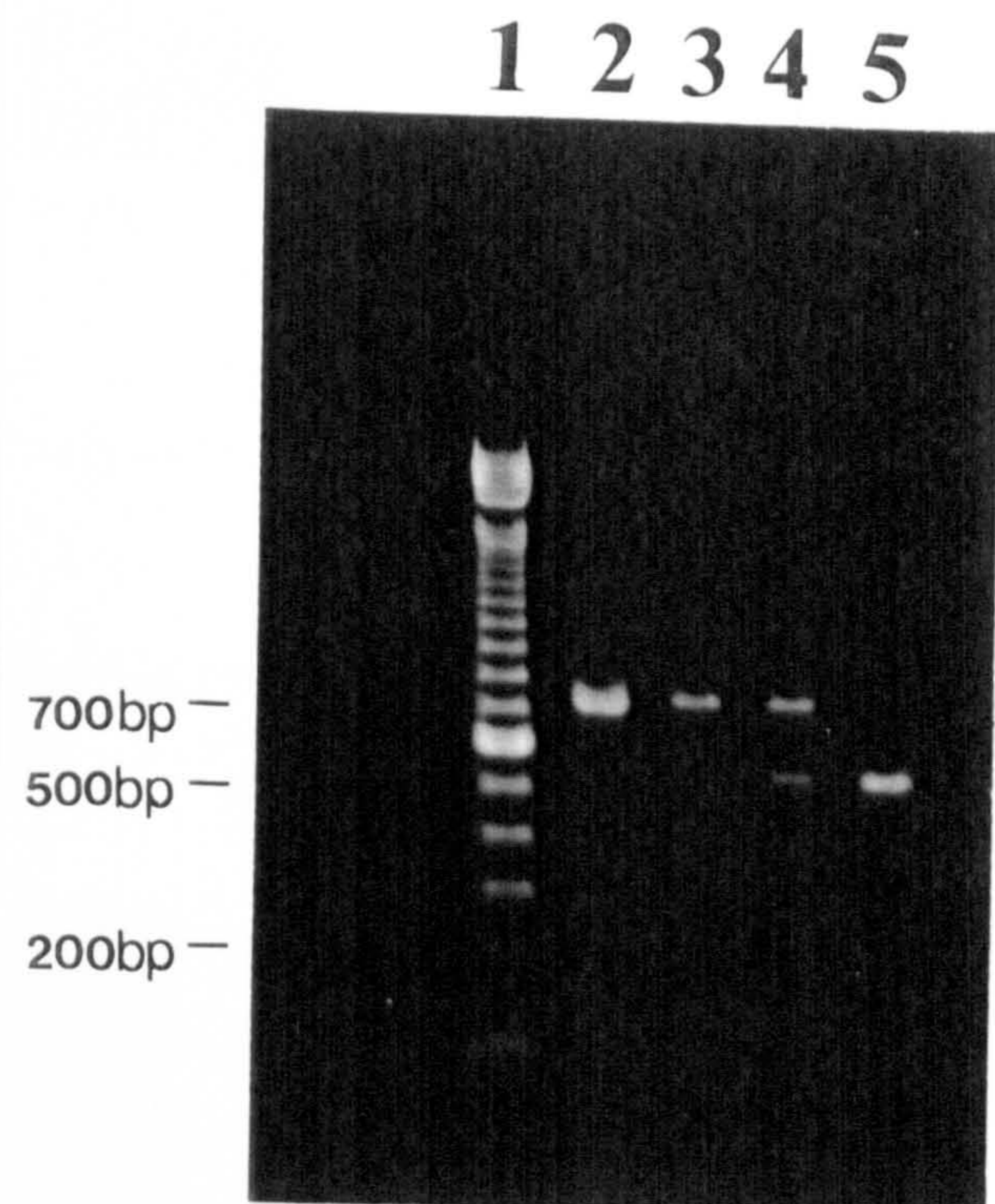


Figure 12. A representative 1% agarose gel of tumor necrosis factor- β polymerase chain reaction products after *Nco*I digestion. Lane 1, 100-bp ladder; lane 2, uncut tumor necrosis factor- β polymerase chain reaction product; lane 3, TNFB*2/TNFB*2; lane 4, TNFB*2/TNFB*1; lane 5, TNFB*1/TNFB*1. The 185-bp product was observed faintly on actual gel.

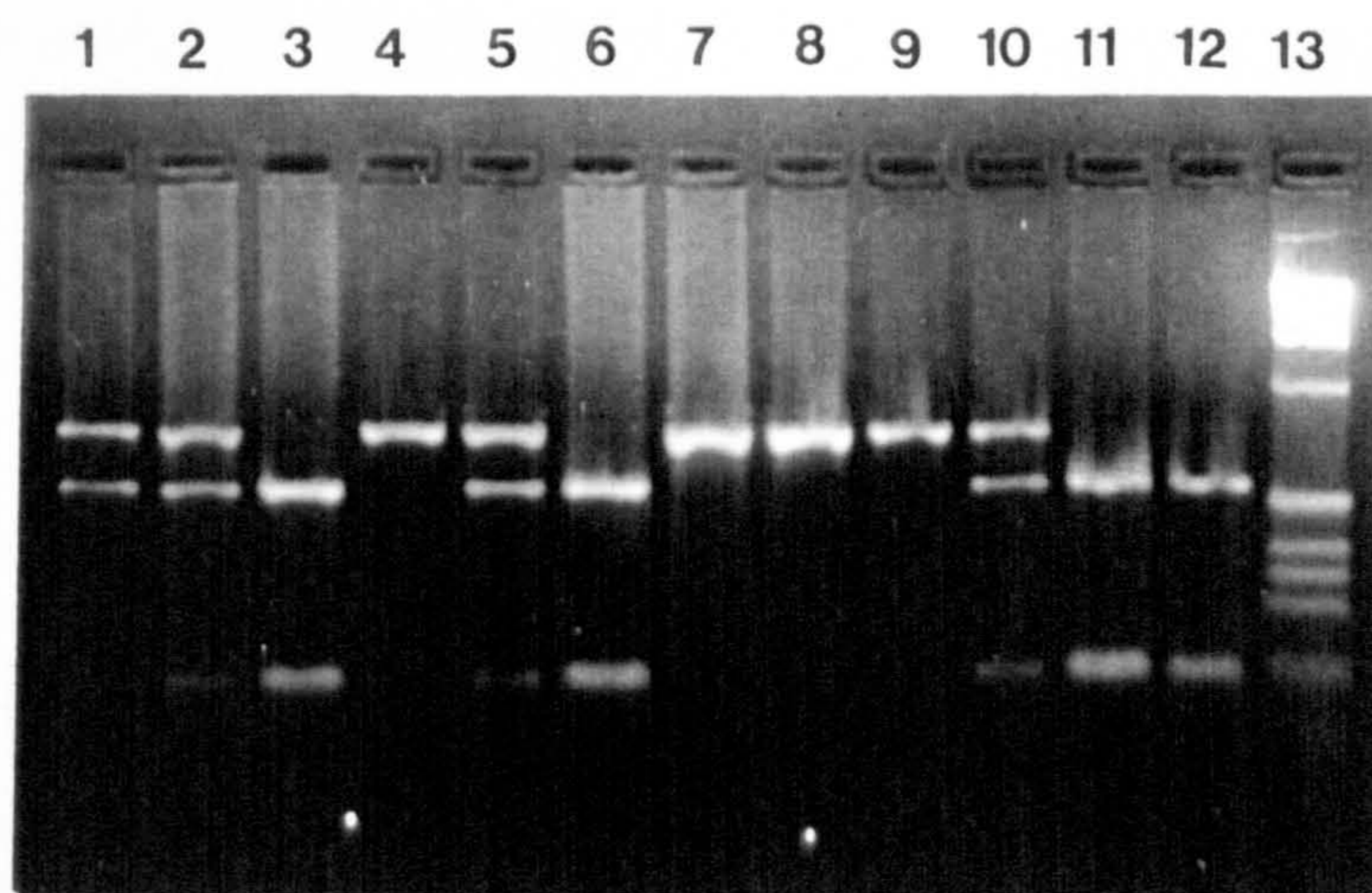


Figure 13. A 0.6% agarose gel of tumor necrosis factor- β polymerase chain reaction product *Nco*I digests of actual patient samples. Lanes 1 and 2, TNFB*2/TNFB*1; lane 3, TNFB*1/TNFB*1; lane 4, TNFB*2/TNFB*2; lane 5, TNFB*2/TNFB*1; lane 6, TNFB*1/TNFB*1; lanes 7-9, TNFB*2/TNFB*2; lane 10, TNFB*2/TNFB*1; lanes 11 and 12, TNFB*1/TNFB*1; lane 13, 1kb ladder.

3.5 PCR and Southern blotting results

3.5.1 Prevalence of *C. trachomatis* DNA in Sheffield/Bristol

The detection of *C. trachomatis* plasmid DNA by polymerase chain reaction and Southern blotting are shown in Table 7, and Graphs 6 and 7. The results for individual patients can be found in Appendix 3.

Table 7. Breakdown of the number of patients and tissue sites sampled positive for *C. trachomatis* plasmid DNA by polymerase chain reaction and Southern blotting for Sheffield/Bristol.

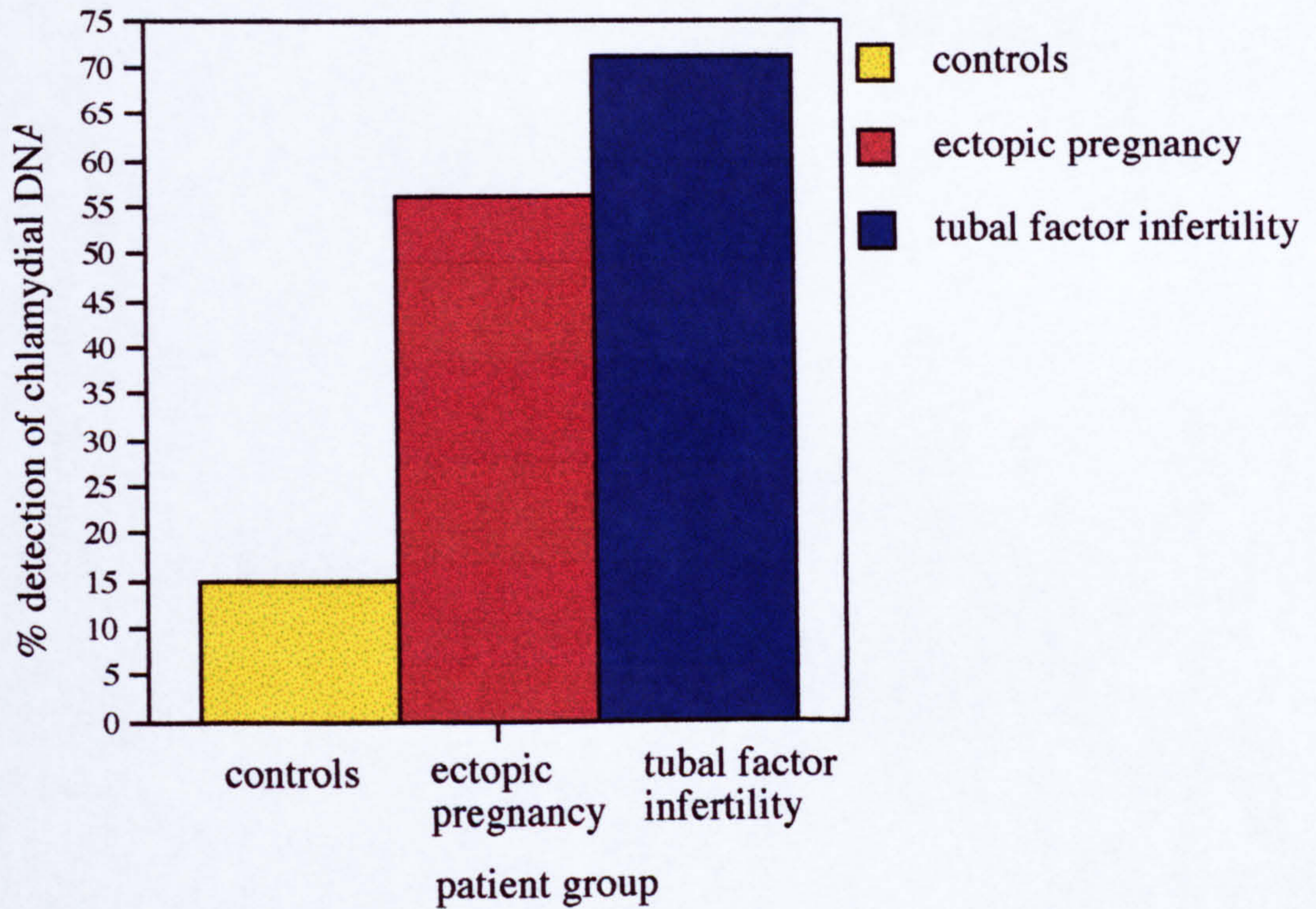
Patient group	% of patients positive for <i>C. trachomatis</i> DNA.	Tissue sample breakdown of positive samples		
		Endometrium	Ovary	Fallopian tube
Study controls mean age= 40yr SD= 6.2 age range= 33-57yr	15% (3/20)	0% (0/19)	5% (1/20)	10% (2/20)
Ectopic pregnancy (EP) mean age= 31yr SD= 5.7 age range= 24-40yr	56% (5/9)	22% (2/9)	22% (2/9)	33% (3/9)
Tubal factor infertility (TFI) mean age= 30yr SD= 3.7 age range= 24-36yr	71% (10/14)	58% (7/12)	38% (5/13)	42% (5/12)

Control group vs. EP
 Control group vs. TFI

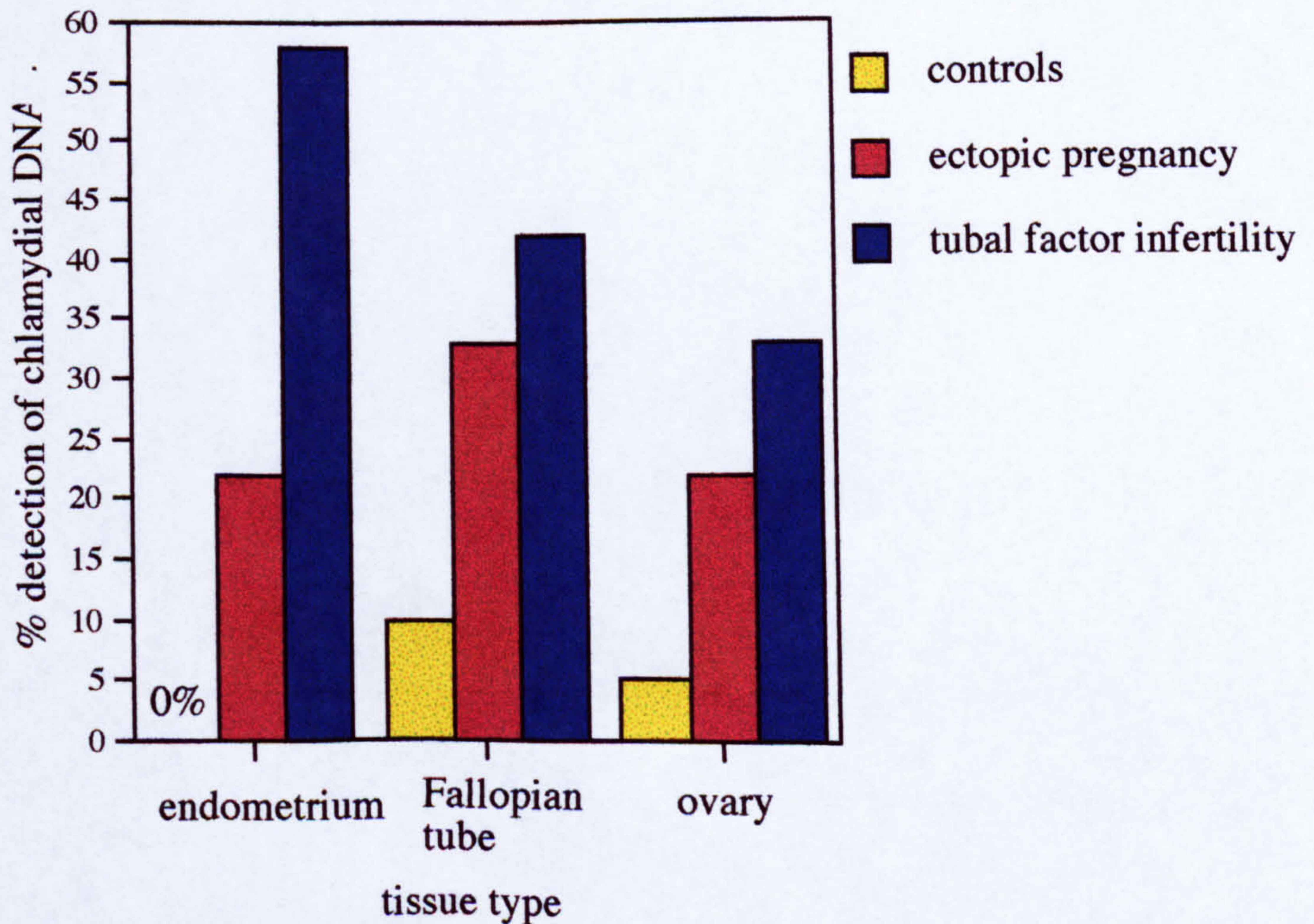
P>0.05
 P<0.05

no significant difference
 significant difference

Graph 6. Percentage detection of *C. trachomatis* DNA by polymerase chain reaction/Southern blotting in the Sheffield/Bristol groups.



Graph 7. Percentage detection of *C. trachomatis* DNA by polymerase chain reaction/Southern blotting in the individual tissuesites, Sheffield/Bristol.



3.5.2 Prevalence of *C. trachomatis* DNA in Trinidad

The detection of *C. trachomatis* plasmid DNA by polymerase chain reaction and Southern blotting are shown in Table 8, Graphs 8 and 9. The results for individual patients can be found in Appendix 4.

Table 8. Breakdown of the number of patients and tissue sites sampled positive for *C. trachomatis* plasmid DNA by polymerase chain reaction and Southern blotting for Trinidad (Mount Hope and Port of Spain).

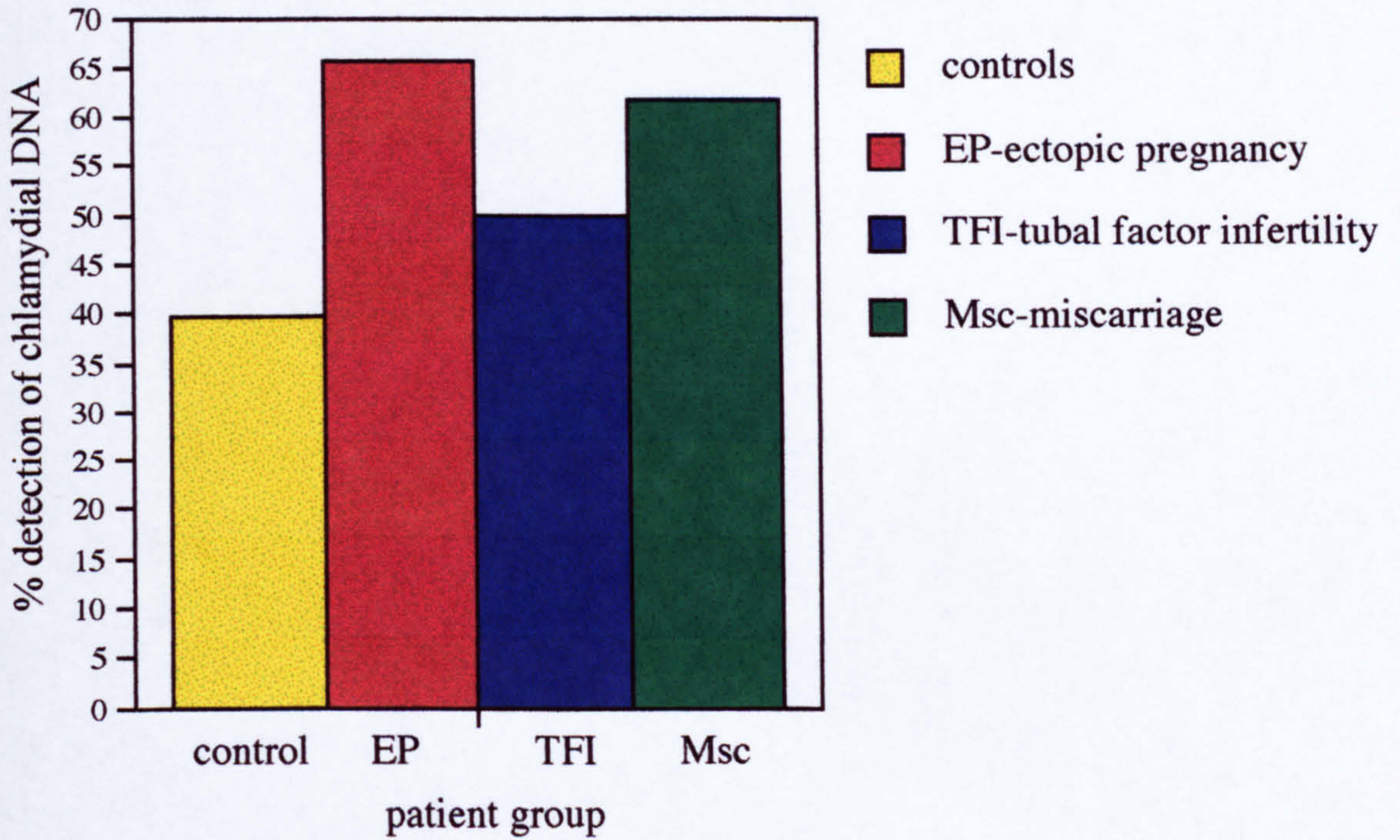
Patient group	% of patients positive for <i>C. trachomatis</i> DNA.	Tissue sample breakdown of positive samples		
		Endometrium	Ovary	Fallopian tube
Study controls mean age= 31.7yr SD= 5.3 age range= 22-44yr	40% (12/30)	6% (1/17)	32% (9/28)	24% (7/29)
Ectopic pregnancy (EP) mean age= 30yr SD= 6.3 age range= 16-40yr	66% (19/29)	42% (8/19)	62% (13/21)	50% (14/28)
Miscarriage (Msc) mean age= 33yr SD= 1.0 age range= 24-41yr	62% (13/21)	38% (3/8)	45% (9/20)	33% (7/21)
Tubal factor infertility (TFI)	50% (1/2)	0% (0/2)	0% (0/2)	50% (1/2)

Control group vs. EP
Control group vs. Msc

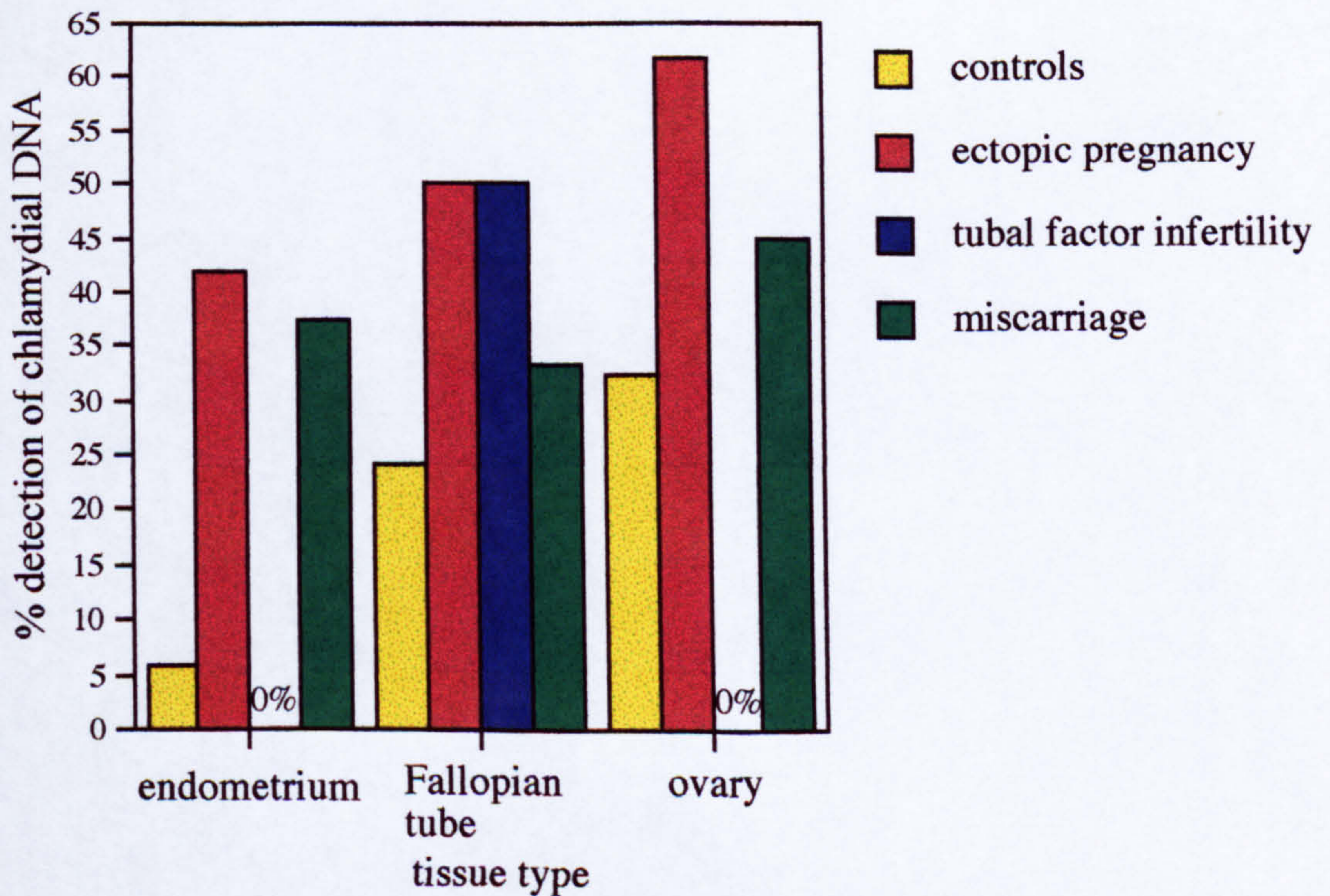
P>0.05
P>0.1

no significant difference
no significant difference

Graph 8. Percentage detection of *C. trachomatis* DNA by polymerase chain reaction/Southern blotting in Trinidad.



Graph 9. Percentage detection of *C. trachomatis* DNA by polymerase chain reaction/Southern blotting in the individual tissue sites sampled in Trinidad.



3.6 MOMP PCR results

3.6.1 MOMP PCR results

The results for the determination of the *C. trachomatis* serovar of MOMP positive samples can be found for individual patients in Appendices 3 and 4.

3.7 TNF polymorphism results

3.7.1 TNF- α results

The distribution of the TNF- α polymorphism -308A (P), as determined by PCR and *Nco*I digestion of the PCR product for Sheffield/Bristol can be found in Tables 9 and 10. The comparable results for Trinidad can be found in Tables 11 and 12. The frequency (f) of the TNF- α 308A polymorphism is expressed as the proportion of P alleles within each patient group. The individual TNF- α results for each patient can be found in Appendices 3 and 4.

Table 9. The detection of TNF- α -308G (N) and TNF- α -308A (P) alleles in Sheffield/Bristol study controls (*C. trachomatis* antibody negative) compared to *C. trachomatis* antibody positive ectopic pregnancy (EP) and tubal factor infertility (TFI) patients.

Patient Group	N:N	N:P	P:P	number (n)	frequency (f) of P
Study Controls	10	3	0	13	0.115
EP	6	2	1	9	0.222
TFI	6	6	1	13	0.308

Table 10. The detection of TNF- α -308G (N) and TNF- α -308A (P) alleles in all the Sheffield/Bristol study controls compared to ectopic pregnancy (EP) and tubal factor infertility (TFI) patients.

Patient Group	N:N	N:P	P:P	number (n)	frequency (f) of P
Study Controls	16	4	0	20	0.100
EP	6	2	1	9	0.222
TFI	7	6	1	14	0.286

Table 11. The detection of TNF- α -308G (N) and TNF- α -308A (P) alleles in Trinidad study controls (*C. trachomatis* antibody negative) compared to *C. trachomatis* antibody positive ectopic pregnancy (EP), tubal factor infertility (TFI) and miscarriage patients.

Patient Group	N:N	N:P	P:P	number (n)	frequency (f) of P
Study Controls	11	0	0	11	0.000
EP+TFI	22	4	0	26	0.077
Miscarriage (Msc)	14	2	0	16	0.063

Table 12. The detection of TNF- α -308G (N) and TNF- α -308A (P) alleles in all the Trinidad study controls compared to ectopic pregnancy (EP), tubal factor infertility (TFI) and miscarriage patients.

Patient Group	N:N	N:P	P:P	number (n)	frequency (f) of P
Study Controls	26	3	0	29	0.052
EP+TFI	25	5	0	30	0.083
Miscarriage (Msc)	16	3	1	20	0.125

3.7.2 TNF- β results

The distribution of the TNF- β polymorphism TNFB*1 (P), as determined by PCR and *Nco*I digestion of the PCR product, for Sheffield/Bristol can be found in Tables 13 and 14. The comparable results for Trinidad can be found in Tables 15 and 16. The

frequency (f) of the TNFB*1 is expressed as the proportion of P alleles within each patient group. The individual TNF- β results for each patient can be found in Appendices 3 and 4.

Table 13. The detection of TNFB*2 (N) and TNFB*1 (P) alleles in Sheffield/Bristol study controls (*C. trachomatis* antibody negative) compared to *C. trachomatis* antibody positive ectopic pregnancy (EP) and tubal factor infertility (TFI) patients.

Patient Group	N:N	N:P	P:P	number (n)	frequency (f) of P
Study Controls	9	4	0	13	0.154
EP	1	6	2	9	0.556
TFI	5	6	2	13	0.385

Table 14. The detection of TNFB*2 (N) and TNFB*1 (P) alleles in all the Sheffield/Bristol study controls compared to ectopic pregnancy (EP), and tubal factor infertility (TFI) patients.

Patient Group	N:N	N:P	P:P	number (n)	frequency (f) of P
Study Controls	11	9	0	20	0.225
EP	1	6	2	9	0.556
TFI	5	7	2	14	0.393

Table 15. The detection of TNFB*2 (N) and TNFB*1 (P) alleles in Trinidad study controls (*C. trachomatis* antibody negative) compared to *C. trachomatis* antibody positive ectopic pregnancy (EP), tubal factor infertility (TFI) and miscarriage patients.

Patient Group	N:N	N:P	P:P	number (n)	frequency (f) of P
Study Controls	6	4	1	11	0.273
EP+TFI	12	9	5	26	0.365
Miscarriage (Msc)	8	5	3	16	0.344

Table 16. The detection of TNFB*2 (N) and TNFB*1 (P) alleles in all the Trinidad study controls compared to ectopic pregnancy (EP), tubal factor infertility (TFI) and miscarriage patients.

Patient Group	N:N	N:P	P:P	number (n)	frequency (f) of P
Study Controls	13	14	2	29	0.310
EP+TFI	14	9	7	30	0.383
Miscarriage (Msc)	9	6	5	20	0.400

3.8 Conclusion

3.8.1 Sheffield/Bristol and Trinidad study

There have been several studies over the past decade that have looked at the prevalence of *C. trachomatis* DNA by PCR in women with ectopic pregnancy (EP), tubal factor infertility (TFI) and controls. The type of specimen used for PCR has varied, with studies investigating the presence of chlamydial DNA in cervical and urethral swabs (Osser *et al.*, 1992), archival cervical smears (Lan *et al.*, 1995), paraffin embedded tubal specimens (Lan *et al.*, 1995), and fresh endometrial biopsies (Lan *et al.*, 1995; Chernesky *et al.*, 1998). To the best of our knowledge there have been no studies reported in the literature that have used fresh endometrial, ovarian and Fallopian tube samples taken at the time EP, at the time of surgery for TFI, or from patients with a recorded history of miscarriage for PCR.

This study showed that PCR could successfully be applied to the detection of *C. trachomatis* DNA from fresh tissue specimens. The incorporation of the human β -globin PCR proved to be a reliable method for showing the successful extraction of human genomic DNA and the absence of PCR inhibitors. The use of multiple controls set up throughout the extraction procedure, and pre-PCR, the use of the GeneAmp Carry Over Prevention System coupled with standard PCR anti-contamination methods; and the use of a specific oligonucleotide to confirm and detect weak positive PCR products, have together given a reliable method for studying the prevalence of *C. trachomatis* DNA by PCR.

An important consideration in the interpretation of the PCR results discussed in the present study is the possibility of sampling error. There is no guarantee that tissue samples taken from patients were taken at the site of chlamydial infection, or that chlamydial DNA was not lost/diluted beyond the threshold sensitivity of the PCR employed giving rise to false negative results. In retrospect perhaps multiple samples of the same tissue site should have been taken to help overcome the potential problem of

sampling error.

The discovery of clinical *C. trachomatis* isolates that lack the 7.5-kb plasmid (Peterson *et al.*, 1990; An *et al.*, 1992; Farencena *et al.*, 1997) has prompted much speculation about the prevalence of such isolates in the general population. The effect these isolates may have on the prevalence rate of *C. trachomatis* if investigated by plasmid PCR alone is unclear, but since only three isolates have been described in the last eight years it may be their presence in the population is rare.

MOMP PCR failed to pick up the majority of plasmid positive samples. While it could be argued that those plasmid positive samples which were MOMP PCR negative were false positives, it is unlikely due to the anti-contamination methods and controls used. It is likely that the amount of chlamydial DNA was very small in extracted samples, since most of the plasmid positive samples tested positive only after Southern blotting.

Plasmid PCR is renowned for its sensitivity due to the presence of multiple copies of the plasmid in *C. trachomatis*. Although nested MOMP PCR as described by Lan *et al.* (1994) is reported to have a sensitivity rivalling that of a single PCR and Southern blotting, or nested plasmid PCR, it has in our hands a sensitivity of approximately 100 times less. Alternately, the presence of high background levels of human genomic DNA may have interfered with the amplification of the MOMP PCR product as was shown for the chlamydial single plasmid PCR.

Chlamydial DNA was detected in 15% of the control Sheffield/ Bristol control patients. The reported prevalence of *C. trachomatis* in the UK has ranged from 3.6-9.1 % of women attending gynaecology/antenatal or family planning clinics (Fish *et al.*, 1989; Hopwood *et al.*, 1990). It is important to remember that the majority of chlamydial epidemiological data is based on either culture or serology which could give a prevalence rate that may not truly reflect the carriage of *C. trachomatis* in the general population.

To date there have been no studies conducted that have determined the prevalence of *C. trachomatis* DNA in Trinidad by PCR. The only data available have been based on

culture (Chout *et al.*, 1995), and DIF (Dowe *et al.*, 1998) in other West Indian countries. The former giving a prevalence of *C. trachomatis* in the cervix of women in Martinique of 27%, and the latter detected *C. trachomatis* in the endocervix in 16% of pregnant Jamaican women studied. In our study chlamydial DNA was detected in 40% of the Trinidad control patients suggesting either a reservoir of persistent *C. trachomatis* in the population, or a very high incidence of asymptomatic/silent chlamydial genital infection..

Studies relating *C. trachomatis* infection and a possible role in miscarriage have given conflicting results. None of the studies investigating the possible role of *C. trachomatis* in miscarriage used DNA detection by PCR. Chlamydial DNA was detected in 62% of the patients in the miscarriage group, and although this was found not to be statistically significant when compared to the control group, it was over 20% greater. Interestingly a substantial proportion of the miscarriage patients tested positive in the endometrium ($P < 0.05$).

The mechanism by which *C. trachomatis* infection of the endometrium could induce miscarriage is unclear, it is possible that the presence of the organism facilitates the immune rejection of the foetus due to the production of TNF- α and IFN- γ . Alternately, TNF- α may damage the placenta directly (Clark and Chaouat, 1989; Hill, 1991), or cause foetal expulsion due to uterine contraction or the necrosis of implanted embryos; or it could act by thrombosing the blood supply to the developing foetus.

Normal pregnancy is characterized by a lack of a strong maternal cell mediated anti-foetal immunity and a dominant humoral immune response (Wegmann *et al.*, 1993; Voisin *et al.*, 1995). Some cytokines are beneficial to the success of pregnancy, whereas certain cytokines may lead to adverse effects on the conceptus either by direct embryotoxic activity or by damaging the placental trophoblast (Hill, 1991): IFN- γ inhibits trophoblast outgrowth *in vitro*, IFN- γ and TNF- α inhibit embryonic and foetal development as well as the proliferation of human trophoblast cell lines *in vitro* (Haimovici *et al.*, 1991). IFN- γ in combination with TNF- α is cytotoxic to rodent embryonic fibroblast-like cells (Suffrys *et al.*, 1989), and TNF- α mediates apoptotic death of trophoblast cells (Yui *et*

al., 1994). The inflammatory cytokines TNF- α , IFN- γ , and IL-2 have all been shown to terminate normal pregnancy when injected into pregnant mice (Chaouat *et al.*, 1990).

The prevalence of *C. trachomatis* DNA in the Sheffield/Bristol EP group was significantly higher than the appropriate control group ($P < 0.05$). Out of the 29 Trinidad EP patients studied many had one or more tissue sites missing and these were therefore unavailable for PCR analysis. Of these patients, 66% had detectable *C. trachomatis* DNA in one or more tissue specimens. This was not statistically significant when compared to the Trinidad control group ($P > 0.05$) but at a significance level of 10% would have been considered significant.

Within both EP groups, chlamydial DNA was detected in all the sample types by PCR, thus stressing the importance of ascending infection in the development of tubal damage that can predispose to tubal pregnancy. Osler *et al.* (1992) studied 33 EP cases for the presence of chlamydial DNA by PCR of DNA extracted from paraffin embedded tubal material removed at the time of salpingectomy. This study did not detect chlamydial DNA in any of the samples and concluded that there was no evidence of a persistent chlamydial infection. Conversely this study did demonstrate an association between the presence of anti-chlamydial IgG and ectopic pregnancy, suggesting a past and not a current chlamydial infection may have been responsible for the development of the EP.

Lan *et al.* (1995) reported similar findings in a retrospective study of forty-eight archival paraffin embedded salpingectomy specimens from thirty-seven women with EP. This study detected chlamydial DNA by PCR in only one patient. However, *C. trachomatis* DNA was detected in genital specimens (cervical smears and/or endometrial biopsies) that had been taken up to six years before the ectopic pregnancy. It is interesting to note that chlamydial DNA was not detected in endometrial, or cervical specimens taken at the time of surgery for EP, implying that a current genital tract *C. trachomatis* infection was not responsible for the EP. It was suggested, that in the cases studied, the EP was a late post-inflammatory complication of an ascending *C. trachomatis* resulting in damage/scarring of the Fallopian tube.

In Sheffield/Bristol and Trinidad, *C. trachomatis* DNA was detected in tissue samples taken at the time of EP. This apparent difference in the detection rate of chlamydial DNA may be due to the use of archival material for DNA extraction, or the amount of paraffin embedded material used for extraction. The presence of chlamydial DNA in a high proportion of both the Trinidad and Sheffield/Bristol EP groups from all sample types may indicate ongoing/persistent infection of *C. trachomatis*. The question of whether ongoing/persistent chlamydial infection plays a role in the development of tubal damage needs to be addressed using such methods as RT-PCR to determine whether the DNA detected was from an actively replicating organism/persistent infection, or from the persistence of DNA after the clearance of infection, although this is thought to last for only a few weeks.

The detection of chlamydial DNA was significantly higher in the Sheffield/Bristol TFI group ($P < 0.05$). Again, like both EP groups, chlamydial DNA was detected in all three sample types. 42% of the Sheffield/Bristol TFI patients had chlamydial DNA in the Fallopian tube implying that at some point the Fallopian tube had been/or was still infected with *C. trachomatis* inducing tubal damage; and 38% of the patients had positive ovarian samples which could indicate silent PID. 58% of patients had chlamydial DNA in the endometrium, this could indicate an active chlamydial infection. Cleary *et al.* (1985), using culture, recovered *C. trachomatis* from the endometrium in 25% of TFI patients studied with serological evidence of *C. trachomatis*. It is possible that the Sheffield/Bristol TFI group still have an active *C. trachomatis* infection, and the question of the role of active infection and inflammatory damage in women with tubal infertility needs to be investigated.

The importance of a positive ovarian sample by PCR is controversial. It may be that the *C. trachomatis* DNA detected in these patients was a contaminant of the peritoneal cavity, as described by Marana *et al.* (1990) who found by culture, 8/34 infertile women tested positive for *C. trachomatis* with two patients testing positive in the peritoneal fluid. If chlamydial contamination of the peritoneal cavity is the answer, it may indicate that those patients with a positive ovarian sample may in fact be suffering from silent/asymptomatic

chlamydial PID. This would imply that a proportion of patients from both the UK and Trinidad in all study groups may have silent/asymptomatic chlamydial PID.

The question of possible chlamydial infection of the ovarian epithelia is intriguing, it may be that such an infection could potentially affect ovarian function/reserve. Positive chlamydial serology has been associated with a poor ovarian response to gonadotropin stimulation during IVF (Keay *et al.*, 1998). Since IVF can be used in the treatment of TFI, TFI patients that have a positive ovarian sample may require alternative IVF management.

Within the EP, TFI groups in both the UK and Trinidad, and in the Trinidad miscarriage group chlamydial DNA was detected in more than one tissue specimen belonging to a particular patient (see Appendices 3 and 4). More importantly the detection of *C. trachomatis* DNA in endometrial samples appears to be suggestive of EP, TFI, or miscarriage although many studies have failed to culture *C. trachomatis* from endometrial samples from significant numbers of patients in comparative groups (Jones *et al.*, 1986b; Henry-Suchet *et al.*, 1987; Shepard *et al.*, 1989). Perhaps multiple sampling of the endometrium by biopsy may provide a screening method for the presence of *C. trachomatis*, whether an active or persistent infection?

3.7.2 Polymorphism detection in the TNF gene

It has been suggested that genetic factors influence susceptibility to the inflammatory sequelae of *C. trachomatis* infection, which has been supported by studies of ocular and genital chlamydial infections in humans (Mabey *et al.*, 1992; Turner *et al.*, 1993; Conway *et al.*, 1996; Conway *et al.*, 1997). It has been shown that *C. trachomatis* induced PID is associated with the expression of HLA A31, C2, and C3 (Kimani *et al.*, 1996); and the expression of major histocompatibility complex (MHC) class II allele DR has been linked to the antibody response to chlamydial heat shock protein 60 (CHSP60) in patients with trachoma (Peeling *et al.*, 1998). In a macaque model of chlamydial PID, Lichtenwalner *et al.* (1997) concluded that susceptibility or the relative risk to the formation of tubal

adhesions was correlated to the expression of MHC class I alleles, consistent with similar reports of chlamydial immunopathology in humans (Kimani *et al.*, 1996).

TNF- α has also been shown to be an important mediator of the inflammation process induced by chlamydial LPS (Ingails *et al.*, 1995), and TNF- α mRNA has been associated with trachomatous inflammation (Bobo *et al.*, 1996), and has been suggested to play a role in chlamydial persistence. TNF- α is also known to be involved in the process of fibrogenesis and tissue remodelling, including the stimulation of prostaglandin E₂, glycosaminoglycan and collagenase production by fibroblasts (Dayer *et al.*, 1985; Elias *et al.*, 1988; Vassalli *et al.*, 1992).

TNF- α may also be protective against active *C. trachomatis* infection, it has been shown to have an anti-chlamydial effect both *in vitro* (Shemer-Avani *et al.*, 1988; Shemer-Avani *et al.*, 1989) and *in vivo* in the *C. trachomatis* mouse pneumonitis model (Williams *et al.*, 1990) TNF- α has also been shown to be secreted at the genital site of *C. psittaci* infection in guinea pigs (Darville *et al.*, 1995), and in women with salpingitis (Toth *et al.*, 1992)

In cases of salpingitis, which can lead to tubal damage predisposing to ectopic pregnancy and tubal factor infertility, an immune mechanism may also contribute to damage of the Fallopian tube. Toth *et al.* (1992) examined fluids from the reproductive tract of seven women with salpingitis, and five controls for the presence of TNF- α . Three women with salpingitis, who were also culture positive for *C. trachomatis* showed TNF- α only in Fallopian tubes showing actual signs of disease. All control patients had negative findings at all genital tract sites. Only one patient had detectable levels of serum TNF- α , suggesting a localized cell-mediated immune activation in response to salpingitis.

In the UK, homozygous TNF- α -308A and TNFB*1 were not identified in the control group. The presence of the TNF- α -308A *Nco*1 polymorphism was found to be associated with TFI, as was the presence of the TNFB*1 *Nco*1 polymorphism in patients with evidence of having/or having had a chlamydial infection. In the EP group the TNF-

α -308A *Nco*1 polymorphism did not appear to be associated with the development of EP, but the presence of the TNFB*1 *Nco*1 was associated with the EP group (with anti-chlamydial, or without anti-chlamydial antibodies).

Polymorphisms in the TNF gene have been shown to affect transcriptional regulation, and in women with TFI increased levels of TNF- α production may induce immune mediated tissue destruction. In trachoma increased levels of TNF- α have been associated with high chlamydial loads and severe follicular inflammation (Bobo *et al.*, 1996) Conversely, if TNF- α production is reduced the infection may not be cleared, or become persistent which could also lead to immune mediated tubal damage.

In Trinidad homozygous TNF- α -308A patients were not detected in the control group, although TNFB*1 homozygotes were. In Trinidad the presence of either *Nco*1 polymorphism in the TNF gene was found not to be associated with either EP or miscarriage.

The data collected in this pilot study suggests in the UK that the TNF- α -308A polymorphism may be linked to TFI, independent of the detection of anti-chlamydial antibody. The TNFB*1 polymorphism, in the same group, appears to be linked to the detection of anti-chlamydial antibody. For the UK EP group, only the TNFB*1 polymorphism appeared to be detected in patients with antibodies against *C. trachomatis*. In the UK, in the small number of patients investigated the TNFB*1 polymorphism may be associated with the development of tubal pathology in response to a chlamydial infection. In Trinidad there appeared to be no association in the detection of TNF gene polymorphisms in any of the study groups, irrespective of anti-chlamydial antibody status.

Although it would be advantageous to be able to identify those women at risk from the adverse sequelae of chlamydial genital tract infection, it may be difficult to pin down a single genetic marker found exclusively in patients with tubal damage. It may be that a much larger genetic study encompassing HLA typing as well as the detection of

polymorphisms in genes of the TNF locus may hold some insight into the possible genetic disposition of some individuals to the deleterious effects of a chlamydial infection.

Chapter 4.0
***In situ* hybridization (ISH)**

4.1 Aims of chapter

The aim of work contained in this chapter was to develop an *in situ* hybridization (ISH) protocol that could be used to determine the prevalence of *C. trachomatis* in paraffin embedded genital tract tissue specimens from women with EP, TFI or miscarriage.

Patients from both Sheffield/Bristol and Trinidad included women presenting with ectopic pregnancy (EP), miscarriage, tubal factor infertility (TFI), and controls undergoing either hysterectomy, or tubal sterilization. Three formalin fixed paraffin embedded tissue samples from each patient (endometrial, ovarian and Fallopian tube) were examined for the presence of *C. trachomatis* by ISH.

Also histological evidence of inflammation in the tissue specimens was investigated to determine whether inflammation correlated with the presence of *C. trachomatis* infection as assessed by the detection of chlamydial DNA.

4.2 Introduction

Serological studies on women with both ectopic pregnancy (EP) and tubal factor infertility (TFI) have demonstrated a strong association with chlamydial infection and tubal damage. However in these patient groups *C. trachomatis* has rarely been cultured from the reproductive tract (Henry-Suchet *et al.*, 1981; Cleary *et al.*, 1985; Thejls *et al.*, 1991; Patton *et al.*, 1994b; Radouani *et al.*, 1997), and the question of chlamydial persistence within genital tract tissues still remains somewhat of an enigma.

Obstruction of the Fallopian tubes resulting in infertility has been associated with chlamydial genital tract infection, but the actual mechanism by which the pathological changes develop is not fully understood. Acute salpingitis caused by *C. trachomatis* infection is associated with acute inflammation in the adjacent perisalpinx and parametrium, resulting in tubal damage, oedema and congestion. Microscopically there is an infiltrate of neutrophil polymorphs which may be followed by the loss of cilia. Chronic salpingitis, which consists of a plasma cell and mononuclear leukocyte infiltrate is not uncommon. In other cases the end result of tubal inflammation may be the formation of a hydrosalpinx, in which the tubes become distended with watery fluid due to the failure of the drainage of excess tubal fluid.

It has been suggested that one of the main mechanisms that may be involved in the immune mediated damage of the Fallopian tubes may be a delayed type hypersensitivity response (DTH) similar to that seen with repeated exposure to *C. trachomatis* in scarring trachoma. It is thereby possible that repeated chlamydial infection of the upper female genital tract, or the persistence of *C. trachomatis* within such tissues is central to the development of DTH. It has been noted that the incidence of tubal obstruction increases with the number of episodes of chlamydial pelvic inflammatory disease (PID) (Svensson *et al.*, 1983).

One of the first investigations into the presence of *C. trachomatis* in Fallopian tubes of women with acute salpingitis was published in 1979 by Moller *et al.* This study did not

find any chlamydial inclusions by electron microscopy and noted that ciliated cells did not show any notable structural changes although the nucleus of ciliated cells did show degenerative alterations with a distorted morphology. Non-ciliated secretory cells showed protrusions with plenty of microvilli indicating an increased luminal surface. In non-ciliated epithelial cells, an increased number of lysosomes were seen suggesting increased secretory activity.

In animal model studies it has been shown that repeated chlamydial infection of the Fallopian tubes with *Chlamydia* resulted in the characteristic pathological changes associated with DTH, when compared against controls infected only once (Patton *et al.*, 1987; Patton *et al.*, 1989a; Tuffrey *et al.*, 1990; Patton *et al.*, 1994a). Although in these studies the route of infection was by direct inoculation into the uterine horns, Rank *et al.* (1995a) demonstrated the same phenomenon with repeated chlamydial infection via the natural vaginal route.

In the guinea pig model of repeated chlamydial infection (Rank *et al.*, 1995a) tubal blockage was associated with mesosalpingeal fibrosis. Scanning electron microscopy, 30 days post-infection confirmed significant alterations in the ciliated and secretory epithelial cells of the tubal mucosa suggestive of tubal dysfunction. Similar observation of tubal damage associated with hydrosalpinx induced by chlamydial infection have been seen in mice (Patton *et al.*, 1989b), rabbits (Patton *et al.*, 1982), and guinea pigs (Sweet *et al.*, 1980; Schachter *et al.*, 1982; Rank *et al.*, 1992; Rank *et al.*, 1995a)

In situ hybridization (ISH) may be defined as the direct detection/ localisation of specific nucleic acid sequences in intact cellular material. The technique of ISH was originally described simultaneously by two research groups working both in the United Kingdom (John *et al.*, 1969), and in the USA (Gall and Pardue, 1969). In clinical situations, ISH has been used to provide cytological information on the location/mutation of genomic sequences in metaphase spreads as well as the direct detection of nucleic acid sequences of microbial origin in paraffin embedded, and frozen material.

The basic principle of ISH involves the pre-treatment of the cellular/tissue preparation to unmask the target nucleic acid; the hybridization of a nucleic acid probe to a complementary region of the target sequence; and finally the detection of the labelled probe followed by the subsequent visualization of the target/probe complex.

In situ hybridization can be performed using probes that have been isotopically labelled, followed by autoradiography for the detection of hybridized duplexes; or by using probes labelled non-isotopically, with for example biotin coupled with a streptavidin detection system. However the use of isotopic probes is time consuming, and quicker and safer approaches using non-isotopic probe labels have been developed. The disadvantage of using biotinylated probes is that biotin has a widespread endogenous tissue distribution contributing to high back ground/non-specific staining. Other labels such as digoxigenin and fluorescein in conjunction with a Fab fragment antibody conjugate system have been found to exhibit equal or superior sensitivity to biotin with lower non-specific background staining.

ISH has been used in the detection of chlamydial DNA in a variety of studies using many different sample types including rectal biopsies (Horn *et al.*, 1988); culture (Horn *et al.*, 1986; Naher *et al.*, 1988); cervical specimens (Horn *et al.*, 1986; Ghirardini *et al.*, 1991); synovial tissue from patients with Reiter's syndrome (Beutler *et al.*, 1995); semen specimens (Yoshida *et al.*, 1994); and prostate biopsies (Corradi *et al.*, 1996).

C. trachomatis DNA was first demonstrated by ISH in Fallopian tube tissue from patients with tubal infertility by Campbell *et al.* (1993) who showed chlamydial DNA in 44% of the TFI patients studied. This was later confirmed in a study by Patton *et al.* (1994) who concluded that chlamydial infection of the salpinx tended to be chronic and asymptomatic. In the monkey model of chlamydial salpingitis and tubal infertility, chlamydial DNA was detected at the sites of inflammation and tissue damage by ISH (Cappuccio *et al.*, 1994). It was suggested that *C. trachomatis* could persist in Fallopian tube specimens, and may be directly involved in the stimulation of the immune-mediated tissue destruction.

4.3 Materials

4.3.1 Reagents

Reagents were purchased from Sigma (Dorset, UK) unless otherwise stated.

4.3.2 *EcoR*1 2.5kb probe preparation

Ampicillin was prepared in sterile water as a stock solution of 50mg/ml, and stored at 4°C. Ampicillin was added to cooled nutrient broth or agar to give a final concentration of 50µg/ml.

E. coli **JM83** carrying the recombinant plasmid pCTL12A was originally acquired from Prof. Ian Clarke, University of Southampton, UK.

Nutrient broth and agar were purchased from Oxoid Ltd. (Hants, UK), and prepared as per the manufacturer's instructions.

Restriction endonucleases and the relevant buffer system were purchased from Gibco Life Technologies Ltd. (Paisley, UK) and stored at -20°C.

4.3.3 Polymerase Chain Reaction (PCR)

OmniGene PCR machine (Hybaid Ltd., Middlesex, UK) with a heated lid was used for all PCR applications. An Omni Gene flat block satellite module was used for non-isotopic *in situ* hybridization.

Sterile aeroguard tips and positive displacement tips were purchased from Alpha Laboratories Ltd., Hampshire, UK.

0.5ml PCR tubes were purchased from GeneMate (Kaysville, UT, USA), and were

validated as being DNase and RNase free. They were autoclaved prior to use.

Stock nucleotides containing dATP, dCTP, dGTP, and dTTP were purchased as a polymerisation mix (Bioline, London, UK). The DNA polymerisation mix contained 20mM of each nucleotide in solution, and was stored at -20°C in a designated DNA-free freezer.

Cloned *Pfu* DNA polymerase was obtained from Stratagene Ltd. (Cambridge, UK), at a concentration of 2.5U/μl. The polymerase was stored at -20°C in a designated DNA-free freezer.

x10 *Pfu* PCR reaction buffer was supplied by Stratagene and contained 200mM Tris-HCl, 20mM MgSO₄, 100mM KCl, 100mM (NH₄)₂SO₄, 1% Triton X-100 and 1mg/ml nuclease-free BSA.

4.3.4 PCR primers

PCR primers were purchased from R & D Systems Ltd. at 0.2mM with no further purification, diluted to 100pmol/μl and aliquots of 100 μl were stored at -20°C. One aliquot was stored at 4°C for routine day to day use.

Nested *Chlamydia trachomatis* PCR primers

The primary PCR primers, T1 and T2, have been previously described (p.98).

						binding position (nucleotide [nt])
T3	Forward	5'	ATC CAT TgC gTA gAT CTC Cg	3'		2622-2641nt
T4	Reverse	5'	gCC ATg TCT ATA gCT AAA gC	3'		2940-2921nt

The binding position of primers T3 and T4 were based on the published sequence of the *C. trachomatis* 7.5kb plasmid (Hatt *et al.*, 1988). T1 and T2 were originally designed by Claas *et al.* (1990), T3 and T4 were in-house primers.

Human β -globin primers (Saiki *et al.*, 1985; Lan *et al.*, 1995)

The PCR primers PC03 and PC06 used for the detection of human genomic DNA were as previously described (p.98).

4.3.5 Isotopic *in situ* hybridization

[α -³⁵S]dCTPaS was purchased from Amersham Life Sciences (Bucks, UK) with a specific activity of >1000Ci/mmol (product code SJ 1305). The storage and handling of the radiolabelled nucleotide was as per the manufacturer's guidelines.

Proteinase K stock solution of 20mg/ml was made in sterile water, and 50 μ l aliquots were stored at -20°C.

1M DTT stock solution was made in sterile water and stored in 1ml aliquots at -20°C.

100% de-ionized formamide was made using AG501-X8 resin (BioRad Ltd., Herts, UK). 20g of resin was initially washed with 20ml of formamide, the liquid was discarded. 400ml of formamide was then added to the resin, and the mixture stirred for 1h. Finally the deionized formamide was filtered twice through filter paper to remove the resin and aliquoted in 50x1ml volumes and stored at -20°C. The remaining 350ml was used to make 95% deionized formamide.

95% deionized formamide

100% deionized formamide	332.5ml
0.1xSSC	17.5ml

The solution was placed in a glass bottle, and the bottle covered in foil due to the light-sensitive nature of the solution.

x10 ISH buffer

20mM sodium acetate	0.164g
3M NaCl	17.532g
10mM EDTA	0.372g
sterile purified water	100ml

The sodium acetate was initially added to the 100ml of sterile water, and the pH of the solution was adjusted to 5.0 before the addition of the rest of the buffer components. The ISH buffer was stored at -20°C.

Proteinase K digestion buffer

100mM Tris-base	12.11g
50mM EDTA	18.61g
sterile purified water	1L

350ml volumes were stored at -20°C. The digestion buffer was pre-warmed to 65°C and 10mM of DTT and 5µg/ml of proteinase K was added before use.

50% dextran sulphate was made by the addition of 5g of dextran sulphate to 10ml of sterile purified water. The solution was heated to facilitate the dissolving of the dextran sulphate before the solution was aliquoted in 350µl volumes and stored at -20°C.

Blocking buffer

100% deionized formamide	500µl
50% dextran sulphate	200µl
x10 ISH buffer	100µl
50xDenhardt's solution (Amersham)	40µl
10mg/ml ssDNA	10µl
purified water	130µl

The blocking buffer was prepared fresh for each ISH run.

Hybridization buffer

100% deionized formamide	250µl
50% dextran sulphate	100µl
x10 ISH buffer	50µl
50xDenhardt's solution (Amersham)	20µl
10mg/ml ssDNA	5µl

The amount of probe used was calculated to give 1×10^5 cpm/10µl of hybridization solution (taking into account ^{35}S has a half life of 87.4 days), the final volume of the hybridization solution was made up to 495µl with purified water.

NTB-2 emulsion was purchased from Kodak (IBI Ltd., Cambridge, UK) and stored in a light-tight container at 4°C away from any radioactive sources.

D-19 developer and Rapid Fix were purchased from Kodak, and prepared as per the manufacturer's instructions. Both solutions once prepared were stored at 4°C and used cold.

4.3.6 Dot blotting

Hybond™-N+ positively charged membrane was purchased from Amersham.

Denaturation solution

1.5M NaCl	87.66g
0.5M NaOH	20.0g
purified water	1000ml

Neutralization solution

1.5M NaCl	87.66g
0.5M Tris-HCl	78.8g
purified water	1000ml

4.3.7 ECL direct hybridization solutions

The ECL direct hybridization kit was purchased from Amersham and stored at 4°C.

ECL direct hybridization buffer

hybridization buffer (supplied)	475ml
liquid block (supplied)	25ml
0.5M NaCl	14.6g

The hybridization buffer and liquid block were warmed to 40°C, the NaCl added and the buffer stirred until all the salt had dissolved. The buffer was aliquoted in 10ml amounts and stored at -20°C for a maximum of 3 months.

20xSSC stock solution

0.3M Na ₃ C ₆ H ₅ O ₇ ·2H ₂ O	88.2g
3M NaCl	175.3g
purified water	1000ml

The pH was adjusted to 7.0, autoclaved and the solution stored at room temperature.

Primary wash buffer

6M urea	360g
0.4% (w/v) SDS	4g
20xSSC	25ml
purified water	975ml

The buffer was stored at 4°C.

Secondary wash buffer

20xSSC	100ml
water	900ml

The buffer was stored at 4°C.

ECL detection reagents (supplied) were mixed in equal volumes in safe-light

conditions and used immediately.

Hyperfilm ECL autoradiography paper was obtained from Amersham and stored in the dark at room temperature.

Ilford PQ universal developer was purchased from Ilford Ltd. (Cheshire, UK), and diluted 1:10 with water before use.

Ilford Ilforspeed fixer was purchased from Ilford Ltd. and diluted 1:4 with water before use.

4.3.8 Non-isotopic *in situ* hybridization (NISH)

The Amersham DNA colour Kit was used for NISH. The anti-fluorescein alkaline phosphatase (AP) conjugate and blocking component were stored at 4°C, and the remainder of the kit at -20°C.

Glassware was cleaned in 7x detergent (ICN Biomedical, Oxfordshire, UK), rinsed in tap water followed by sterile purified water and finally dried at 80°C for a minimum of 24h.

Phosphate buffered saline (PBS) was prepared by dissolving a PBS tablet (Oxoid) in 100ml of water, which was then autoclaved and stored at room temperature.

TBS buffer

10mM Tris-HCl	15.76g
400mM NaCl	23.38g
purified water	1000ml

The pH was adjusted to 7.5, the solution was then autoclaved and stored at room temperature.

Pepsin digestion buffer

0.2M HCl	350ml
pepsin (500µg/ml)	0.7ml

The pepsin (activity of 3200-4500U/mg protein) was prepared as a stock solution of 500µg/ml in purified water and stored in 1ml aliquots at -20°C. The pepsin digestion buffer containing 1µg/ml pepsin was prepared fresh for each ISH run.

ISH hybridization buffer

As supplied in Amersham DNA Colour Kit.

4xSSC
2x Denhardt's solution
600µg/ml herring testes DNA
rate enhancing compound

The hybridization buffer was supplied as a 2x concentrate, for ISH it was diluted 1:1 with deionized formamide. The working ISH buffer was aliquoted in 1ml amounts and stored at -20°C.

ISH wash 1

20xSSC	25ml
0.1% (w/v) SDS	0.5g
purified water	475ml

The buffer was stored at room temperature.

ISH wash 2

20xSSC	12.5ml
0.1% (w/v) SDS	0.5g
purified water	475ml

The buffer was stored at room temperature.

ISH Blocking solution

TBS	500ml
0.5% (w/v) blocking component (supplied)	2.5g

The blocking solution was gently warmed until the blocking component had fully dissolved and was then stored at 4°C. Before use the blocking solution was pre-warmed to 37°C.

Antibody solution

TBS	500ml
0.5% (w/v) BSA fraction V (Winlab)	2.5g

The solution was stored at 4°C. Prior to use the anti-fluorescein alkaline phosphatase conjugate (supplied in the Amersham DNA Colour Kit) was diluted to 1:500 in the antibody solution.

Detection buffer

100mM Tris-HCl	15.8g
100mM NaCl	5.8g
50mM MgCl ₂	4.8g
purified water	1000ml

The Tris-HCl and NaCl were pre-mixed with water and the pH adjusted to 9.5, finally the MgCl₂ was added and the solution stored at 4°C.

Substrate Solution

detection buffer	10ml
nitroblue tetrazolium (NBT, supplied)	45µl
5-bromo-4-chloro-3-indolyl phosphate (BCIP, supplied)	35µl

The substrate solution was prepared fresh for each NISH run.

1% (w/v) Methyl green counterstain was prepared by washing the appropriate amount of methyl green (BDH) in chloroform to remove impurities, and was finally diluted to the correct concentration in water.

4.3.9 Patient specimens

Specimens were collected from Sheffield (Jessop Hospital for Women), Bristol (St. Michael's Hospital) and Trinidad (Mount Hope Women's Hospital; St. Augustine and the General Hospital, Port of Spain). From Sheffield/Bristol, three study groups were examined, women presenting with ectopic pregnancy (EP), women undergoing surgery for tubal factor infertility (TFI), and control patients. From Trinidad, four study groups were considered, women presenting with EP, women with a history of miscarriage, women undergoing surgery for tubal factor infertility, and control patients. The specimens taken from the miscarriage group were taken retrospectively. The study controls from Sheffield/Bristol were hysterectomy patients and controls from Trinidad were patients undergoing sterilization. The study had Ethics Committee approval at all sites (UK and Trinidad).

Three tissue samples were collected from each patient in the study; an endometrial sample; a sample of the Fallopian tube (ampulla, isthmus or fimbriae); and an ovarian core biopsy. For the patients belonging to the EP groups, tissue samples were taken at the time of surgery, and Fallopian tube samples were taken from the site of the EP. Tissue samples were fixed immediately in 10% clear neutral buffered formalin for a maximum of 24h and a minimum of 2h for histological examination, and *in situ* hybridization. Samples were coded with a patient reference number and the sample type.

4.4 Methods

4.4.1 Histological processing

Histology Processing Schedule

Samples were fixed for a maximum of 24h in clear neutral buffered formalin. Samples were then removed to a Shandon Citadel 2000 automatic processor for the following:- 2h in 70% alcohol x3; 2h in 95% alcohol x3; 2h in absolute alcohol x2; 2h in xylene x2; the samples were then immersed in paraffin wax for 2h and then in paraffin wax under vacuum for a further 2h. Samples were finally orientated and embedded in paraffin wax prior to sectioning

APES coating of slides

This was as previously described by van Prooijen-Knegt *et al.* (1983). Briefly, clean glass microscope slides (BDH Chemicals, Poole, UK) were immersed in 1% (v/v) lissol for 30min, washed under running tap water for a further 30min, rinsed twice in distilled water and finally dried overnight at 37°C. The slides were then coated in a freshly prepared 1% (v/v) 3-aminopropyltriethoxysilane (APES) in acetone, rinsed twice in acetone followed by washing twice in distilled water. Finally the slides were dried overnight at 37°C, and stored in a dust free environment at room temperature.

Sectioning 5µm sections were cut onto APES coated slides and dried at 37°C. Coated slides were finally stored in foil and used within 1 month.

Haematoxylin and Eosin staining

5µm sections were also cut onto glass slides for haematoxylin and Eosin staining (H&E staining). The sections were initially dewaxed in xylene twice for 2min, rehydrated through graded ethanol (100% x2, 95% x2, 70% x2) and finally rinsed in water for 1min. Slides were then dipped in Gill's Haematoxylin (BDH) for 2min, washed in tap water, placed in Scott's tap water substitute and then washed again in tap water. Slides were then placed in 1% Eosin (w/v) for 5min, rinsed briefly in water, dehydrated through

graded ethanol (2x70%, 2x95%, 2x100%) before being placed in xylene. Finally the sections were mounted in picolyte (Phase Separation Ltd., Clywd, UK) and dried at room temperature.

4.4.2 Isotopic probe preparation

326-bp human β -globin probe preparation

The double stranded DNA probe for the detection of human β -globin DNA was generated via PCR using primers PCO3 (Saiki *et al.*, 1985), and PCO6 (Lan *et al.*, 1995).

The PCR was performed on 1 μ l of human placental DNA diluted to 1 μ g/ μ l in a final reaction mix of 50 μ l. The final reaction mix contained 1.0mM MgCl₂; x 1 *Pfu* PCR buffer; 200 μ M of dNTP; 50pmol of each primer; and 2.5U *Pfu* DNA polymerase. The PCR consisted of 25 cycles of amplification, comprising denaturation at 94°C for 40sec, primer annealing at 55°C for 40sec, and primer extension at 72°C for 1min. Pre-PCR the tubes were incubated at 95°C for 4min, and post PCR at 72°C for 8min. The expected PCR product of 326-bp was visualised under UV light on a 0.8% agarose gel after ethidium bromide staining. The remaining PCR product was purified through a QIAquick column (Qiagen Ltd., Crawley, UK) as per the manufacturer's instructions to remove primers, *Pfu* and dNTP's. Finally, the probe concentration was measured at 260nm, aliquoted in 10 μ l amounts and stored at -20°C.

***Eco*R1 2.5kb probe preparation**

The recombinant plasmid pCTL12A containing pUC18 and *Pst*I digested pLGV440 (originally from *C. trachomatis* L1/440/LN) in *E. coli* JM83 was initially grown on ampicillin nutrient agar (Hatt *et al.*, 1988). A single colony was inoculated into 5ml nutrient broth and the culture shaken at 37°C overnight. The recombinant plasmid pCTL12A was recovered from 3ml of the resulting cellular suspension using the Wizard Plus Miniprep DNA purification system (Promega, Southampton, UK) as per the manufacturer's instructions.

The final purity of the plasmid preparation, and confirmation of the plasmid size was achieved by overnight digestion with *Pst*I. The digest was run on a 0.6% agarose gel, two fragments were observed, one fragment measured approximately 2.7kb (linearized pUC18) the other 7.5kb (linearized chlamydial plasmid). The 7.5kb fragment was extracted from the gel and the DNA purified using the Qiagen QIAquick gel extraction kit as per the manufacturer's protocol. The purified 7.5kb fragment was then digested with *Eco*R1 overnight and the digest run on a 0.6% agarose gel. Two fragments were visualized after ethidium bromide staining and UV transillumination, one fragment was found to be approximately 5kb, the remaining fragment was 2.5kb. The 2.5kb fragment was removed from the gel and purified as previously discussed.

Finally the DNA concentration of the 2.5kb *Eco*R1 fragment was measured using a spectrophotometer, and stored at -20°C.

4.4.3 Isotopic ISH

ISH probe labelling

The manufacture and storage of the radioactive probes was carried out in a designated radioactive area. Each probe was diluted in sterile purified water to a final concentration of 25ng in 10µl, denatured by boiling for 10min, and snap cooled on ice. The labelling reagents (Amersham Multiprime DNA labelling system) were thawed on ice prior to use. To 25ng of probe the following were added; 10µl of buffer (supplied); 2µl of primer containing random hexanucleotides; 2µl of Klenow fragment (2U/µl); 2.5µl of radiolabelled dCTP; and the total volume was made up to 50µl with sterile purified water. The labelling reaction was left to proceed at room temperature overnight.

The initial specific activity of the labelled probes was calculated as per the manufacturers instructions to be approximately 8×10^8 dpm/ug of DNA. Finally the radioactively labelled probes were stored at -20°C for a maximum of 5 weeks.

Isotopic ISH methodology

The isotopic methodology was based on the work of Campbell *et al.* (1993) and Patton *et al.* (1994).

Pre-hybridization

The sections were de-waxed in xylene for 15min, rehydrated through graded ethanol (3x100%; 2x95%; 1x70%; 1xwater) and then soaked in 0.2N HCl for 15min at room temperature. The sections were then rinsed in water and washed gently in approximately 1L of 2xSSC for 30min at room temperature. Sections were then lightly digested with the proteinase K digestion buffer for 5min and rinsed in water before being post-fixed in 10% formalin for 5min. The slides were then washed in water and left for 1min in 0.1M triethanolamine (pH 8.0), excess buffer was removed carefully by blotting. Acetic anhydride (final concentration of 0.25% in 0.1M triethanolamine) was added to a clean staining dish, the sections left to soak for 10min and then rinsed in water. Slides were then washed in 2xSSC for 30min at room temperature and rinsed in water before being dehydrated through graded ethanol (1x70%; 2x95%; 3x100%). Sections were then soaked in 95% formamide at 65°C for 15min, followed by 10min in ice cold 0.1xSSC for 10min and rinsed in water. Finally the slides were dehydrated through graded ethanol and left to drain.

Blocking of non-specific hybridization was achieved using a blocking buffer (1ml) which was boiled for 10min, and then chilled on ice for 5min before the addition of 20µl of 1M DTT. The final blocking buffer was then vortexed, 10-30µl (depending in the section size) was applied over each section and a glass coverslip placed over the top. Sections were then placed in a box containing a moist paper towel and incubated at 42°C for 2h. After incubation the coverslips were floated off in 2xSSC (2min), rinsed in water and dehydrated through graded ethanol. The sections were then ready for hybridization with the isotopic probe.

Hybridization

All the steps proceeding the pre-hybridization steps were carried out in a designated radio-

isotope lab, radioactive waste was disposed of as per the University of Sheffield's guidelines. Appropriate shielding, safety precautions and monitoring were used at all times. The hybridization buffer containing the radioactively labelled probe was boiled for 10min, and chilled immediately on ice for 10min before the addition of DTT to a final concentration of 20mM. An appropriate volume of hybridization buffer to cover each section was applied over the dehydrated tissues using an ISH EasiSeal (Hybaid). The EasiSeal was sealed and the slides left to hybridize overnight at 42°C in a moist chamber.

Post-hybridization

The EasiSeal was carefully removed, and the slides subjected to stringency washing in three changes of 4xSSC containing 10mM DTT (first wash, 15min; second wash, 15min; final wash, 30min). The slides were then washed in 1L of 2xSSC containing 10mM DTT for 30min at 37°C, followed by 1L of 0.1xSSC containing 10mM DTT for 30min at 42°C. Finally the slides were washed in 0.1xSSC containing 10mM DTT for 30min at 37°C, rinsed in water and dehydrated through graded ethanol containing 300mM ammonium acetate and left to air dry.

Under the appropriate safe-light conditions in a dark room the NTB-2 emulsion (Kodak) was melted in a beaker of hot water. 10ml of emulsion was diluted 1:1 with 600mM sodium acetate in a dipping chamber and the mixture allowed to aerate for 20min. The mixture was not shaken as this could introduce air bubbles into the emulsion which affects slide coating. Before the commencement of slide dipping a plain glass slide was dipped into the diluted emulsion to remove any air bubbles. Sections were dipped into the emulsion smoothly and evenly twice. The slides were allowed to dry vertically in a light-tight box for 3h before being transferred to a slide box containing a desiccant. The slide box was wrapped in multiple layers of foil followed by a black plastic bag and left to develop for 3 days at 4°C.

Developing hybridization signal

Under safe-light conditions the slides were removed from the box to slide carriers. The slides were developed for 3min in Kodak D-19 developer, rinsed in 2% acetic acid for

30sec to stop the developing reaction, and finally fixed for 5min in Kodak Rapid Fix. The sections were then washed in running water for 5min and then left to air dry if H&E counterstaining was not to be done the same day. After counterstaining the sections were examined by light microscopy.

One major problem encountered with the isotopic ISH procedure was the development of high levels of non-specific background staining. This occurred both on positive and negative control slides and tissue sections making the discernment of a true *C. trachomatis* or β -globin positive signal impossible. Both probes were tested for specificity using the Amersham ECL direct kit. Each probe was labelled using the kit and then used to hybridize to dot blots containing *C. trachomatis* DNA, purified *C. trachomatis* 7.5kb plasmid DNA, pUC18 DNA and human DNA. The *EcoR1* 2.5kb *C. trachomatis* probe was shown to hybridize against only the *C. trachomatis* DNA and purified *C. trachomatis* plasmid DNA (Figure 14). The human β -globin probe was found to hybridize only to human DNA (Figure 15).

Stringency washes and temperatures were increased, as was the concentration of acetic anhydride used in the pre-hybridization step, all of which had little effect on reducing the levels of background signal.

Finally the emulsion itself was investigated. As previously described the emulsion was prepared using 600mM ammonium acetate in complete darkness. Three plain glass slides were dipped in the diluted emulsion one was placed immediately in a light-tight box, one was exposed under safe-light conditions before storing in a light-tight box, the last slide was exposed to white light. All three slides were developed in total darkness. All the slides showed the development of a background signal. It was concluded from this that either the emulsion had been exposed to an unknown radioactive source, or that the darkroom conditions were inadequate. The same effect was observed with a new batch of emulsion that had been stored in a different location.

Due to the problems encountered with the radioactive ISH, a non-radioactive *in situ*

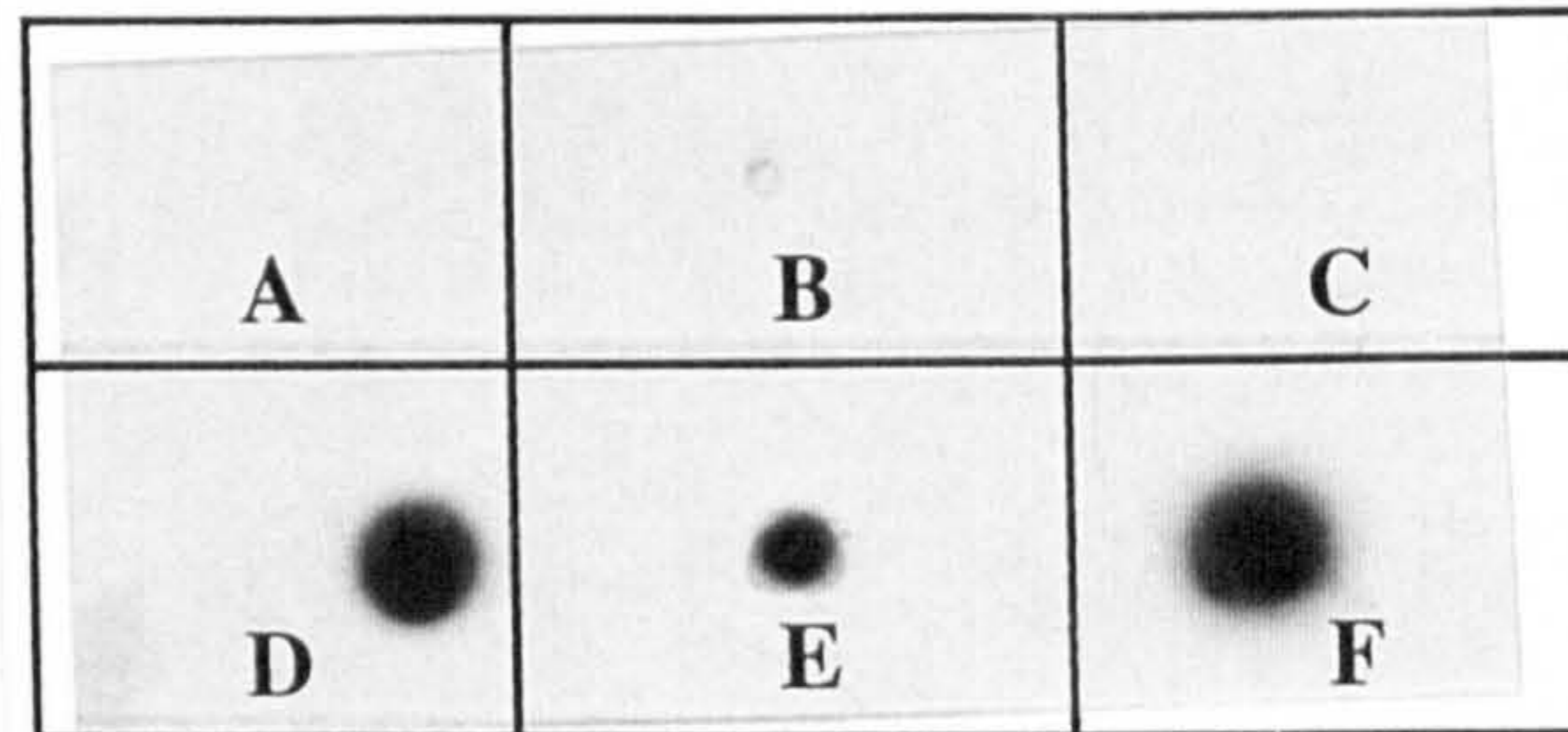
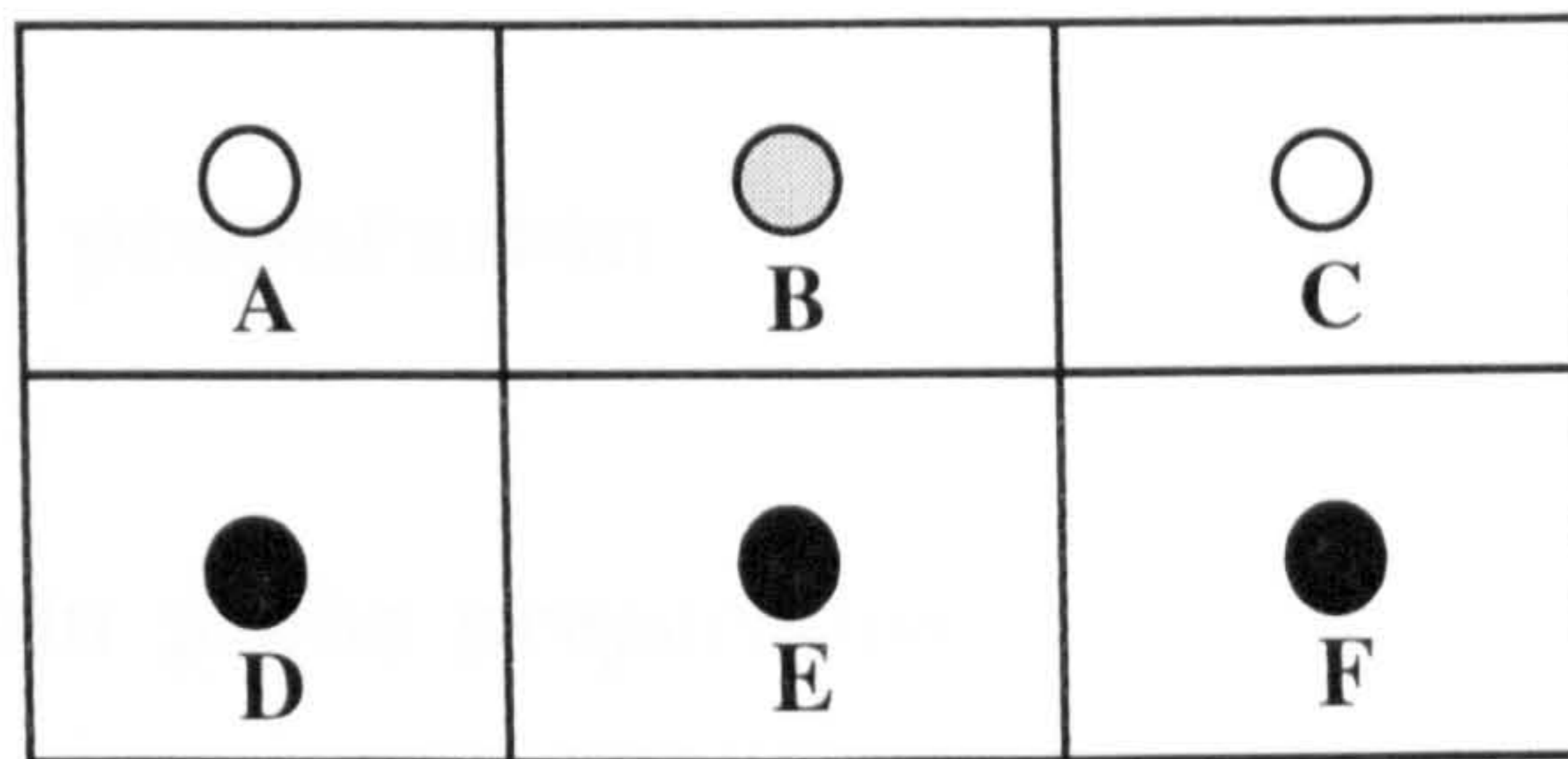


Figure 14. Dot blot using the 2.5kb *EcoR1* *C. trachomatis* probe. A-pUC18 DNA; B-LGV1 DNA; C-human DNA; D-pCTL12A; E, 7.5kb *C. trachomatis* plasmid fragment; F, 2.5kb *EcoR1* *C. trachomatis* fragment.

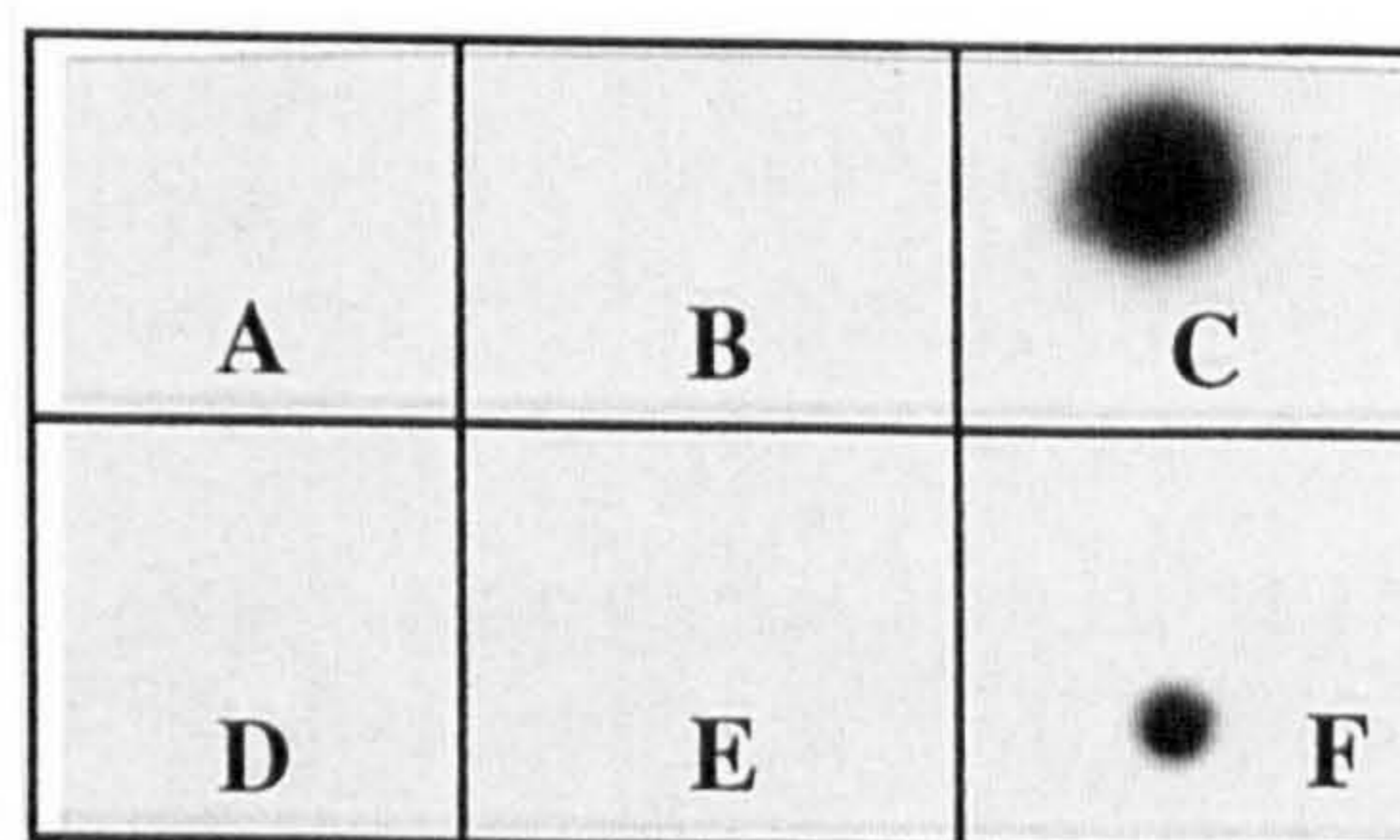
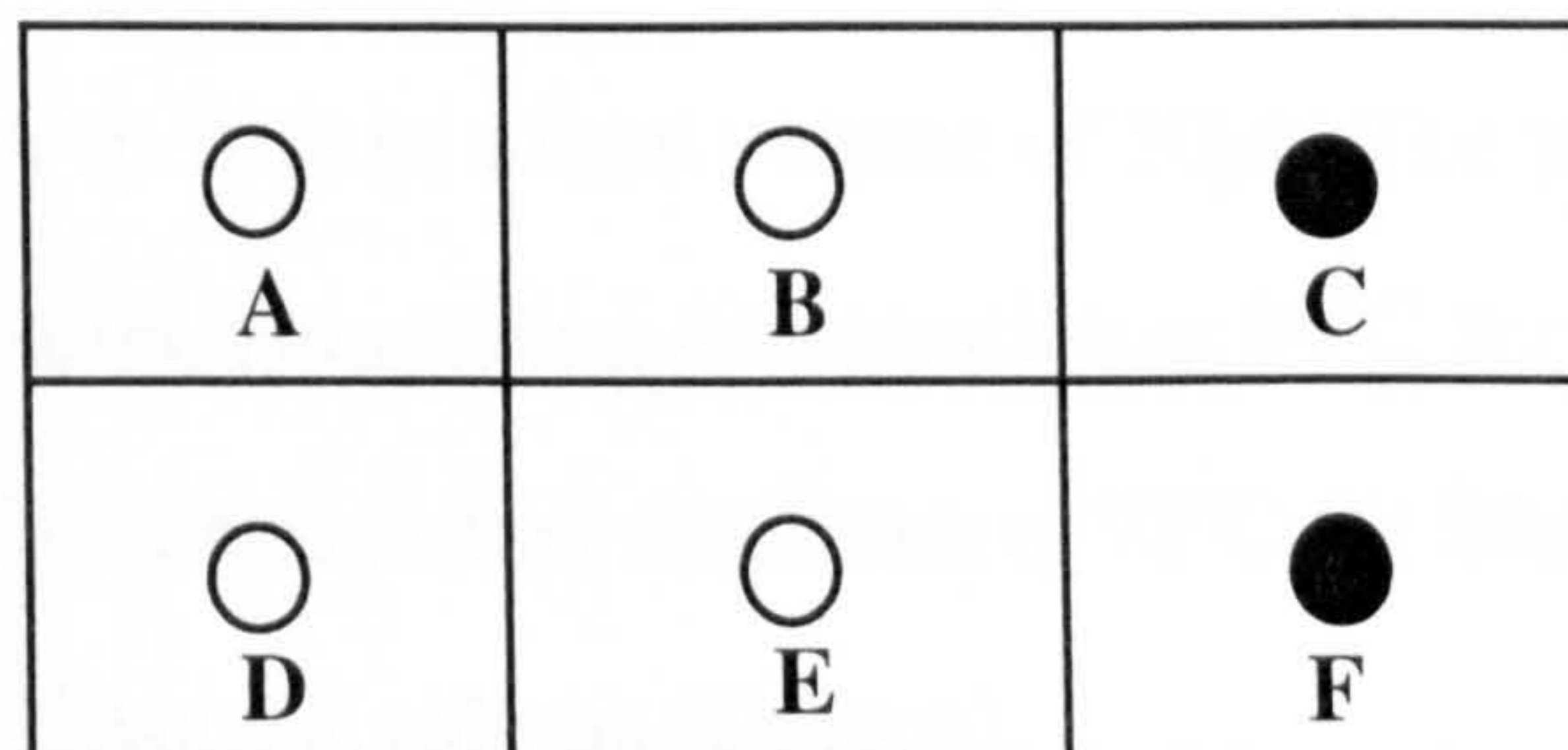


Figure 15. Dot blot using the 326-bp human- β globin specific probe. A-pUC18 DNA; B-LGV1 DNA; C-human DNA; D-pCTL12A; E, 7.5kb *C. trachomatis* plasmid fragment; F, 326-bp human- β globin polymerase chain reaction product.

hybridization protocol (NISH) was developed.

4.4.4 NISH probe preparation

326-bp human β -globin probe preparation

The human β -globin PCR product as previously described was also used for NISH

320-bp *C. trachomatis* probe preparation

The DNA probe for the detection *C. trachomatis* was a double stranded 320-bp DNA PCR product produced via a nested PCR. The primary PCR used the Claas *et al.* primers, T1 and T2 with *Pfu* DNA polymerase and *Pfu* reaction buffer (Stratagene). The secondary PCR used in-house primers (T3 and T4) internal to the primary 517-bp PCR product.

The primary PCR reaction mix contained 2.5mM MgCl₂; x1 *Pfu* PCR buffer; 200 μ M of dATP, dCTP, dGTP; and dTTP; 100pmol of each primer; 2.5U *Pfu* DNA polymerase and 50ng of LGV1 DNA (p.107) in a final volume of 50 μ l. The primary PCR consisted of 25 cycles of amplification, comprising denaturation at 94°C for 30sec, primer annealing at 55°C for 30sec, and primer extension at 72°C for 80sec. The PCR product was purified through a QIAquick column (Qiagen).

The secondary PCR was identical to the primary, except primers T3 and T4 were used, and 3 μ l of primary PCR product was used as template. Finally, the secondary PCR product of 320-bp was visualised on a 0.8% agarose gel under UV light to verify product size (Figure 16), and the remaining PCR product was purified through a QIAquick column. The DNA concentration of the probe was measured at 260nm, and stored at -20°C.

4.4.5 Preparation of dot blots

The sensitivity of both NISH probes was initially investigated by solution phase

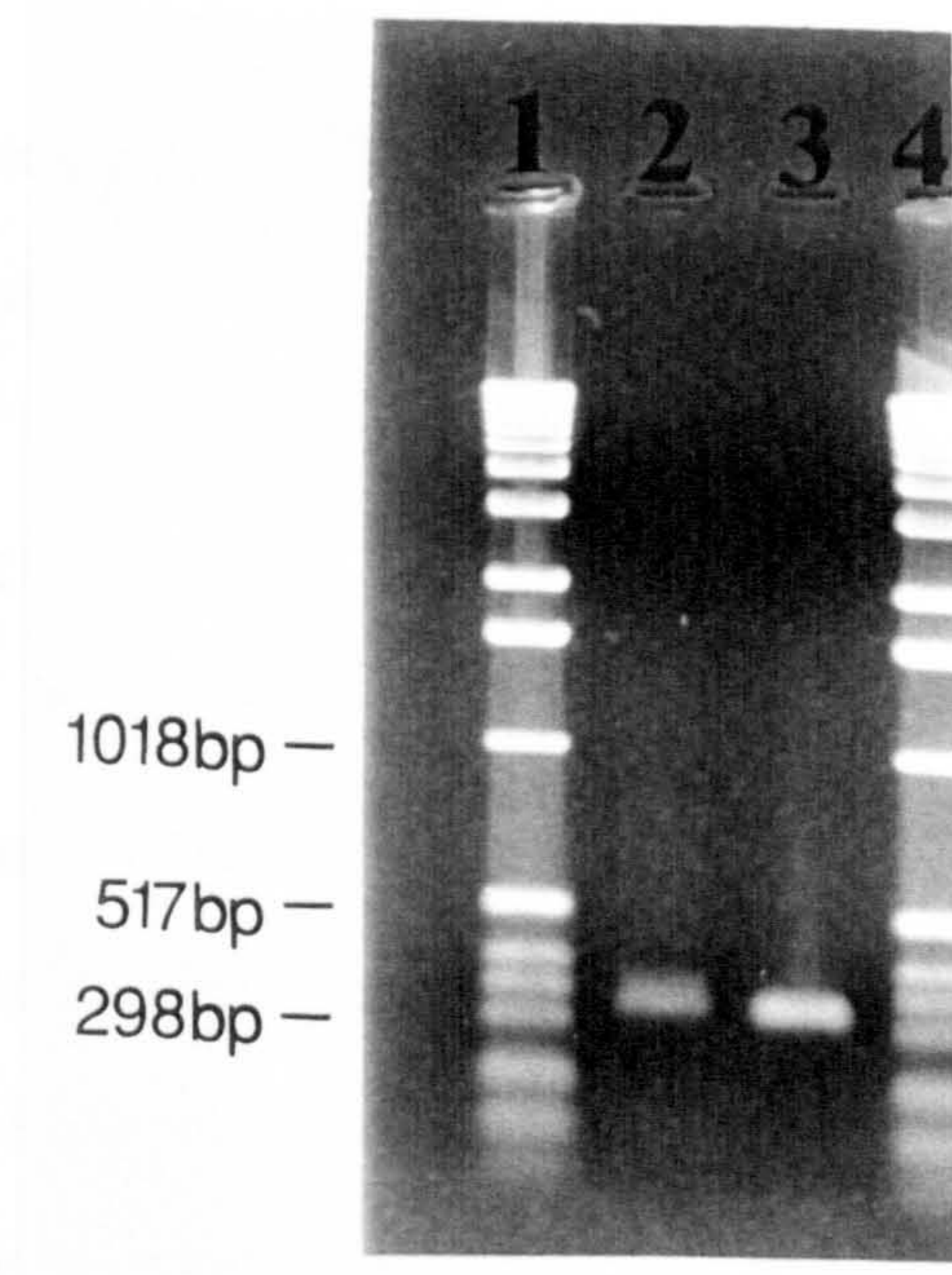


Figure 16. A representative 0.8% agarose gel showing Lane 1, 1kb ladder; Lane 2, 326-bp human β -globin polymerase chain reaction product; Lane 3, 320-bp *C. trachomatis* secondary polymerase chain reaction product; Lane 4, 1kb ladder.

hybridization. Human placental DNA was initially diluted from 1µg/µl to 0.03ng/µl in purified sterile water. 2µl of each dilution was dotted onto a piece of Hybond-N+ membrane and the membrane left to air dry. The membrane was then placed on a piece of filter paper pre-soaked in denaturation solution for 5min, followed by 5min on a filter paper pre-soaked in neutralization solution. The membrane was then left to air dry. Prior to hybridization the membrane was UV cross-linked.

The 320-bp *C. trachomatis* secondary PCR product was diluted in a similar manner (3.9ng/µl to 0.007ng/µl), and a dot blot prepared as above.

4.4.6 ECL direct nucleic acid labelling and detection

ECL direct labelling of probes

Each probe was diluted in sterile purified water to give 25ng DNA in 10µl, denatured by boiling for 5min, and snap cooled on ice. 10µl of DNA labelling reagent (supplied) was added to 10µl of probe, and mixed thoroughly. An equivalent volume (10µl) of glutaraldehyde solution (supplied) was then added, the mixture vortexed briefly, centrifuged and then incubated for 10min at 37°C. The labelled probe was either used immediately or stored at -20°C in 50% (v/v) glycerol.

ECL direct hybridization

10ml of hybridization buffer was pre-warmed to 42°C for 1h in a clean hybridization tube, the membrane was then added and left to pre-hybridize for a further 1h. A small volume of hybridization buffer was withdrawn to which 10µl (70ng) of probe was added, the hybridization buffer plus probe was then returned and left to hybridize overnight at 42°C in a hybridization oven.

The blot was removed from the hybridization buffer, placed in a clean hybridization tube with 20ml of pre-warmed primary wash buffer and left at 42°C for 10min. The primary wash buffer was discarded and 20ml of fresh primary one buffer added and left at 42°C for a further 10min. The blot was removed and placed in a clean container, covered in an

excess of secondary wash buffer and incubated at room temperature with agitation for 5min. The wash buffer was discarded, fresh secondary buffer added and the blot incubated for a final 5min.

Signal generation and detection

Under safe-light conditions in a dark room the two ECL detection reagents were mixed in equal volumes, the membrane drained of excess wash buffer and covered in the detection solution and left for 1min. The membrane was drained again, placed on an acetate in an X-ray developing cassette and covered with another acetate. An equivalent sized piece of ECL Hyperfilm was placed over the acetate-covered membrane and the cassette locked and left for a minimum of 5min. The film was then developed, fixed and washed and left to air dry. Depending on the signal strength another piece of film was placed over the membrane and left for a maximum of 24h.

Probe sensitivity by solution phase hybridization

The 326-bp human β -globin probe could detect down to 0.9ng of human DNA (Figure 17), and the 320-bp *C. trachomatis* probe 0.4ng of *C. trachomatis* DNA by solution phase hybridization (Figure 18). The detection threshold for NISH would be less than solution phase hybridization due to steric hindrance.

4.4.7 NISH

NISH probe labelling

Each probe was diluted in sterile purified water to a final concentration of 25ng/ μ l, denatured by boiling for 10min, and snap cooled on ice. The labelling reagents (Amersham DNA Colour Kit) were thawed on ice. To 2 μ l of probe (50ng) the following were added; 10 μ l of nucleotide mix containing fluorescein-11-dUTP; 5 μ l of nonamer primers; 1 μ l of Klenow fragment (4U/ μ l); and 42 μ l sterile purified water. The labelling reaction was left to proceed at 37°C for 1h. Finally the fluorescein labelled probes were stored at -20°C in the dark for a maximum of 6 months.

● A	● B	● C	● D	● E	● F	● G	● H
○ I	○ J	○ K	○ L	○ M	○ N	○ O	○ P

● A	● B	● C	● D	● E	● F	● G	● H
○ I	○ J	○ K	○ L	○ M	○ N	○ O	○ P

A-2 μ g **B**-1 μ g **C**-0.5 μ g **D**-0.25 μ g **E**-0.125 μ g
F-0.062 μ g **G**-0.031 μ g **H**-0.015 μ g **I**-7.8ng **J**-3.9ng
K-1.9ng **L**-0.9ng **M**-0.4ng **N**-0.2ng **O**-0.1ng
P-0.06ng

Figure 17. The determination of the detection threshold of the 326-bp human β -globin probe after solution phase hybridization.

● A	● B	● C	○ D	○ E
○ F	○ G	○ H	○ I	○ J

● A	● B	● C	○ D	○ E
○ F	○ G	○ H	○ I	○ J

A-7.8ng **B**-3.9ng **C**-1.9ng **D**-0.9ng **E**-0.4ng
F-0.2ng **G**-0.1ng **H**-0.06ng **I**-0.03ng **J**-0.015ng

Figure 18. The determination of the detection threshold of the 320-bp polymerase chain reaction *C. trachomatis* probe after solution phase hybridization.

ISH positive control slides

C. trachomatis positive control slides were prepared by growing LGV1 on coverslips as previously described (p.61). The presence of LGV1 was confirmed by immunofluorescence. Coverslips were then drained of maintenance media, washed briefly in PBS and fixed in acetone for 1min. The acetone was removed and the coverslips mounted on glass sides (cells facing upwards) with glue. The slides were then stored at 4°C.

Determination of digestion time

Formalin fixation prevents DNA and RNA degradation by endogenous nuclease activity and retains tissue/cellular morphology. Unfortunately cross-linking fixatives such as formalin used prior to paraffin embedding effectively mask nucleic acid sequences. To unmask nucleic acid sequences a proteolytic digestion step is necessary, but control of this process is essential as over-digestion can weaken protein structures that localise the target sequence leading to the diffusion/loss of a positive signal. If the digestion is inadequate the target sequences will not be unmasked and no signal will be evident.

To investigate the effect of digestion time, duplicate sections of an ovarian and Fallopian tube sample were de-waxed in xylene for 15min. The sections were then re-hydrated through graded ethanol (2x100%, 2x95%, 2x70%) and washed in PBS for 5min. One ovarian and one endometrial section were then digested for 15min in the pepsin digestion buffer at 37°C, the remaining two section were digested for 30min. All the sections were then rinsed in sterile water to prevent over-digestion and immersed in 20% (v/v) acetic acid for 15sec to remove endogenous alkaline phosphatase activity from within the sections. All sections were gently washed for 5min in PBS, dehydrated through graded ethanol (2x70%, 2x95%, 2x100%) and left to air dry.

The human β -globin specific probe was boiled for 5min, snap cooled on ice for 10min and diluted to 100ng/ml in ISH hybridization buffer. 65 μ l of the resulting mixture was placed over each section using an ISH EasiSeal (Hybaid), air bubbles were carefully removed, and the chamber sealed. The slides were then heated to 95°C for 10min using

an OmniGene flat block satellite module.

The slides were left to hybridize overnight at 42°C, the EasiSeal was removed and the slides subjected to stringency washing (ISH wash 1 for 2x5 min; ISH wash 2 for 2x10 min at 42°C) to remove non-specifically bound probe. The slides were then washed for 5min in TBS to remove any residual SDS, blocked for 1h in ISH blocking solution at 37°C, rinsed in TBS for 1min and then drained. The area surrounding each section was carefully dried and the section traced with a Pap Pen (Binding Site, Birmingham, UK) to provide a hydrophobic barrier to keep the antibody solution over the section. 70µl of antibody solution was placed over each section and the slides incubated in a humidified box at 37°C for 1h. The slides were then washed in TBS for 3x5min and left to drain.

Finally 150µl of substrate solution was placed over each section and the slides left to develop in the dark for 4h at room temperature. The reaction was terminated using water, and the slides were left to air dry prior to counterstaining. Figures 19 and 21 illustrate the effect of sufficient pepsin digestion leading to the development of a positive signal in the nucleus of the cells. Figures 20 and 22 show both the loss of cellular architecture, and a positive signal localized to the nucleus due to the over-digestion of the tissue sections. A digestion time of 15min was finally decided upon for further ISH runs.

NISH methodology

Study slides were de-waxed and pre-treated as described above. The *C. trachomatis* labelled probe was boiled for 5min, snap cooled on ice for 10min and diluted to 400ng/ml in ISH hybridization buffer. 65µl of the resulting mixture was placed over each section using an ISH EasiSeal, air bubbles were carefully removed and the chamber sealed. The slides were then heated to 95°C for 10min. The human β-globin specific probe was diluted to 100ng/ml in the same manner and used as a positive digestion control. Other controls included a *C. trachomatis* positive control slide probed with the *C. trachomatis* specific probe (Figure 23); a negative McCoy cell control slide probed with the *C. trachomatis* specific probe (Figure 24); a negative control slide (tissue section with neither probe) to determine the presence of non-specific background staining (Figure 25); a tissue

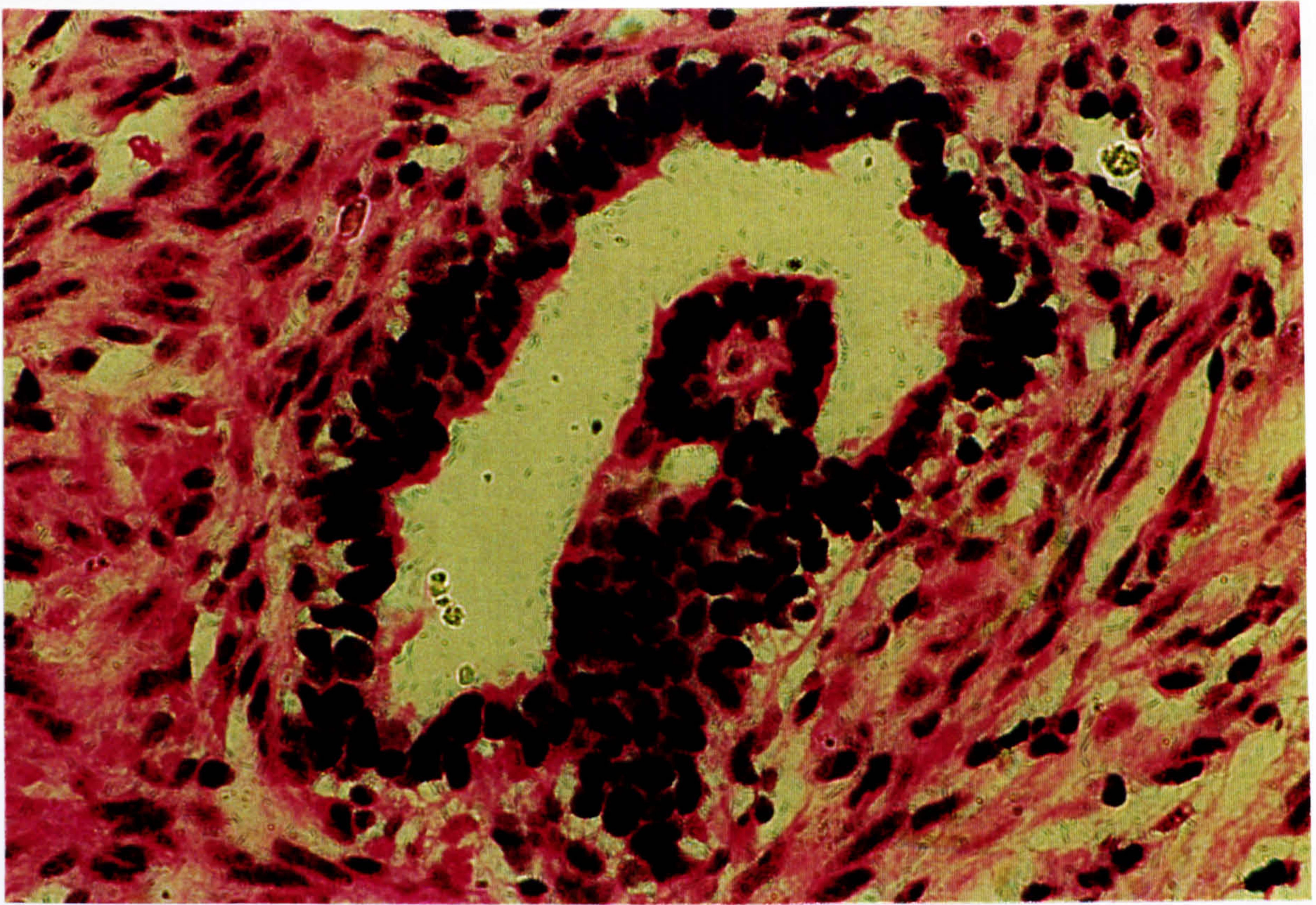


Figure 19. Eosin counterstained Fallopian tube section (mag.x400) after 15min pepsin digestion, probed with human β -globin specific probe. Section showing retention of morphology, and a localized non-isotopic *in situ* hybridization signal in the nucleus.

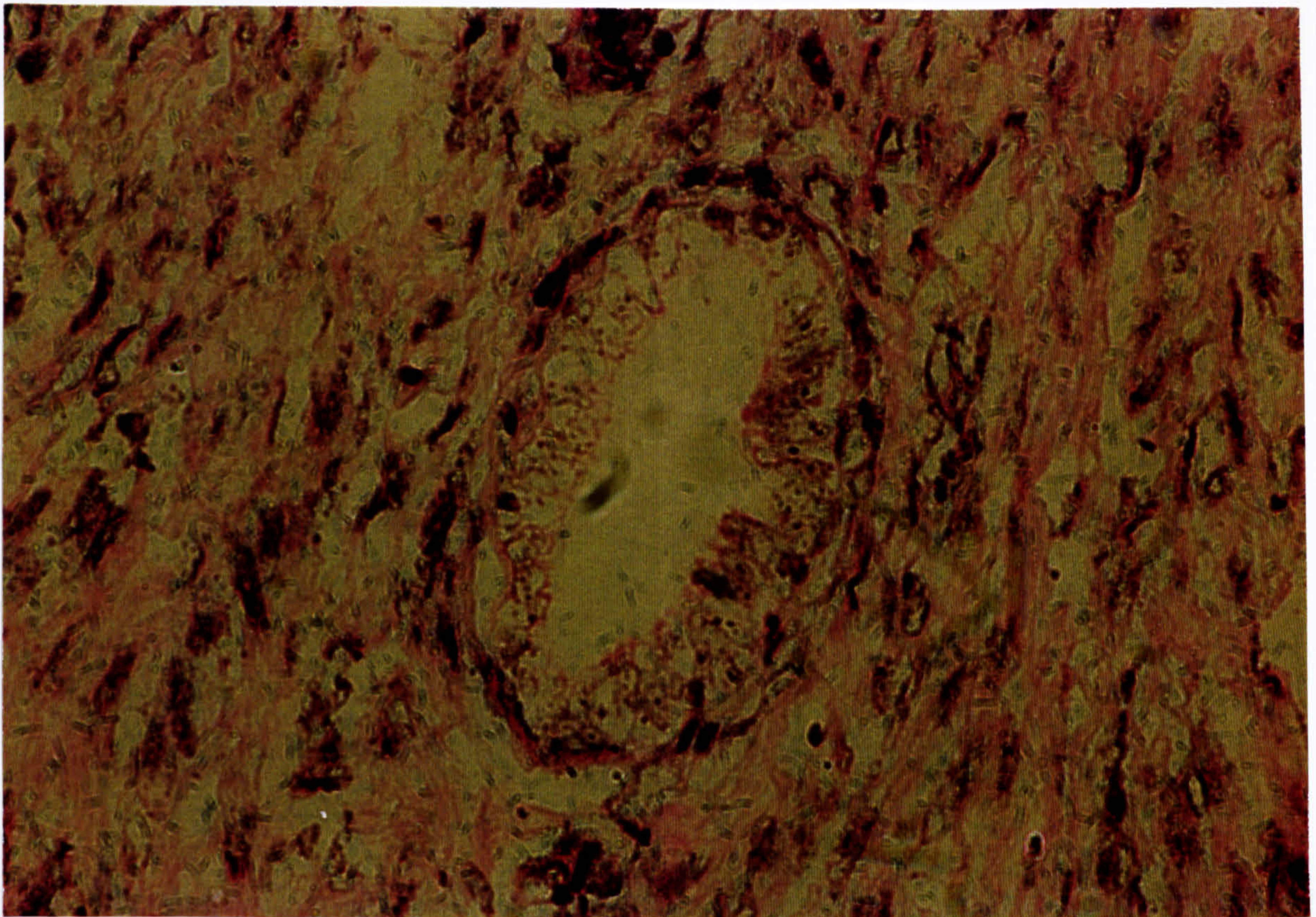


Figure 20. Eosin counterstained Fallopian tube section (mag.x400) after 30min pepsin digestion, probed with human β -globin specific probe. Section showing loss of cellular morphology, and non-isotopic *in situ* hybridization signal.

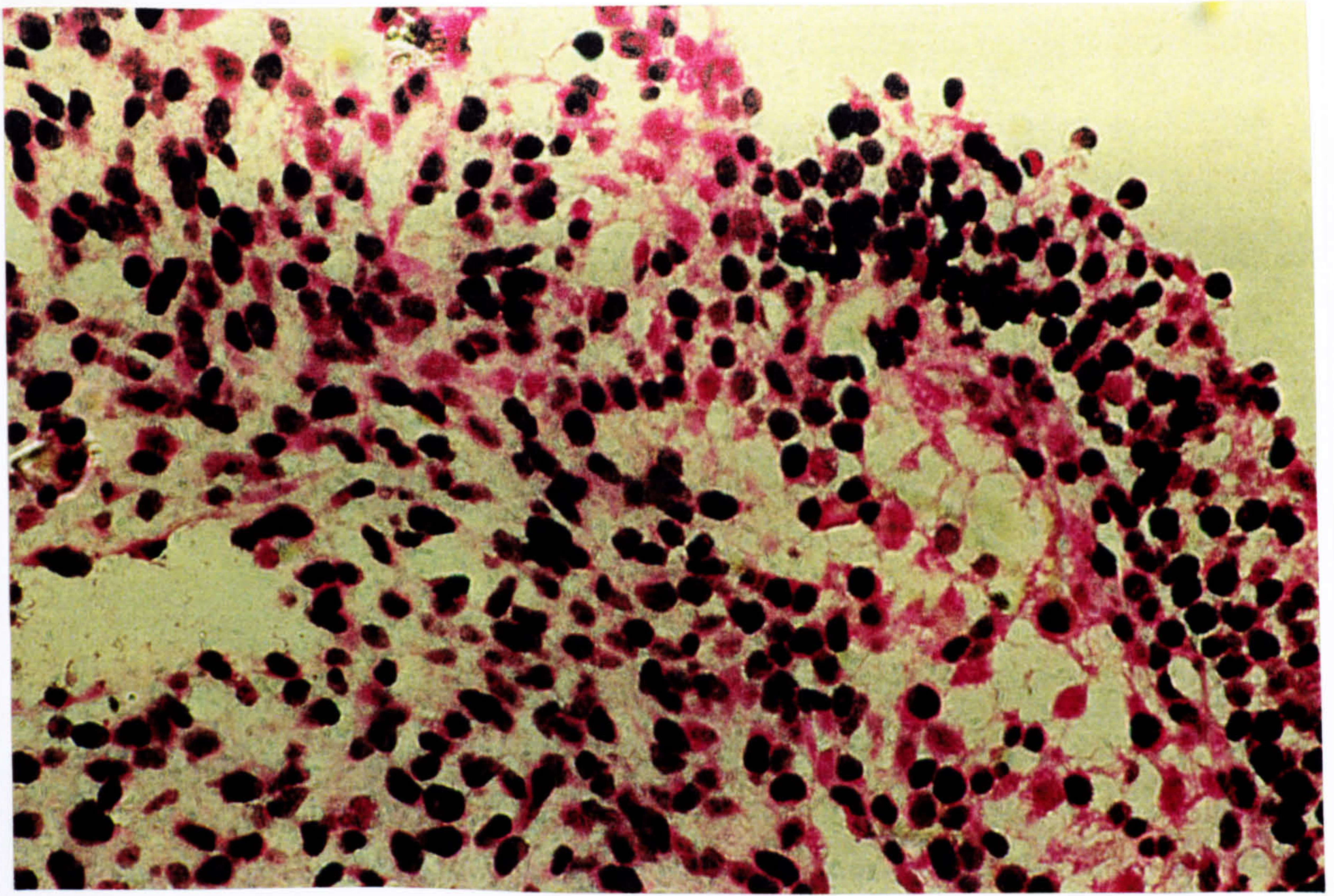


Figure 21. Eosin counterstained ovarian section (mag.x400) after 15min pepsin digestion, probed with human β -globin specific probe. Section showing retention of cellular architecture, and a localized non-isotopic *in situ* hybridization signal in the nucleus.

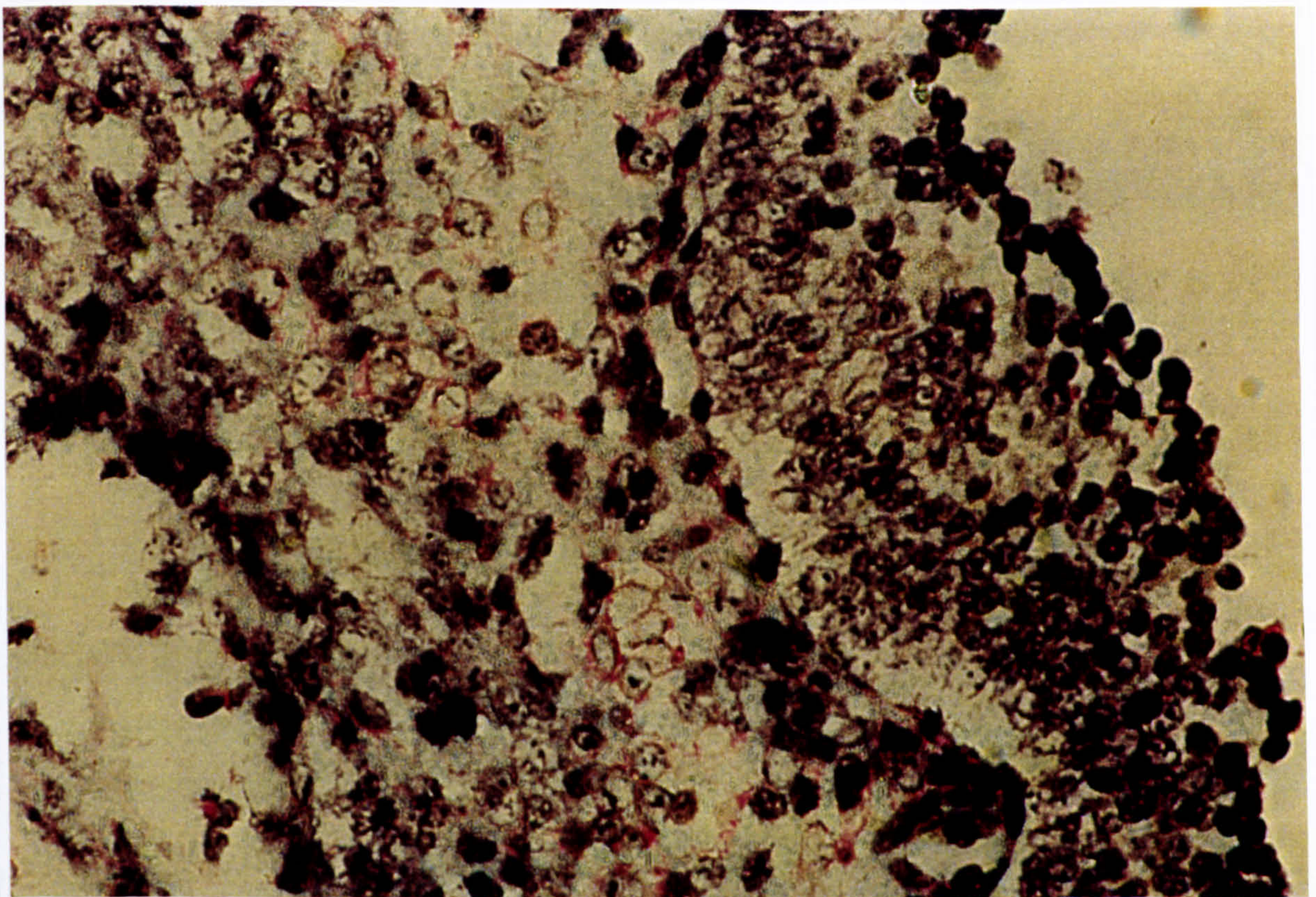


Figure 22. Eosin counterstained ovarian section (mag.x400) after 30min pepsin digestion, probed with human β -globin specific probe. Section showing loss of cellular architecture, and non-isotopic *in situ* hybridization signal.

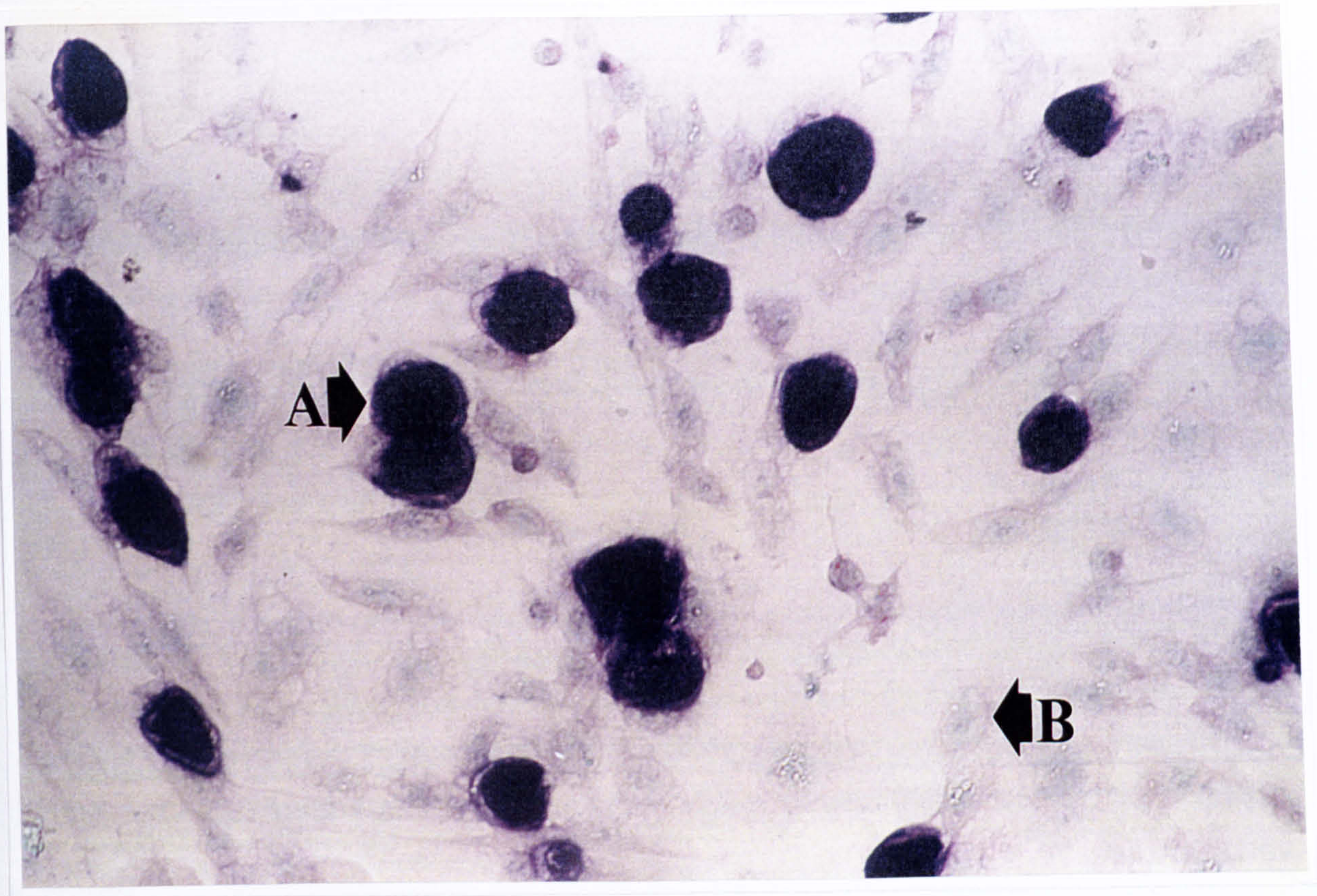


Figure 23. Methyl green counterstained McCoy cells infected with LGV1 and probed with the 320-bp *C. trachomatis* specific probe (mag.x1000). A- positive non-isotopic *in situ* hybridization signal identifying a chlamydial inclusion; B-uninfected McCoy cell showing no non-isotopic *in situ* hybridization signal.

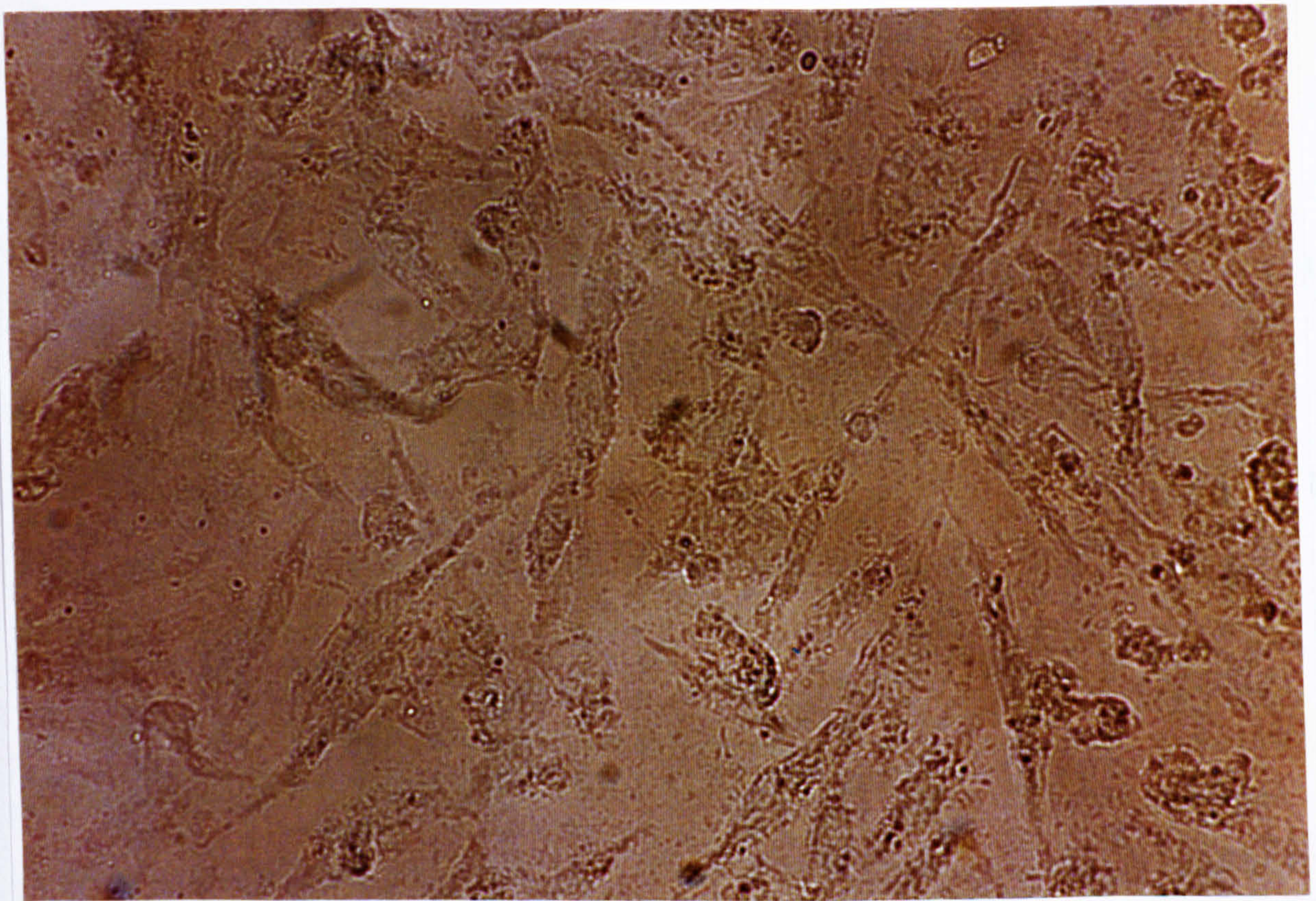


Figure 24. Methyl green counterstained uninfected McCoy cells probed with the 320-bp *C. trachomatis* specific probe (mag.x1000) after non isotopic *in situ* hybridization.

section of a lymph node biopsy of a patient with confirmed LGV (Kellock *et al.*, 1997) (Figure 26).

The slides were left to hybridize overnight at 42°C, the EasiSeal was removed and the slides subjected to stringency washing (ISH wash 1 for 2x5 min; ISH wash 2 for 2x10 min at 42°C) to remove non-specifically bound probe. The slides were then washed for 5min in TBS to remove any residual SDS, blocked for 1h in ISH blocking solution at 37°C, rinsed in TBS for 1min and then drained. The area surrounding each section was carefully dried and the section traced with a Pap Pen. 70µl of antibody solution was placed over each section and the slides incubated in a humidified box at 37°C for 1h. The slides were then washed in TBS for 3x5min and left to drain.

Finally 150µl of substrate solution was placed over each section and the slides left to develop in the dark for 4h at room temperature. The reaction was terminated using water, and the slides were left to air dry prior to counterstaining.

Methyl green counterstaining

After NISH the sections were rehydrated through graded ethanol (100%x2, 95%x2, 70%x2) and finally rinsed in water for 1min. Slides were immersed in 1% methyl green for 1min, rinsed briefly in water, dehydrated through graded ethanol (2x70%, 2x95%, 2x100%) before being placed in xylene. Finally the sections were mounted in picolyte and allowed to dry at room temperature. A positive signal was observed as a blue/purple /black precipitate within a cell as viewed under light microscopy.

4.4.8 Statistical analysis

The prevalence rate of *C. trachomatis* DNA by non-isotopic *in situ* hybridization for each study group was compared to the appropriate control group using the Fisher Exact Probability Test (2 tailed). A probability (P) value of <0.05 was considered to be statistically significant.

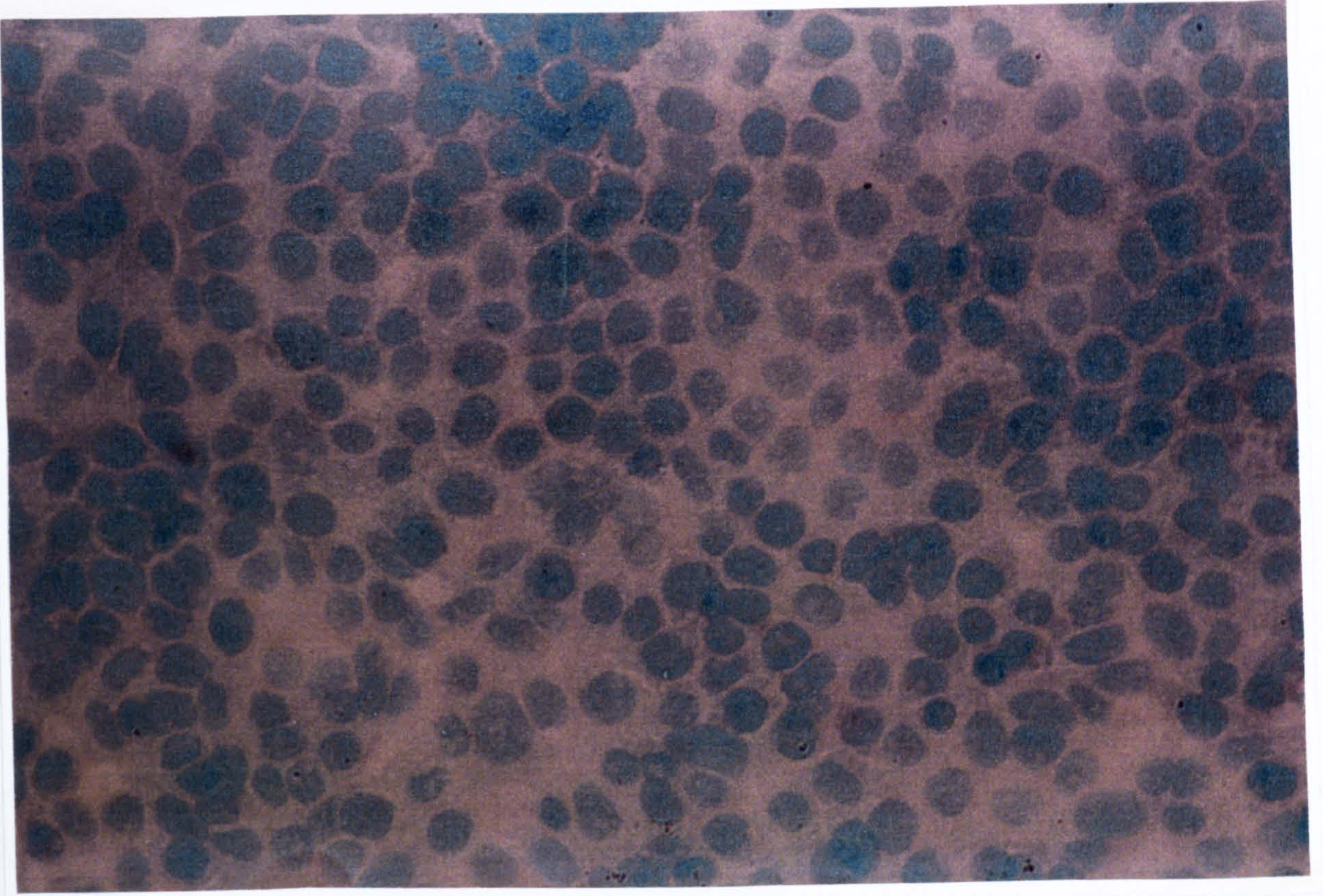


Figure 25. Methyl green counterstained endometrial section (mag.x1000) with neither probe after non-isotopic *in situ* hybridization.

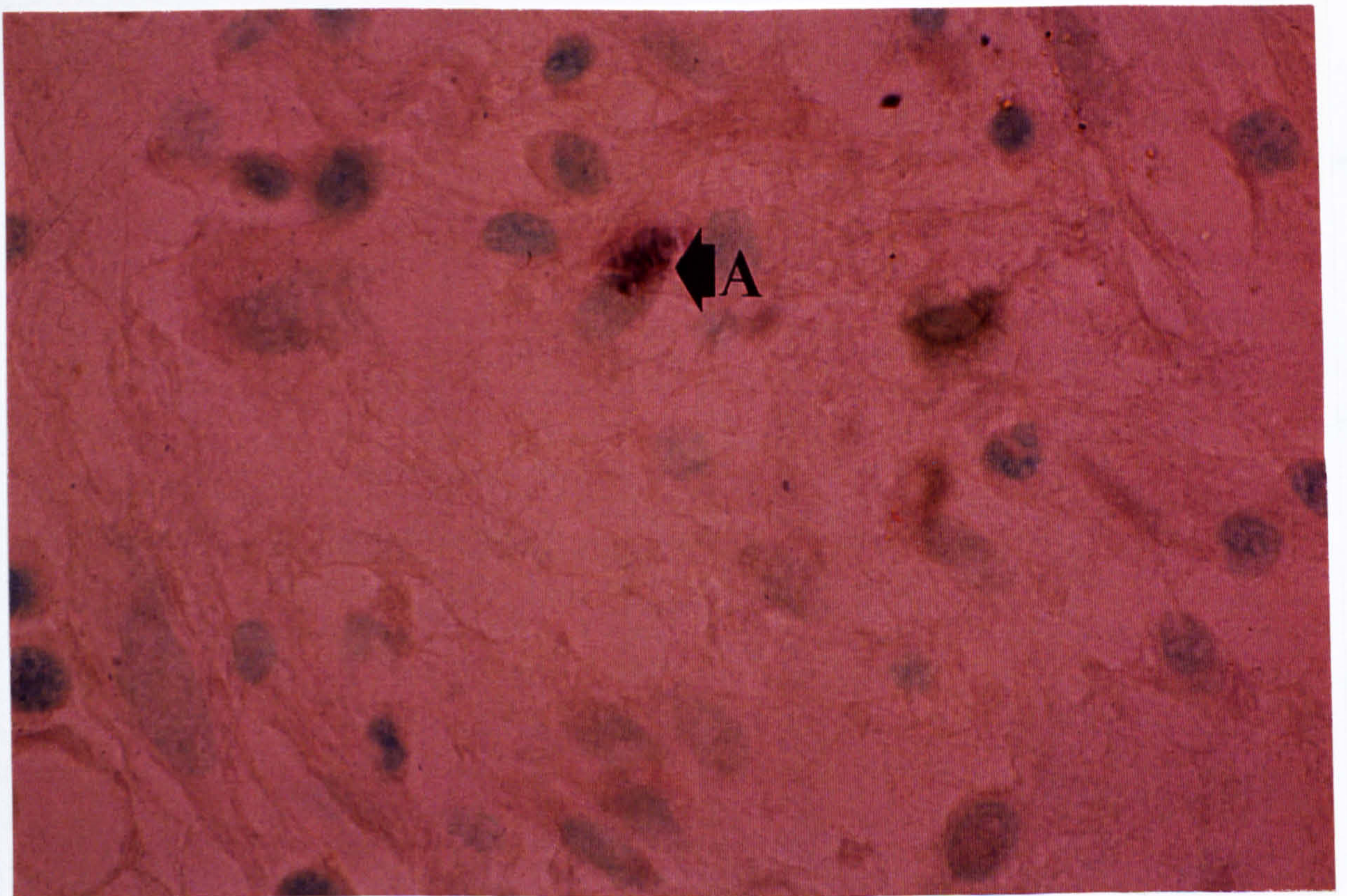


Figure 26. Methyl green counterstained section of a human lymph node biopsy from a patient diagnosed with LGV after non-isotopic *in situ* hybridization with the 320-bp *C. trachomatis* specific probe (mag.x1000). A- positive non-isotopic *in situ* hybridization signal demonstrating a chlamydial inclusion.

4.5 NISH results

4.5.1 Prevalence of *C. trachomatis* DNA in Sheffield/Bristol

The detection of *C. trachomatis* DNA by non-isotopic *in situ* hybridization (NISH) is shown in Table 17, and in Graphs 10 and 11. NISH results, and histological findings for individual patients can be found in Appendix 3.

Table 17. Breakdown of the number of patients and tissue sites sampled positive for *C. trachomatis* DNA by non-isotopic *in situ* hybridization for Sheffield/Bristol.

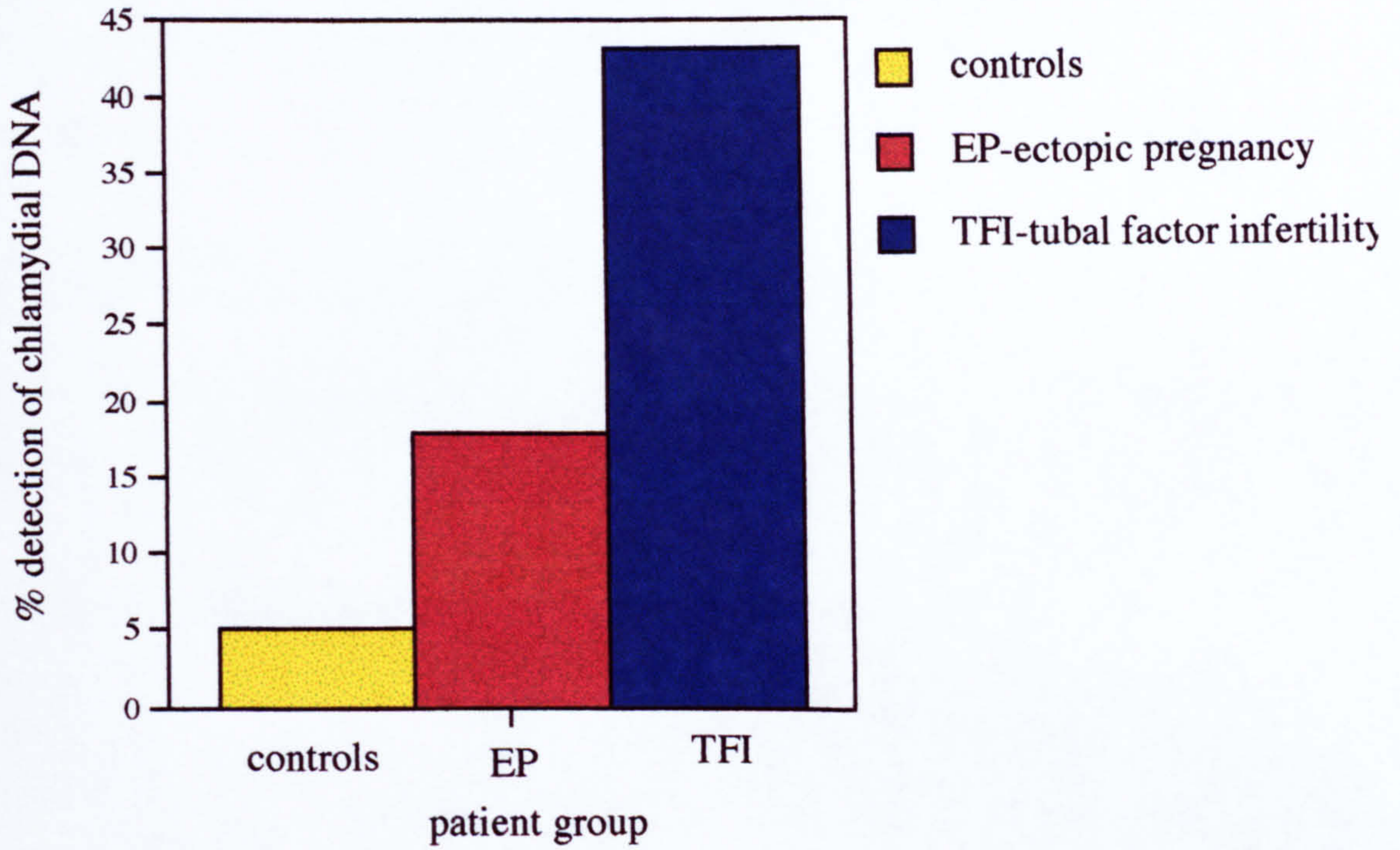
Patient group	% of patients positive for <i>C. trachomatis</i> DNA.	Tissue sample breakdown of positive samples		
		Endometrium	Ovary	Fallopian tube
Study controls mean age= 40yr SD= 6.2 age range= 33-57yr	5% (1/19)	0% (0/17)	0% (0/14)	7% (1/15)
Ectopic pregnancy (EP) mean age= 31yr SD= 5.7 age range= 24-40yr	18% (2/11)	18% (2/11)	0% (0/9)	13% (1/8)
Tubal factor infertility (TFI) mean age= 30yr SD= 3.7 age range= 24-36yr	43% (6/14)	15% (2/13)	0% (0/12)	45% (5/11)

Study group vs. EP
Study group vs. TFI

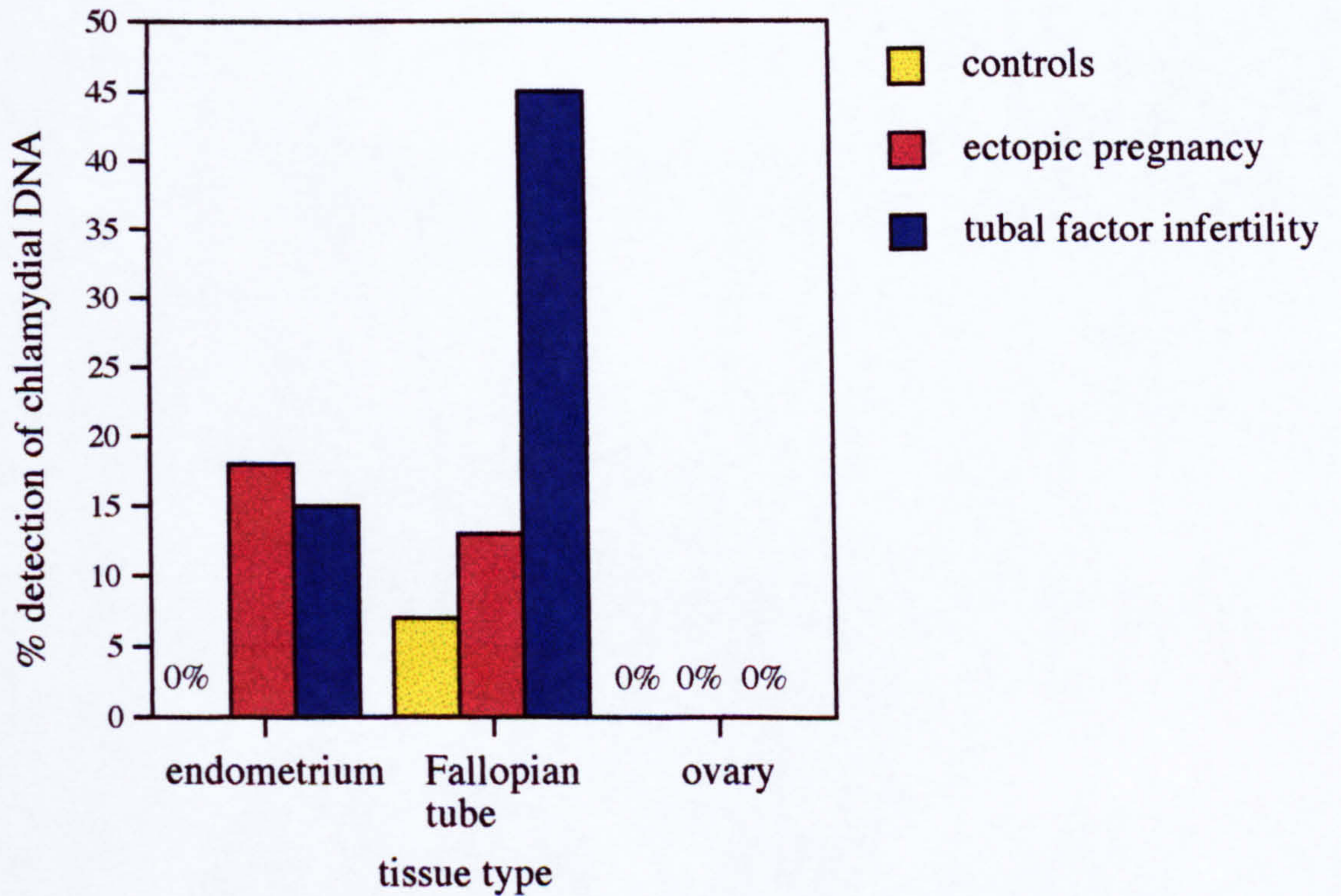
P>0.1
P<0.05

no significant difference
significant difference

Graph 10. Percentage detection of *C. trachomatis* DNA by non-isotopic *in situ* hybridization in Sheffield/Bristol.



Graph 11. Percentage detection of *C. trachomatis* DNA by non-isotopic *in situ* hybridization in the individual tissue sites sampled from Sheffield/Bristol.



4.5.2 Prevalence of *C. trachomatis* DNA in Trinidad

The detection of *C. trachomatis* DNA by non-isotopic *in situ* hybridization (NISH) is shown in Table 18, and in Graphs 12 and 13. NISH results, and histological findings for individual patients can be found in Appendix 4.

Table 18. Breakdown of the number of patients and tissue sites sampled positive for *C. trachomatis* DNA by non-isotopic *in situ* hybridization for Trinidad.

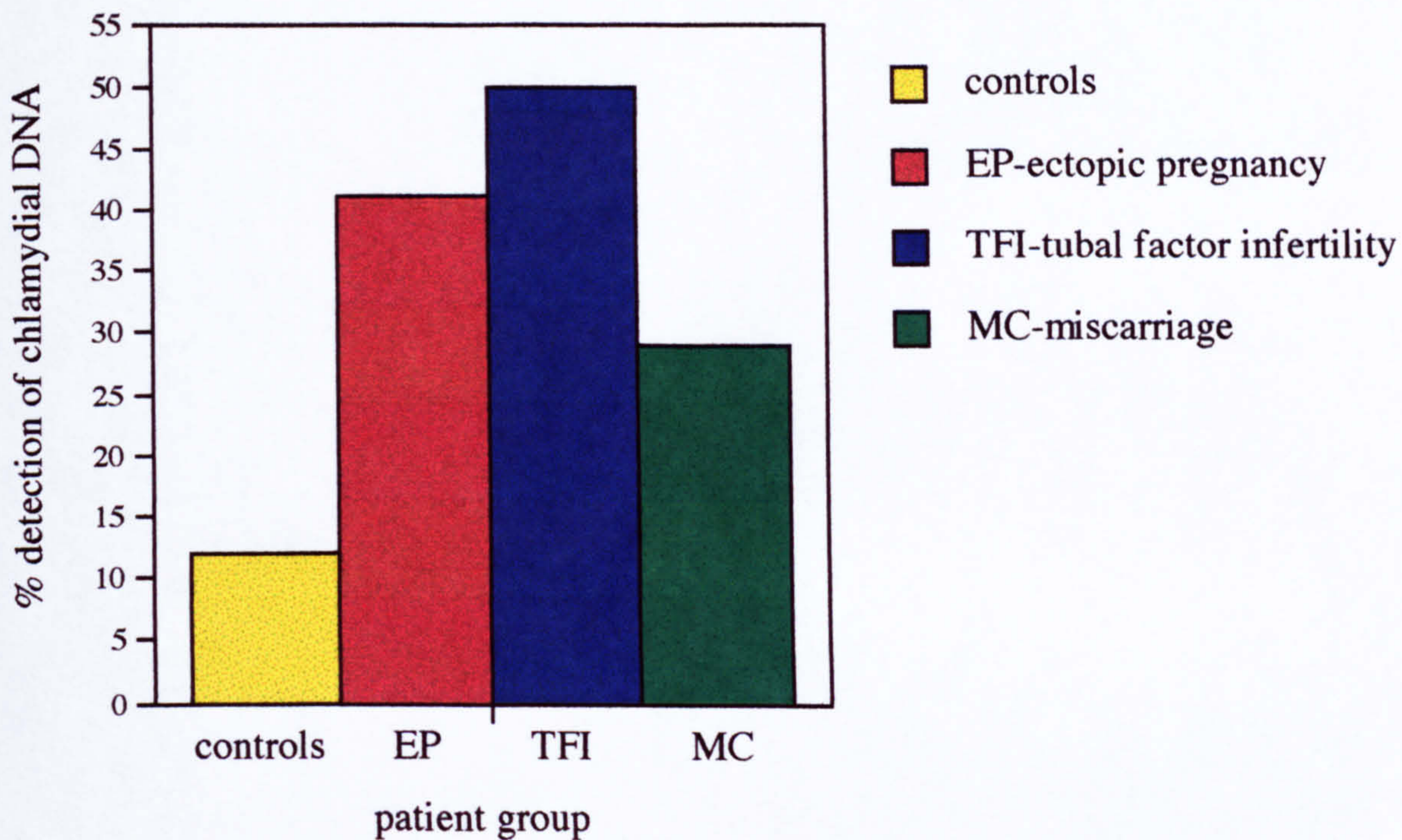
Patient group	% of patients positive for <i>C. trachomatis</i> DNA.	Tissue sample breakdown of positive samples		
		Endometrium	Ovary	Fallopian tube
Study controls mean age= 31.7yr SD= 5.3 age range= 22-44yr	12% (2/17)	0% (0/14)	0% (0/13)	13% (2/15)
Ectopic pregnancy (EP) mean age= 30yr SD= 6.3 age range= 16-40yr	41% (7/17)	30% (3/10)	0% (0/13)	29% (5/17)
Miscarriage (Msc) mean age= 33yr SD= 1.0 age range= 24-41yr	29% (2/7)	20% (2/7)	0% (0/5)	14% (1/7)
Tubal factor infertility (TFI)	50% (0/1)	0% (0/1)	0% (0/1)	50% (1/1)

Study group vs. EP
Study group vs. Msc

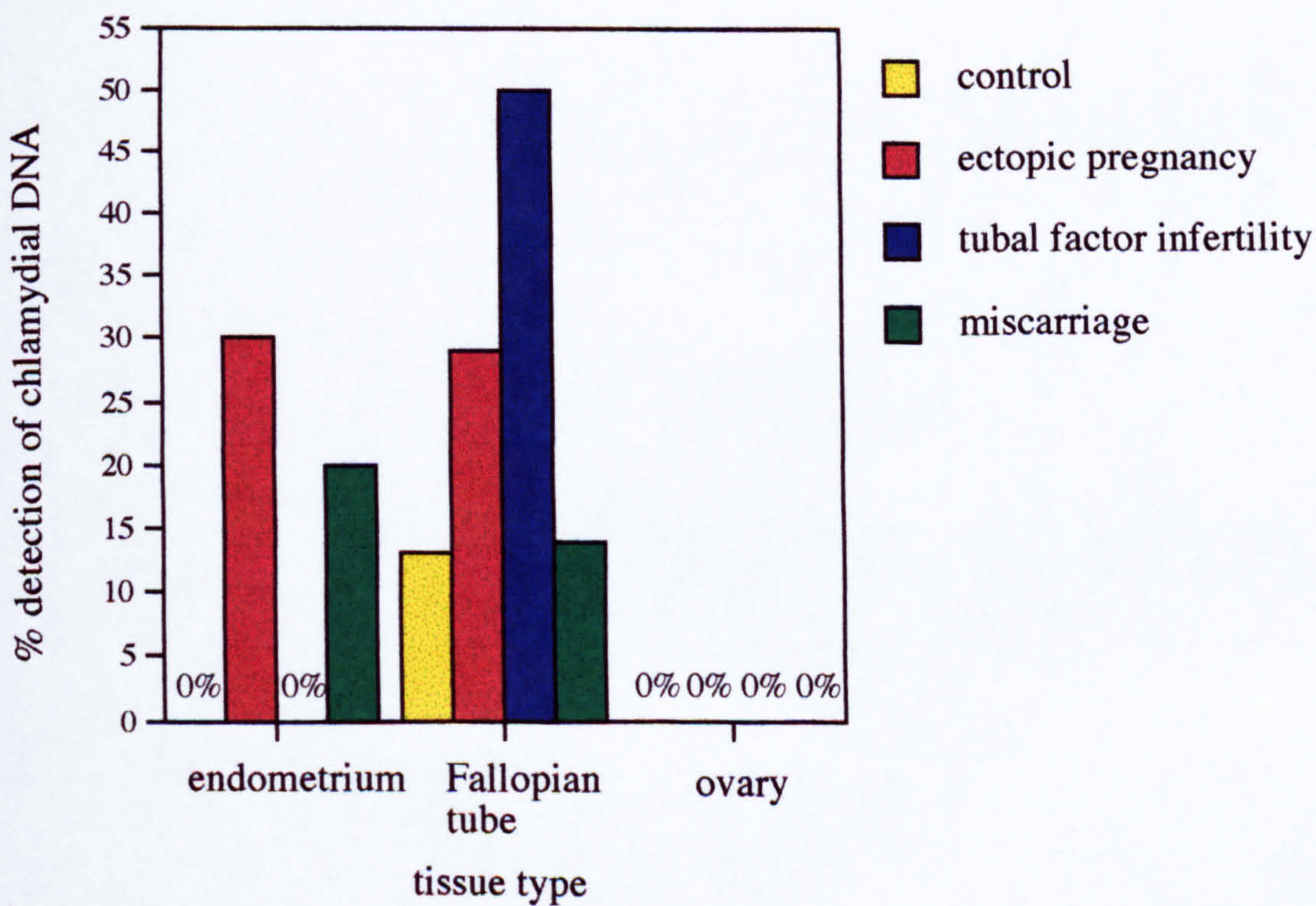
P>0.1
P>0.5

no significant difference
no significant difference

Graph 12. Percentage detection of *C. trachomatis* DNA by non-isotopic *in situ* hybridization in Trinidad.



Graph 13. Percentage detection of *C. trachomatis* DNA by non-isotopic *in situ* hybridization in the individual tissue sites sampled in Trinidad.



4.6 Conclusion

4.6.1 Sheffield/Bristol and Trinidad study

Multiple episodes of PID are more likely to cause tubal scarring, and the histopathological changes associated with infertility and ectopic pregnancy, than a single episode of PID (Westrom *et al.*, 1992). Tubal damage may occur as a result of an acute inflammatory response to a *C. trachomatis* infection of the Fallopian tube. It has been suggested that a mechanism by which tubal damage is mediated may be a delayed-type hypersensitivity response (DTH), analogous to the immune response to trachoma in which repeated chlamydial infection leads to conjunctival scarring.

In the primate model of chlamydial infection a single inoculation of *C. trachomatis* has been shown to produce a self-limited infection of the lower reproductive tract. Repeated chlamydial genital infection in both guinea pig and primate models has been reported to induce local tissue destruction, adhesion formation and tubal occlusion (Patton *et al.*, 1987; Cappuccio *et al.*, 1994; Rank *et al.*, 1995a; van Voorhis *et al.*, 1997). These findings are similar to those found in human salpingitis.

Studies by Patton *et al.* (1994b) showed that distally occluded Fallopian tubes in infertile women were strongly associated with previous chlamydial infection as assessed by serology and histopathological findings, even in the absence of culturable *C. trachomatis*. The same group reported similar findings in women with post-infectious tubal infertility who had also been treated with antibiotics regarded as being effective against *C. trachomatis*. *C. trachomatis* was detected in tubal specimens from 22/25 of these women by culture, immunohistochemistry or ISH. The result of this study suggested that *C. trachomatis*, or chlamydial antigens may persist in genital tract tissues despite antimicrobial treatment, thereby stimulating tubal inflammation and prompting tubal fibrosis and scarring.

Chlamydial DNA was detected by NISH in 5% of the Sheffield/Bristol controls and 12%

of the Trinidad controls. In the Trinidad miscarriage group the number of patients with detectable chlamydial DNA was found not to be statistically significant ($P>0.5$). In the EP group from Sheffield/Bristol *C. trachomatis* DNA was detected in 18% of the patients from Sheffield/Bristol ($P>0.1$) and 41% of EP patients from Trinidad ($P>0.1$), although the detection of chlamydial DNA was not statistically significant at either site. Finally, in the Sheffield/Bristol TFI group there was a statistically significant detection of chlamydial DNA by NISH ($P<0.05$).

Infection of the endometrium is less common than elsewhere in the female genital tract, this being attributed mainly to regular endometrial shedding. The endometrium, or uterine mucosa consists of an epithelial lining and an endometrial stroma. The epithelial lining consisting of a simple columnar epithelia through which the uterine glands extend. Once the structural changes associated with the endometrial secretory phase become evident three endometrial zones can be identified, the compact and the spongy layers (both of which are lost during menstruation), and the basal layer (which is not lost during menstruation) containing the blind ends of the endometrial glands. *Chlamydia* infecting the endometrial epithelia would therefore be subjected to the regular menstrual shedding of the compact and spongy layers.

In all of the study groups NISH localized chlamydial DNA mainly to the secretory cells of the endometrial glands (Figure 27), but due to the orientation of the sections it was impossible to determine through which endometrial layer the chlamydial DNA was observed. If the chlamydial infection was localised to the compact and spongy layers it is likely that chlamydia are lost with menstrual shedding, but if the infection had already ascended to the Fallopian tubes there is a possibility that the endometrium could be re-infected from chlamydia carried in tubal fluids.

On the other hand if the chlamydia infected the blind ends of the endometrial glands in the basal layer they would remain throughout the menstrual cycle, and therefore may have the opportunity to infect deeper tissues such as the connective tissue stroma found immediately under the basal layer or the myometrium. It is also possible that a chlamydial

infection of the endometrial glands may affect the production of protein components needed for egg implantation leading to infertility in women with previous pelvic inflammatory disease.

In the Trinidad study, chlamydial DNA was detected in 29% of the patients with the endometrium. This chlamydial infection, whether current or persistent, could possibly modulate implantation results. However, an immune response elicited by the

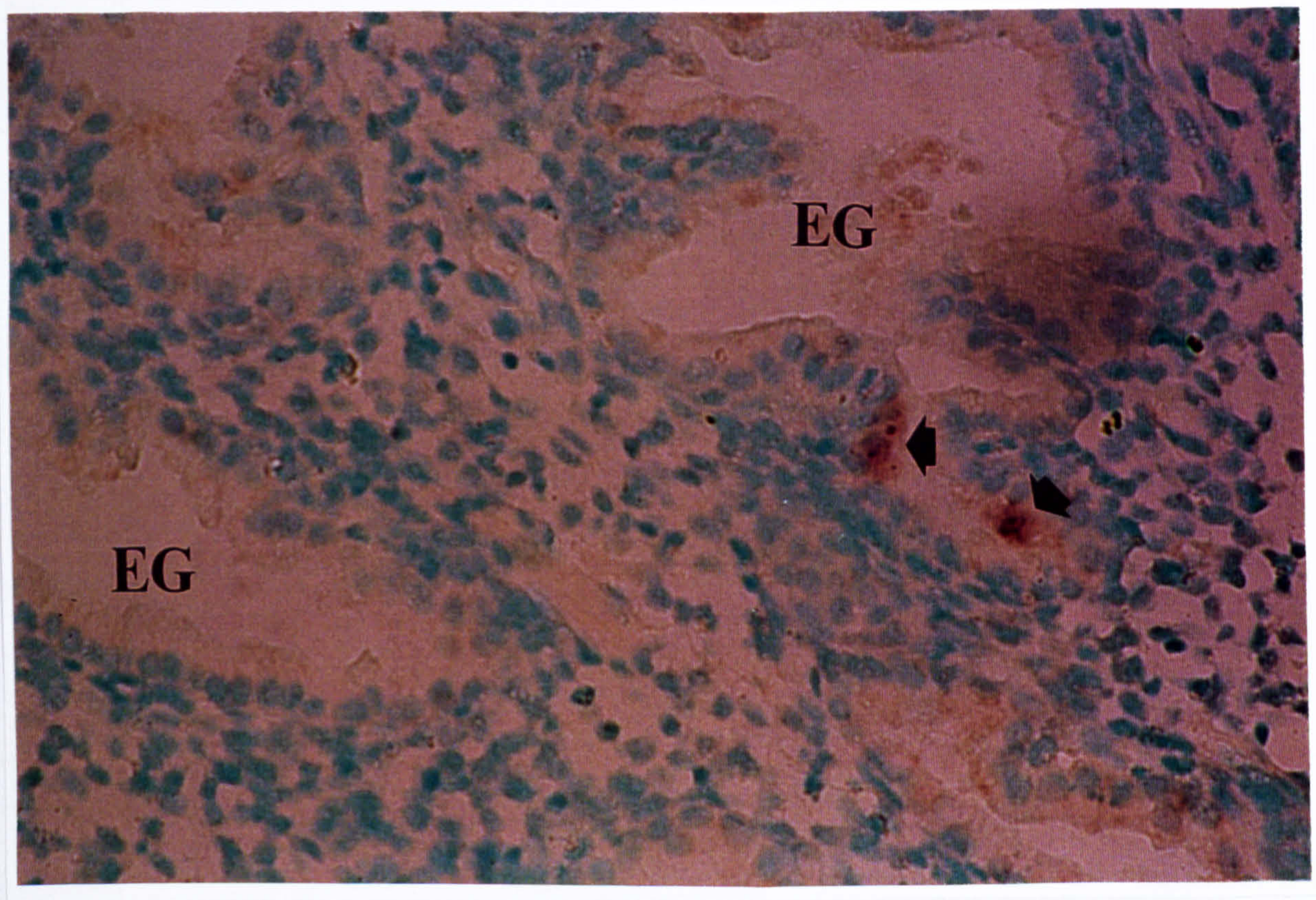


Figure 27. Methyl green counterstained endometrial section (mag.x400) with 320-bp *C. trachomatis* probe after non-isotopic *in situ* hybridization. Arrow heads indicate the location of chlamydial DNA. EG-endometrial gland.

infection of the endometrial glands may effect the production of protein components needed for egg implantation thus leading to infertility in women with possibly no evident tubal damage.

In the Trinidad miscarriage group chlamydial DNA was detected in 20% of the patients in the endometrium. How chlamydial infection, whether current or persistent, could possibly mediate miscarriage remains uncertain, but an immune component elicited by *C. trachomatis* may be an underlying cause. In a recent study by Gencay *et al.* (1997) chlamydial DNA was demonstrated by *in situ* hybridization in paraffin embedded placental material from a stillborn foetus in the 36th week of gestation. The mother was shown to have anti-chlamydial antibodies and had been culture positive three years previous. It is possible that chlamydial infection of the placenta of the developing foetus contributes to immune rejection and hence miscarriage.

Chlamydial DNA was not detected in any endometrial tissue section by NISH in any of the controls groups. However, it has to be noted that many of the Trinidad patients either did not have endometrial tissue samples taken at the time of surgery, or the samples were unsuitable for NISH due to inadequate fixation /processing (Figures 28 and 29).

The cellular constituents of the Fallopian tube include the tubal columnar epithelia (consisting of ciliated and non-ciliated secretory cells) which rests upon a cellular lamina propria. The Fallopian tube is also part of the secretory immune system with the preferential secretion of IgA from normally present IgA plasma cells in the lamina propria. The epithelium also contains IgA receptors and secretory components (Kutteh *et al.*, 1990).

Chlamydial DNA found in the Fallopian tubes in the both the UK (13%) and Trinidad (29%) EP groups, was generally localized to the ciliated epithelium (Figure 30).

Infection of the ciliated cells of the Fallopian tube may effect the wave-like motion of the cilia which are responsible for the movement of the fertilized ovum towards the endometrium. This could predispose to the tubal implantation of the fertilized ovum due

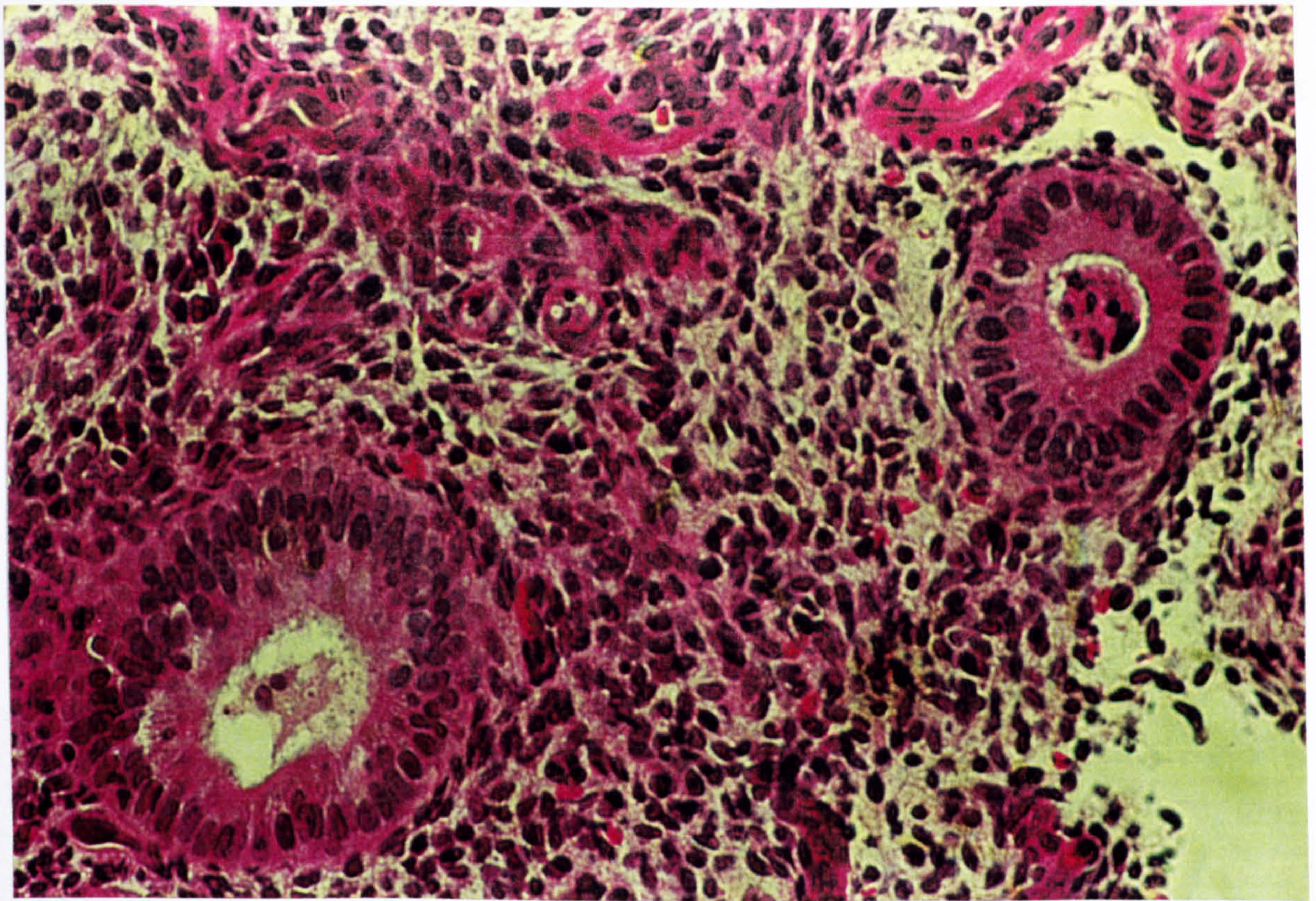


Figure 28. Haematoxylin and eosin stained endometrial section (UK sample) showing good formalin fixation and histological processing (mag.x400).

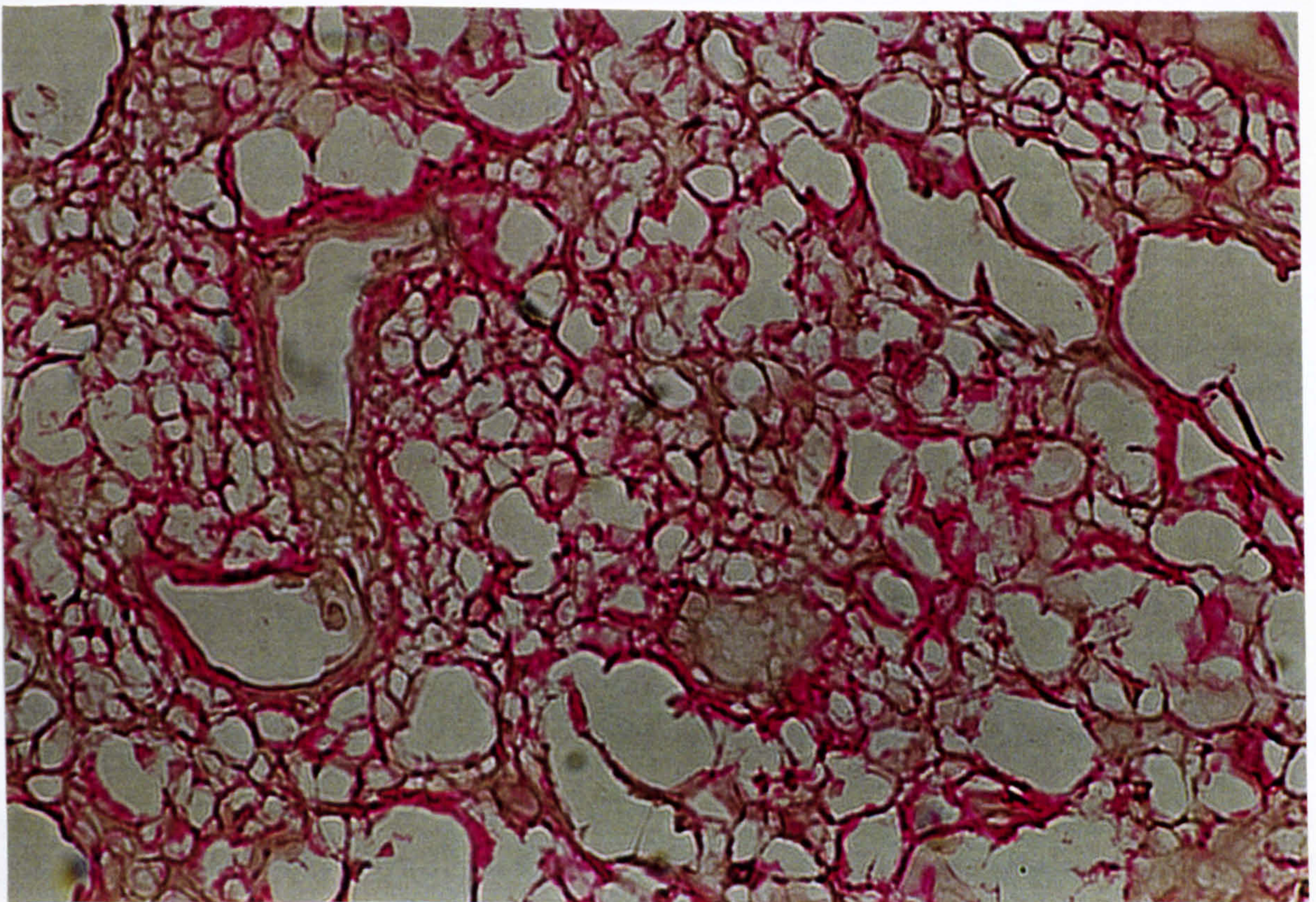


Figure 29. Haematoxylin and eosin stained endometrial section (Trinidad sample) showing loss of tissue morphology due to inadequate formalin fixation and histological processing (mag.x400).

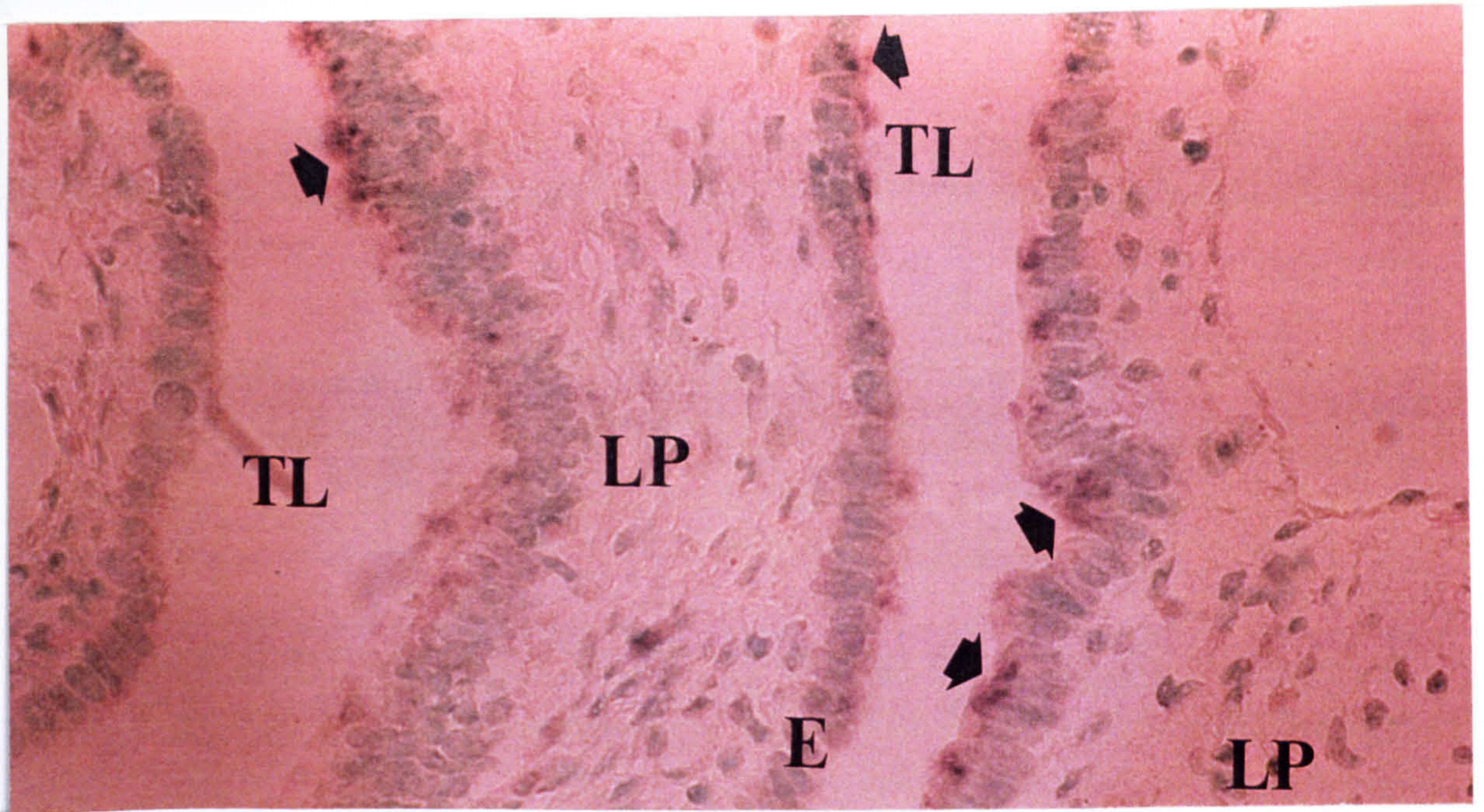


Figure 30. Methyl green counterstained Fallopian tube section from an ectopic pregnancy patient after non-isotopic *in situ* hybridization with the 320-bp *C. trachomatis* probe (mag.x400). Arrow heads indicate the localization of chlamydial DNA (purple). E- tubal epithelium; TL-tubal lumen; LP-lamina propria.

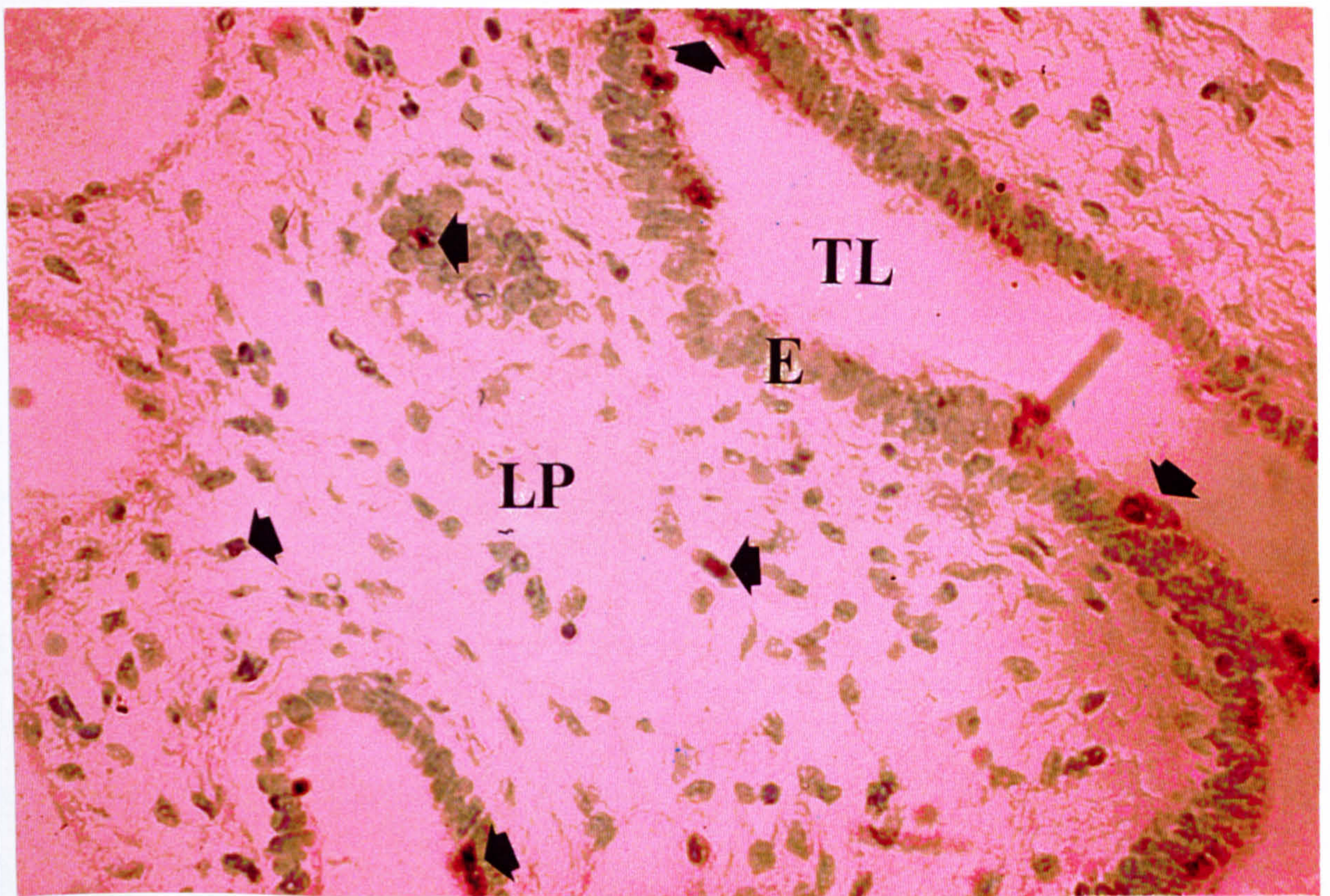


Figure 31. Methyl green counterstained Fallopian tube section from a tubal factor infertility patient after non-isotopic *in situ* hybridization with the 320-bp *C. trachomatis* probe (mag.x400). Arrow heads indicate the localization of chlamydial DNA (purple). E- tubal epithelium; TL-tubal lumen; LP-lamina propria.

to the mechanical failure of the cilia.

The majority of the TFI patients tested positive in the Fallopian tube (45%), mainly in the epithelia and occasionally in the lamina propria of the mucosal folds and sub-mucosa (Figures 31). The presence of chlamydial DNA in the tubal epithelia and sub-mucosa has also been shown in the primate model of chlamydial salpingitis after a single inoculation, and in deeper tissues after repeated infection (Cappuccio *et al.*, 1994).

The localization of chlamydial DNA to the Fallopian tube epithelia is not surprising since epithelial cells are natural targets of chlamydial infection. Using NISH chlamydial DNA was rarely detected in the deeper tissue of the Fallopian tubes. Patton *et al.* (1994b) using isotopic ISH showed that *C. trachomatis* DNA was found in the sub-mucosa or adhesions in women with post-infectious tubal infertility. The inability of NISH to detect chlamydial DNA in deeper Fallopian tube tissues may be due to the relative insensitivity of the non-isotopic method when compared to the method of Campbell *et al.* (1993), and Patton *et al.* (1994b).

Infection of the tubal epithelium could potentially damage normal tubal function by damaging ciliated cells. Scanning electron and transmission electron microscopy of Fallopian tube material from repeatedly *C. trachomatis* infected monkeys has shown enlarged non-ciliated cells, bald patches devoid of microvilli, the formation of connective bridges between non-ciliated cells, the presence of club tipped ciliated cells on the mucosal surface and intracellular epithelia damage to both secretory and ciliated cells (Patton *et al.* 1987). Also evidence of organized fibrous adhesions forming bridges between mucosal folds were observed in the monkey model, a phenomenon which is also seen in study TFI patients who showed a decreased tubal lumen.

In the guinea pig model of PID with intravaginal inoculation of *C. psittaci* GPIC, only 40% of animals that had chlamydia present in the oviducts developed tubal pathology (Rank *et al.* 1992b). Stacey *et al.* (1990) showed that chlamydial elementary bodies could be detected in apparently healthy Fallopian tubes. Both studies suggest that *C.*

trachomatis in the upper genital tract does not always correlate with active clinical disease.

Histological examination of H&E stained Fallopian tube sections from TFI patients showed that in nearly all patients there was evidence of tubal fibrosis, and the loss of mucosal folds and a reduced tubal lumen (Figures 32 and 33). In some patients the folds of the Fallopian tube had been lost, coupled with the total lack of ciliated epithelial cells and damaged, flattened non-ciliated secretory epithelial cells (Figure 34). Such damage could potentially affect the secretion of tubal fluid, the importance of which is unknown but which may create a specialized microenvironment for fertilization and early embryonic development.

The localization of chlamydial DNA to the tubal epithelium brings into question the role of active vs. persistent infection in the long term development of tubal damage. It has been suggested that a persistent infection, or the persistence of chlamydial antigens contributes to tissue damage due to immune stimulation. The NISH and histology results suggest that active chlamydial infection (as determined by the presence of chlamydial DNA in the tubal epithelia and the absence of chlamydial DNA in deeper Fallopian tube tissues) in the TFI patients studied may be in part responsible for the observed tubal disease.

None of the patients from either the Sheffield/Bristol or Trinidad groups had detectable chlamydial DNA in the ovarian tissue sections. It has been shown that positive chlamydial serology is associated with a poor ovarian response to gonadotropin stimulation during IVF (Keay *et al.*, 1998). How a chlamydial infection could potentially affect ovarian reserve/function is unknown. *C. trachomatis* infects epithelial cells and it may be that it is able to infect the ovarian epithelia. The inability to localize chlamydial DNA in the ovarian samples may have simply been a reflection of the small size of the sample and the insensitivity of the NISH employed.

Histological examination of H&E stained tissue specimens for the presence of inflammation was inconclusive. Out of all the samples examined only 9% (19/212) showed evidence of significant inflammation, but only three samples had inflammation

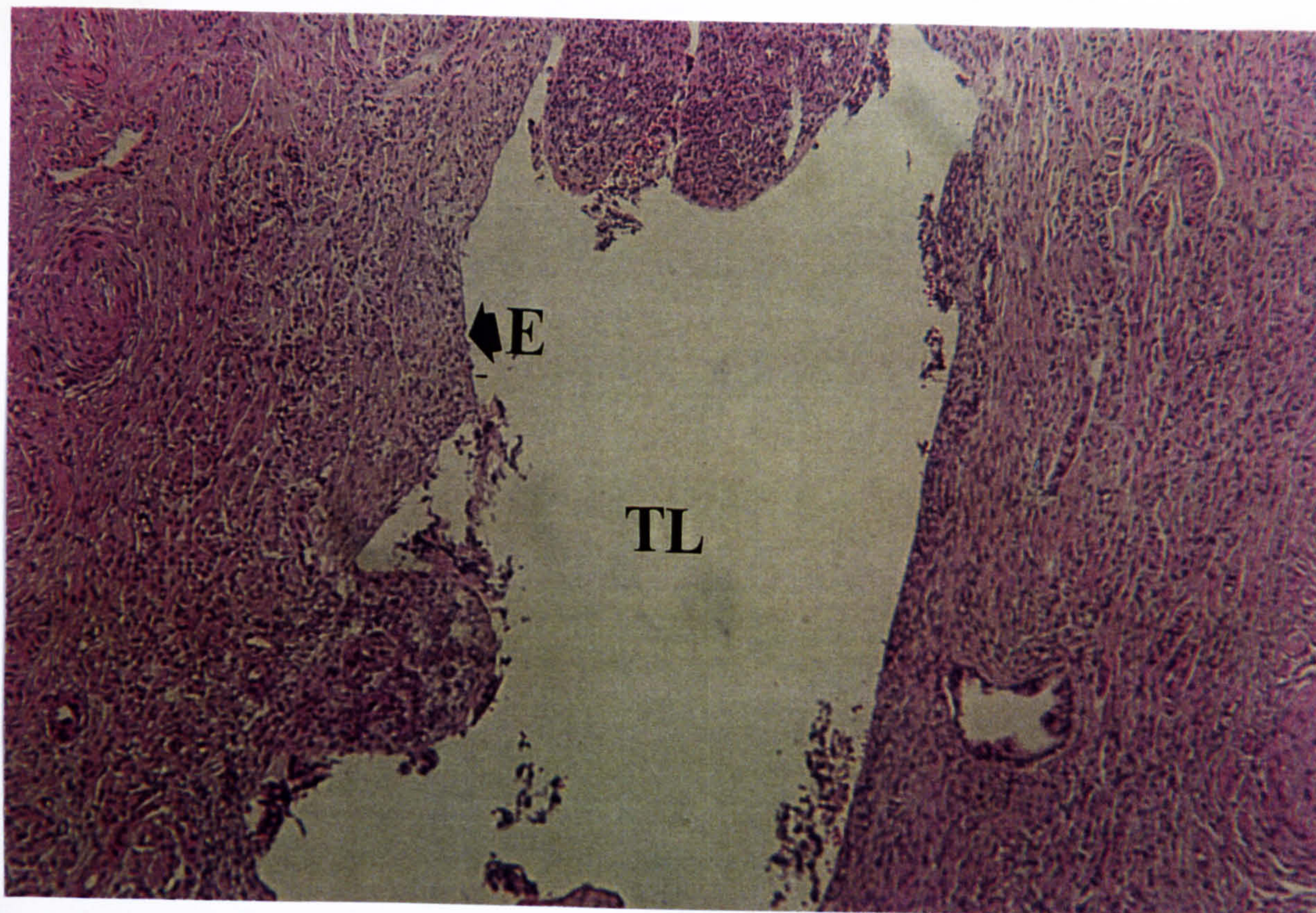


Figure 32. Haematoxylin and eosin stained normal Fallopian tube cross-section (mag.x25) showing a marked tubal epithelium (E) and mucosal folds. TL-tubal lumen.

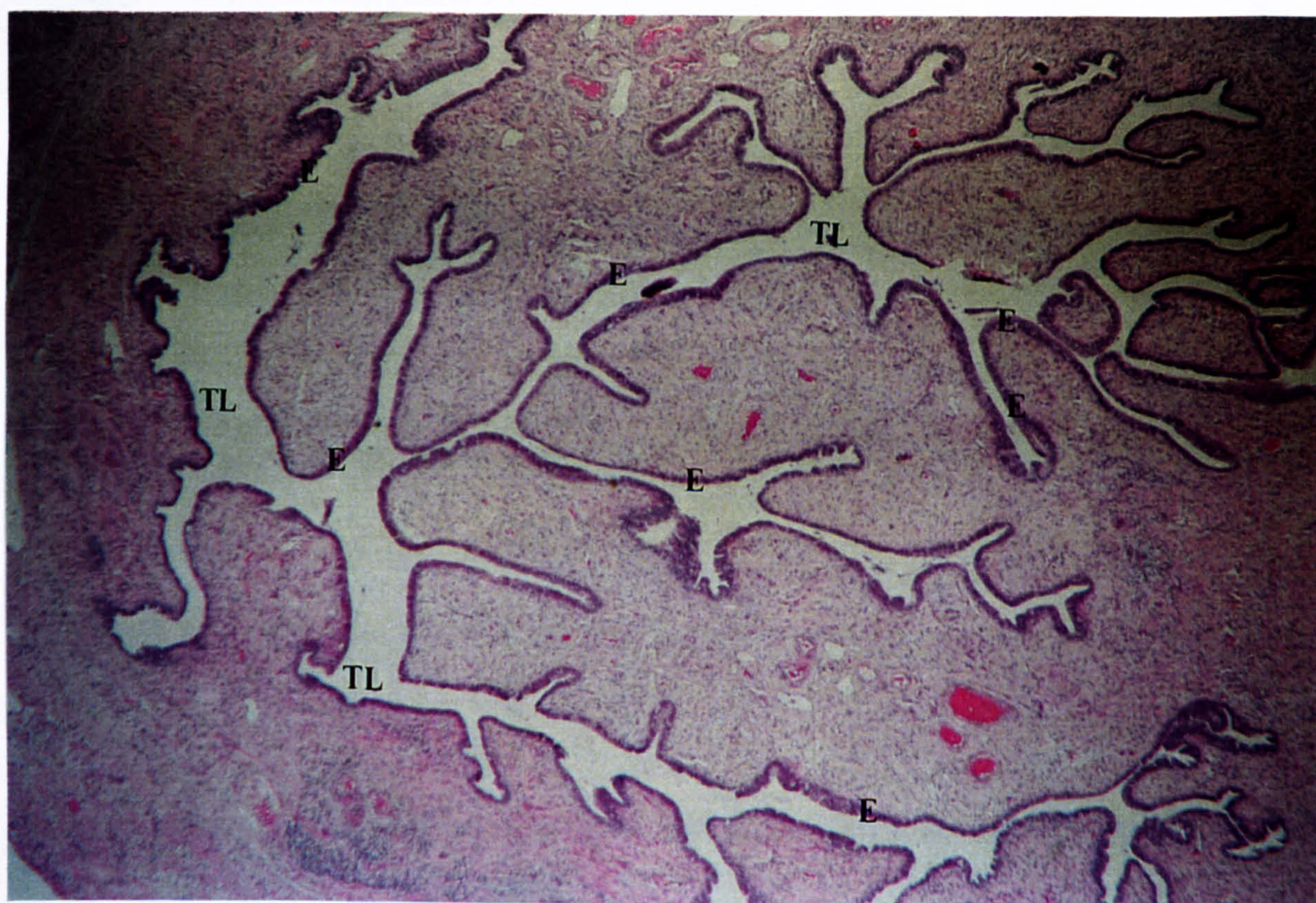


Figure 33. Haematoxylin and eosin stained Fallopian tube cross-section from a tubal factor infertility patient (JHW34-anti-chlamydial IgG positive) showing loss of mucosal folds, damaged epithelium (E) and reduced tubal lumen (mag.x160). TL-tubal lumen.

and detectable chlamydial DNA by NISH. Two of the endometrial and chlamydial DNA positive samples were Fallopian tube samples. Endometrial biopsy with IUD and both samples showed mild chronic inflammation.

NISH proved to be a reliable method for the detection of *C. trachomatis* DNA in paraffin embedded biopsy material from women with EP. Immunohistochemical cytochemistry

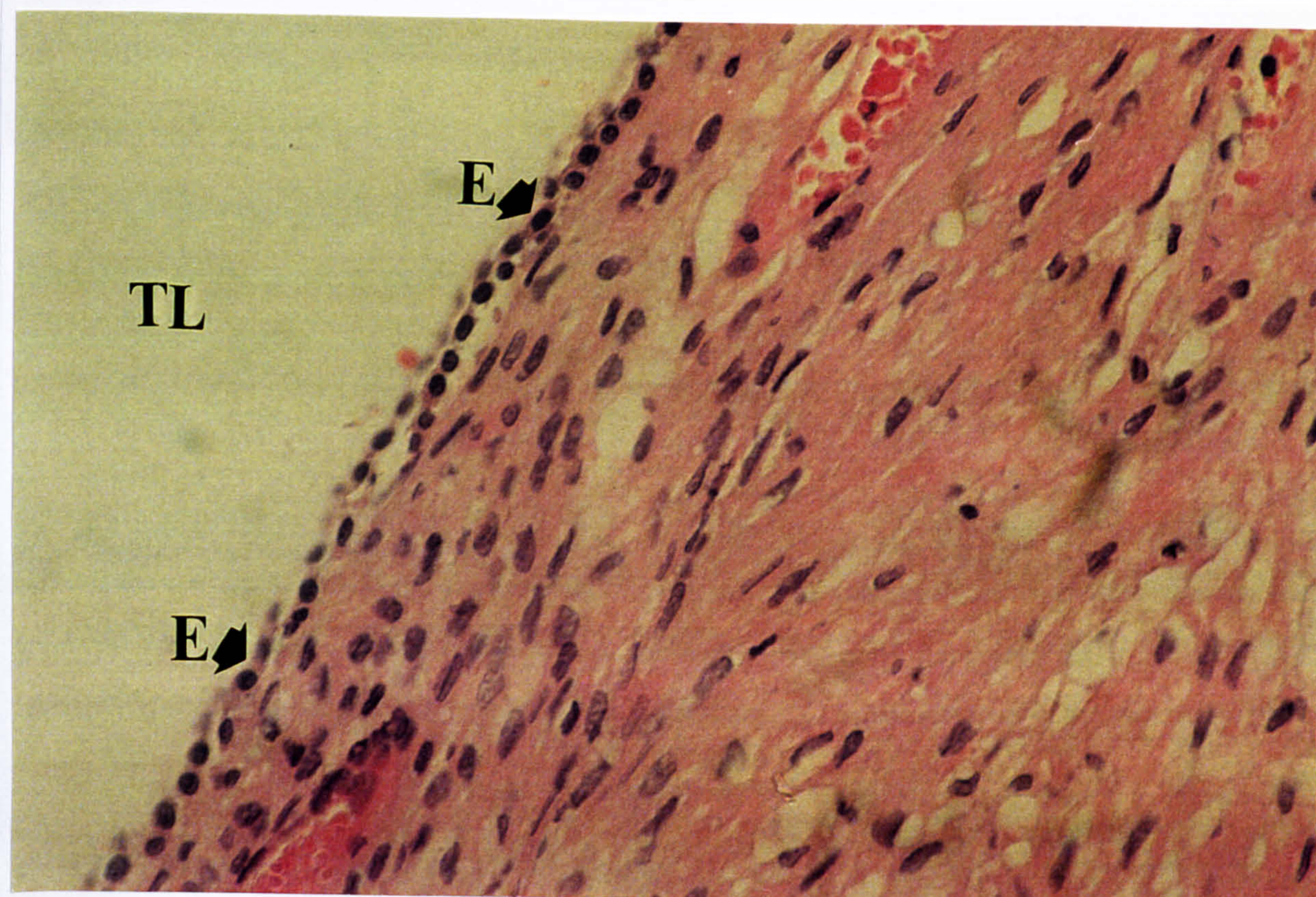


Figure 34. Fallopian tube section from a tubal factor infertility patient (BR09- anti-chlamydial IgG positive and chlamydial DNA positive in endometrial, Fallopian tube and ovarian samples by polymerase chain reaction) after haematoxylin and eosin staining (mag.x400) showing lack of ciliated epithelium, and flattened damaged secretory epithelial cells. TL-tubal lumen; E- tubal epithelium.

and detectable chlamydial DNA by NISH. Two of the inflammation and chlamydial DNA positive samples were Fallopian tube samples from patients presenting with EP and both samples showed mild chronic inflammation.

NISH proved to be a reliable method for the detection of *C. trachomatis* DNA in paraffin embedded biopsy material from women with EP, miscarriage and TFI although sampling error may have generated false negative results. Although ISH has many advantages in that it allows the direct localization of chlamydial DNA to particular cells types, the method itself can be very labour intensive and insensitive when compared to other molecular techniques such as PCR and LCR.

Chapter 5.0
General discussion

5.0 Discussion

There have been many studies over the past two decades in which the relationship between *C. trachomatis* infection and the incidence of ectopic pregnancy (EP), miscarriage, and tubal factor infertility (TFI) have been investigated. Methods of investigation have included the polymerase chain reaction (PCR), *in situ* hybridization, and serology, but to date no single study has utilized all three methods to investigate the prevalence of *C. trachomatis* infection in women with reproductive abnormalities.

The primary aim of this study was to evaluate the role of *C. trachomatis* in women with EP, miscarriage and TFI using PCR, non-isotopic *in situ* hybridization (NISH) and serology when compared to controls. Also, the influence of polymorphisms in the TNF gene were investigated to establish if there was an association between chlamydial infection and the development of tubal pathology that could predispose to EP and TFI.

An important feature of this study was the use of three fresh tissue specimens from each patient (endometrial, Fallopian tube and ovarian) for PCR and NISH. These tissue samples were obtained at the time of surgery for EP, or at the time of tubal reconstruction in the case of TFI patients. In conjunction with the collection of fresh tissue specimens from each patient, a blood sample was also taken and the serum used for serology. The individual patient results (serology, PCR, NISH, histology) and their clinical details can be found in Appendix 3 (Sheffield/Bristol) and Appendix 4 (Trinidad).

There were four study centres enrolled, two in the UK (Jessop Hospital for Women, Sheffield; St. Michael's Hospital, Bristol), and two in Trinidad (Mount Hope Women's Hospital, St. Augustine; and the General Hospital, Port of Spain). Ethical committee approval was granted at each individual study site. In total 130 patients were recruited, these included 50 study controls, 43 EP patients, 21 miscarriage patients, and 16 TFI patients. The study controls from the UK were hysterectomy patients, and the study controls from Trinidad were women undergoing sterilization. Serum samples from

antenatal patients were also collected from both the UK (n=28), and Trinidad (n=64) in order to establish a baseline prevalence of anti-chlamydial IgG and IgM in both areas.

A secondary study was designed to investigate the role of *C. trachomatis* (as determined by the presence of anti-chlamydial IgG) in women who responded poorly to gonadotropin stimulation prior to *in vitro* fertilization (IVF). Two IVF centres were enrolled, one at the University of Bristol (UK) and the other at the University of Southampton (UK). In total 242 IVF patients were investigated for the presence of anti-chlamydial IgG. The IVF patients were further sub-divided into those patients that responded 'well' to gonadotropin stimulation prior to IVF (n=148), and those who responded 'poorly' (n=94).

For the primary study, two distinct geographic areas were studied (UK and Trinidad), each with different socio-economic climates, and with differing risk factors for the acquisition of a sexually transmitted disease (STD). To date, in Trinidad there have been no studies that have evaluated the presence of *C. trachomatis* in the general population. The only information that is available in the West Indies has been from Jamaica and Martinique where the reported prevalence of *C. trachomatis* has been reported as being 16% by DIF (Dowe *et al.*, 1998) and 27% by culture of the cervix and urethra (Chout *et al.*, 1995) respectively. In the UK, the reported prevalence of *C. trachomatis* ranges from 3.6-9.1% (Fish *et al.*, 1989; Hopwood *et al.*, 1990; cit. CMO's Expert Advisory Group, 1998).

In the Sheffield/Bristol study control group, 15% (3/20) had detectable anti-chlamydial IgG, and 10% (2/20) had anti-chlamydial IgM. Similar serology results were found in the antenatal controls with 14% (4/28) having detectable IgG against *C. trachomatis* and 7% (2/28) with IgM. What is interesting is that even though the study controls were approximately 13 years older (which should in effect add an element of negative bias in the acquisition of a *C. trachomatis* infection) than the antenatal controls, the prevalence of anti-chlamydial IgG and IgM were almost identical. The IgG results are comparable to those previously reported in the UK. However, the prevalence of anti-chlamydial IgM in

our study is approximately three times greater than has been previously reported in the UK (Moore *et al.*, 1993).

The detection of chlamydial DNA in the Sheffield/Bristol study control group was 15% (3/20) by PCR and 5% (1/19) by NISH. Overall, the prevalence of *C. trachomatis* by PCR was the same as the detection of anti-chlamydial IgG, but no control patients had both chlamydial DNA detected by PCR and anti-chlamydial antibody. One patient (JHW20) had detectable chlamydial DNA (by NISH in the Fallopian tube) and anti-chlamydial IgG, plus histological evidence of chronic inflammation at the site of DNA localization. It is possible that this particular patient was suffering from asymptomatic PID.

Four patients had detectable anti-chlamydial antibody, but no chlamydial DNA in any of the tissue sites available for testing. The question therefore remains whether these patients had a cervical chlamydial infection, a site not investigated in this present study. Two of the four antibody positive/chlamydial DNA negative patients, only had detectable anti-chlamydial IgM, which may represent a newly acquired chlamydial infection in the lower genital tract and or the loss of chlamydial DNA during sampling or DNA extraction.

In the Trinidad study control group, 36% (10/28) had detectable anti-chlamydial IgG, and 11% (3/28) IgM. In the Trinidad antenatal control group the prevalence of anti-chlamydial IgG was 23% (15/64), and 8% (5/64) for IgM. The prevalence of anti-chlamydial IgG was higher in the study controls, although this was found not to be statistically significant ($P>0.1$). The difference in the detection of anti-chlamydial IgG in the two Trinidad control groups may simply be a reflection of the differing mean ages (the study controls being approximately seven years older than the antenatal controls), or due to differences in the populations sampled.

The detection of chlamydial DNA by PCR was 40% (12/30) and 41% (7/17) by NISH. However, only seven control patients (25%) had both detectable antibody against *C.*

trachomatis, and chlamydial DNA. Six patients had only antibody alone, which could possibly indicate asymptomatic chlamydial infection of the lower genital tract.

The detection of *C. trachomatis* in the Trinidad study control group was higher than the reported prevalence of *C. trachomatis* in other West Indian countries. In Martinique, the reported prevalence rate was 27% by culture (Chout *et al.*, 1995), which may in fact represent an underestimation of the carriage of *C. trachomatis* in the female population, due to the relative insensitivity of the technique when compared to molecular techniques such as PCR. Culture of the lower genital tract would not indicate a past exposure to *C. trachomatis*, or persistent or active upper genital tract infection.

In both the Sheffield/Bristol and Trinidad control groups the detection of antibody (either IgG and IgM) did not correlate well with the detection of chlamydial DNA. It may be that in those patients with detectable chlamydial DNA but no detectable antibody against *C. trachomatis*, an inadequate antibody response could have contributed to the establishment of an upper genital tract infection, or the persistence of *C. trachomatis* or DNA in genital tissue specimens. Alternatively, antibody levels may have fallen below the threshold value of the ELISA system developed.

The Trinidad control group data suggest a population with an increased rate of silent/ asymptomatic or persistent chlamydial infection. Does Trinidad therefore have higher EP compared to the UK? The first report of ectopic pregnancies in the Caribbean was in 1963 (Douglas, 1963) which revealed an incidence of 1 in 28 live births for the Jamaican population studied. The high EP rate was associated at the time with the high incidence of PID in Jamaica. To our knowledge there have been no similar studies reported from Trinidad.

The incidence of EP in the UK (1988-1993) has been reported as being 10.8 per 1000 total conceptions (cit. CMO's Expert Advisory Group, 1998). In France the incidence of EP has been reported as being 20.2 per 1000 live births (Coste *et al.*, 1994b), in Finland 28 per 1000 live births (Makinen, 1993), and 22 per 1000 live births in the USA (CDC,

1992). The 1963 EP rate in Jamaica equated to 35.7 EPs per 1000 live births, approximately three times greater than the UK.

Within the Sheffield/Bristol EP group, anti-chlamydial IgG was detected in 67% (6/9) of the patients, and IgM in 22% (2/9). *C. trachomatis* DNA was detected in 56% (5/9) of the EP patients by PCR and in 18% (2/11) by NISH. When individual patients were considered, only two patients had both antibody against *C. trachomatis* and detectable chlamydial DNA, four patients had detectable antibody alone and three had demonstrable chlamydial DNA only. Again the detection of anti-chlamydial antibody did not correlate well the detection of chlamydial DNA by either PCR or NISH.

Of the 11 EP patients examined from the UK all had evidence of having/or having had a chlamydial infection. Five of the EP patients had had at least one previous EP, of which three also had a history of PID. In the five women with a previous EP, the current EP may be a direct result of tubal damage elicited by a previous chlamydial infection. Of the remaining six EP patients, all had evidence (either by the presence of chlamydial DNA or anti-chlamydial antibody) of having had or still having a *C. trachomatis* infection, which may have been responsible for the development of the current EP. Only two EP patients had anti-chlamydial IgM, but neither patient had detectable chlamydial DNA and it is possible that in these women the current EP was not due to tubal damage caused by *C. trachomatis*. However, they may have had a chlamydial infection of the lower genital tract, as determined by the detection of anti-chlamydial IgM.

In Trinidad, anti-chlamydial IgG was detected in 62% (13/21), IgM in 38% (8/21), and chlamydial DNA in 66% (19/29) by PCR and 41% (7/17) by NISH. It must be noted that many of the Trinidad EP patients had incomplete sample sets. Of the 30 Trinidad EP patients, 16 had both detectable antibody against *C. trachomatis* and detectable chlamydial DNA. Of the 22 EP patients with clinical details available, nine had a recorded history of PID, and two had pelvic adhesions at the time of surgery suggestive of PID. Of the women with a history of PID, who had serum samples available for testing, six had

detectable IgG and two had anti-chlamydial IgM. These results suggest that *C. trachomatis* might have been responsible for the reported episodes of PID.

What is interesting in the Trinidad EP group is the relatively high incidence of IgM (38%) when compared to the Trinidad study controls (8%), and the antenatal controls (11%). Although this difference was found not to be statistically significant at the 5% level ($P>0.05$). A similar association was also seen in the UK EP group ($P>0.1$), but the incidence of anti-chlamydial IgM was again found not to be statistically significant, possibly due to the limited number of patients studied in the UK. Within the two EP groups the detection of IgM in a large proportion of individuals tested warrants further study.

The high incidence of IgM in this group, in women with evidence of having or having had a chlamydial infection suggests that in these women an active chlamydial infection may be in part responsible for the EP. NISH identified chlamydial DNA in the ciliated epithelium of the Fallopian tube of three EP patients with anti-chlamydial IgM, suggesting that active chlamydial infection may have been responsible for damaging the cilia resulting in the implantation of the fertilized ovum in the tube.

In both the UK and Trinidad EP groups, chlamydial DNA was detected in all the sample types studied (ovarian sample by PCR only). From both the UK and Trinidad EP groups, of the total number of patients with ovarian samples available for testing by PCR ($n=30$), 57% (17/30) were positive for the presence of *C. trachomatis* DNA. Since no direct experimental evidence of chlamydial infection could be found using NISH, it can only be assumed that the detected chlamydial DNA by PCR was a contaminant of the pelvic cavity. Conversely it may reflect the insensitivity of NISH when compared to PCR. The discovery of such a high proportion of EP patients with detected chlamydial DNA in the pelvic cavity underlines the importance of ascending infection in the possible development of EP.

In the Trinidad miscarriage group anti-chlamydial IgG was detected in 39% (7/18) and IgM in 11% (2/18), both of which were found not to be statistically significant when compared to the appropriate control groups. Conversely in the miscarriage group the detection of chlamydial DNA was 62% (13/21) by PCR and 29% (2/7) by NISH, and although neither were statistically significant, there was an increased detection of *C. trachomatis* DNA.

Of the 21 miscarriage patients, 24% (5/21) had a recorded history of PID, of these five patients, four had evidence of having or having had a chlamydial infection. All four patients had either/or positive Fallopian tube samples (by PCR or NISH) or ovarian samples (by PCR only) suggestive of an upper genital tract chlamydial infection. It is well documented that chlamydial PID tends to be asymptomatic in nature, and although many of the patients in all the Trinidad groups have had reported episodes of PID, it remains to be determined how many others have suffered from chlamydial PID without the appropriate treatment. If evidence of chlamydial PID is the detection of chlamydial DNA in the Fallopian tubes (PCR and NISH) or ovarian samples (PCR only) it would indicate that a large proportion (54%) of the Trinidad patients (with no recorded history of PID) irrespective of group may have chlamydial PID.

Considering only the Trinidad miscarriage serological data, the incidence of anti-chlamydial IgG and IgM were found not to be statistically significant. Although the detection of chlamydial DNA was not statistically significant it was certainly higher than the control group (PCR 62% vs. 40%, $P > 0.1$; and NISH 29% vs. 12%, $P > 0.5$). These results suggest that in the case of miscarriage the detection of a presumed chlamydial infection (as determined by the detection of chlamydial DNA) may be an important factor.

Of the eight endometrial samples available for testing by PCR and NISH from the miscarriage group, 50% (4/8) were positive for chlamydial DNA. It is possible that chlamydial infection, especially of the endometrium may contribute to miscarriage in as yet unknown way, possibly by triggering an adverse immune response detrimental to the developing foetus. A recent study by Gencay *et al.* (1998) showed the detection of *C.*

trachomatis DNA in the placenta of a miscarried stillborn foetus. Whether *C. trachomatis* infection of the placenta actually resulted in the miscarriage remains uncertain, but *C. psittaci* infection of the placenta has been shown to cause natural abortion in animals. The detection of chlamydial DNA in miscarried foetal material may shed light on the possible role of *C. trachomatis* in miscarriage.

In the Sheffield/Bristol TFI group, of the ten PCR positive patients, seven had positive endometrial samples, five had positive ovarian samples and five positive Fallopian tube samples. Two patients (BR08, BR09) had all three sites positive by PCR, and detectable anti-chlamydial IgG but no IgM indicating that the damage associated with TFI may be from a previous infection.

Of the 23 Fallopian tube samples from TFI patients available for PCR and NISH, 43% (10/23) were positive for chlamydial DNA. Histology revealed that many of the TFI patients had evidence of tubal fibrosis indicative of a past infection coupled with the lack of tubal epithelia and a reduced tubal lumen. Tubal occlusion resulting from hydrosalpinx and peritubal adhesion formation and tubal scarring are the underlying pathological changes associated with TFI following acute salpingitis.

Of the nine chlamydial DNA positive patients who had serum samples available for testing, seven had detectable IgG, and none had anti-chlamydial IgM. Patient BR07 was histologically diagnosed as having salpingitis isthmica nodosa, the development of which has been attributed to inflammation due to an infection. This patient had anti-chlamydial IgG, and a positive ovarian sample by PCR suggestive of ascending infection and possibly Fallopian tube infection. The question of whether *C. trachomatis* was responsible for the development of this condition remains uncertain.

Only two out of the nine TFI patients with detectable chlamydial DNA and serum sample available for testing, had no demonstrable anti-chlamydial antibodies. Campbell *et al.* (1993) also found two out of 16 TFI patients who were positive for chlamydial DNA by immunoperoxidase staining to be negative for anti-chlamydial IgG by MIF. The

Campbell *et al.* study failed to detect anti-chlamydial IgM in any of the 16 women examined with distal tubal occlusion.

A possible explanation of the Campbell *et al.* results and the results of the Sheffield/Bristol TFI group is that tubal damage is associated with repeated past chlamydial infection, and circulating antibody levels may have declined below the threshold value of the serological tests used. It may also be that in the TFI patients with detectable chlamydial DNA but no detectable antibody response against *C. trachomatis*, an inadequate antibody response may have been elicited during infection resulting in tubal damage from prolonged chlamydial infection/persistence.

Of the 13 ovarian samples tested by PCR, five (38%) were positive for chlamydial DNA. No evidence of chlamydial infection could be demonstrated by NISH indicating that the chlamydia detected was a contaminant from the pelvic cavity. However, it has to be stressed that our study did show that PCR was a more sensitive method for the detection of chlamydial DNA than NISH, and the failure of NISH to detect chlamydial DNA in ovarian samples may be a reflection of this apparent lack of sensitivity.

Since chlamydial DNA could not be localized in the ovarian epithelium by NISH, but was detected by PCR in some patients it could only be assumed that due to ascending infection *C. trachomatis* had found its way into the fluid of the pelvic cavity surrounding the ovary and as such was a contaminant of the ovarian sample; or it could be argued that chlamydial DNA was not detected in ovarian samples due to the relative insensitivity of NISH when compared to PCR.

The effect of a chlamydial infection in the pelvic cavity, and its possible effect on the ovary and ovarian function remains to be elucidated. Experimental evidence has indirectly suggested that pelvic infection and adhesion formation can affect ovarian response (Madehaven *et al.*, 1985; Molloy *et al.*, 1987; Bowman *et al.*, 1993; Czemichy, 1996), but the actual mechanism is unknown. Further work on the

possibility of *C. trachomatis* infection of the ovary using primary tissue culture, and *in situ*-PCR may help resolve this conundrum.

The results of our study suggest that *C. trachomatis* does play an important role in the development of both TFI and EP. In the case of miscarriage the role of chlamydia is less clearly defined and may be related to the induction of maternal immune rejection of the foetus facilitated by a chlamydial infection. The IVF study showed that women undergoing IVF, who also had detectable levels of anti-chlamydial IgG were statistically more likely to respond poorly to gonadotropin ovarian stimulation prior to IVF, producing fewer follicles. This may be due to the presence of pelvic adhesions or hydrosalpinx, both of which have been shown to affect ovarian reserve in animal models.

It is possible that due to the less invasive nature of serology, the detection of anti-chlamydial IgG could be used as an indicator of adverse tubal pathology of chlamydial aetiology. However, it is important that further work on the comparison between the molecular and serological detection of *C. trachomatis* in TFI and EP patients be completed.

The identification of genes that make humans more susceptible to certain infectious diseases is difficult due to such factors as genetic heterogeneity and the effect of environmental factors. The TNF polymorphism data as determined by our study suggest that in the UK a polymorphism in the TNF- β gene may be associated with the development of tubal damage in women with evidence of having/or having had a chlamydial infection (as determined by the presence of anti-chlamydial IgG). In Trinidad neither of the *Nco*1 polymorphisms in the TNF gene were found to be associated with either EP or miscarriage in patients with or without anti-chlamydial antibody. This may be due to environmental factors and the actual populations studied. Due to the limited number of patients studied in both the UK and Trinidad, a larger and more comprehensive genetic study on the role of polymorphisms in the TNF gene and the development of tubal damage due to *C. trachomatis* needs to be undertaken.

The importance of persistent chlamydial infection also needs to be addressed, our study showed the presence of chlamydial DNA in tissue samples from women without a detectable antibody response against *C. trachomatis*. The question of whether the DNA detected was from a past infection and the antibody levels had declined below the threshold value of the serological tests employed, or whether the DNA was from a persistently infecting chlamydia needs clarification. Methods such as reverse transcriptase (RT) PCR, RT-ISH or RT-*in situ* PCR may be useful in determining the presence of metabolically active chlamydia, although RT-ISH and RT-*in situ* PCR may be of more use by identifying and localizing persistently infecting chlamydia within tissue sections. Also of importance would be the continued investigation of the role of *C. trachomatis* in miscarriage by evaluating the maternal immune/foetal immune response in pregnant women with chlamydial infections.

What has been established in this present study is that *C. trachomatis* plays an important role in diseases of the upper female genital tract, and the subsequent development of tubal pathology that could predispose to both EP and TFI. However, even when different modern molecular methods were employed together, many questions still remain unanswered allowing plenty of scope and opportunity for further research.

Chapter 6.0

Appendices

6.1 Appendix 1. ELISA development

6.1.1 IgG ELISA development

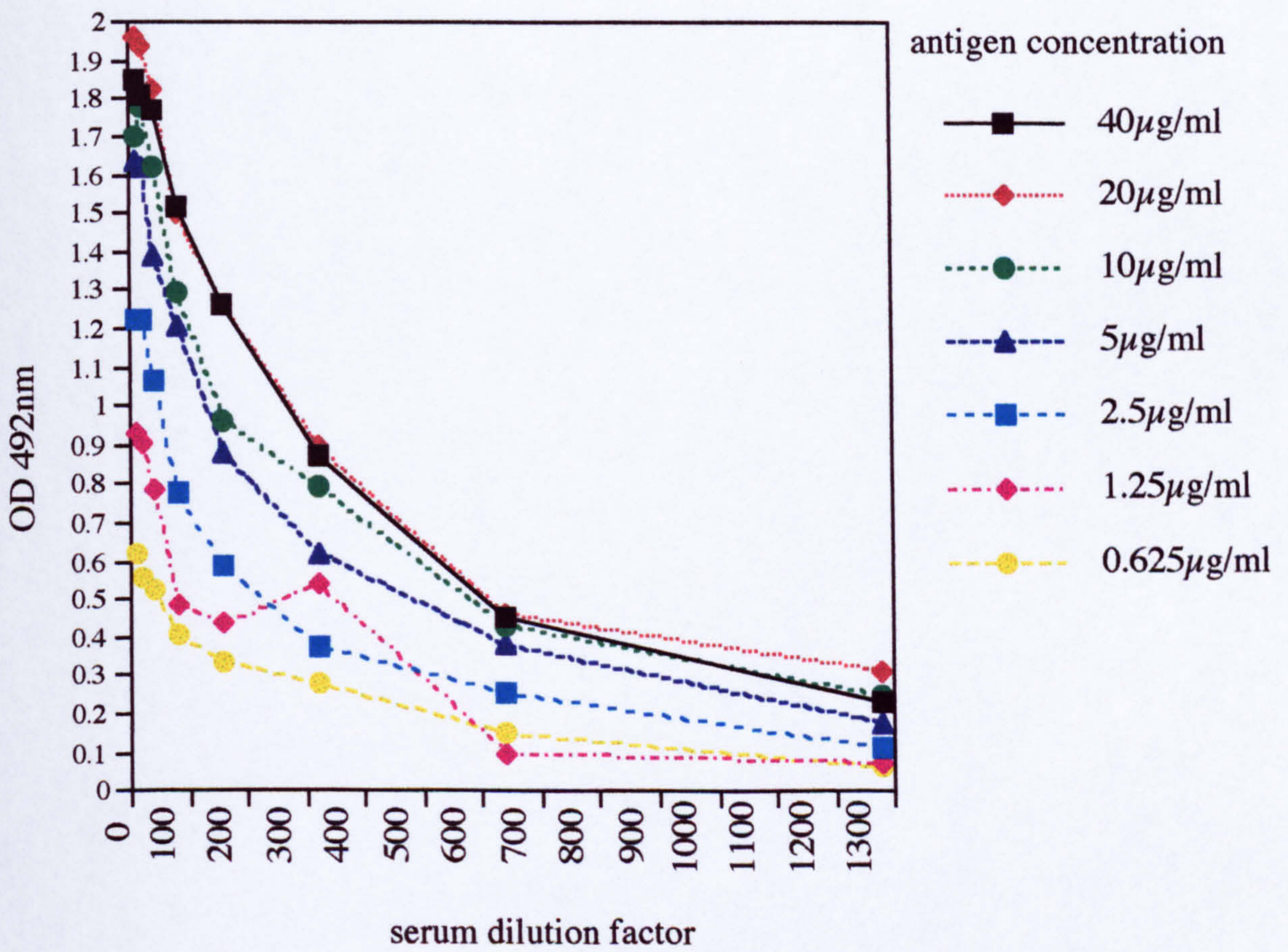
Titration of LGV1 antigen

A basic chequerboard ELISA was used to establish the optimal antigen concentration for the developmental detection of chlamydial IgG from both study patients and from sera of known chlamydial titres.

The *C. trachomatis* antigen preparation was diluted from 40µg/ml to 0.625µg/ml in coating buffer by doubling dilutions, and 50µl of each dilution was dispensed into eight wells on an EIA plate. The plate was left overnight at 4°C in a moist chamber, and was then washed three times in PBS-Tween. A serum sample with a known MIF titre of 1:512 for *C. trachomatis* was diluted from 1:10 to 1:1280 by doubling dilutions in serum diluent; 50µl of each dilution was placed into each of the wells containing the different antigen concentrations, and the plate incubated for 1h at 37°C in a moist chamber. The plate was then washed three times in PBS-Tween, 50µl of HRP-conjugated anti-human IgG pre-diluted 1:2000 in serum diluent was then added to each well and the plate incubated again at 37°C for 1h. Following incubation the plates were washed three times to remove unbound antibody that could contribute to the generation of a background signal, and excess liquid removed by inverting the plate on tissue paper. 50µl of substrate buffer was placed in each well and the plate left to develop at room temperature for 15min. The enzyme reaction was stopped by the addition of 50µl of 2M H₂SO₄ to each well, and the absorbance was read immediately at 492nm in an ELISA reader.

A decreasing absorbance (OD) with increasing serum dilution, and decreasing antigen concentration was seen (Graph 14). A final antigen concentration of 10µg/ml and a serum dilution of 1:80 was initially chosen to develop the ELISA methodology.

Graph 14. IgG enzyme-linked immunoassay with a *C. trachomatis* specific serum (IgG microimmunofluorescence titre 1:512) with 1:2000 IgG conjugate.



Effect of different detergents

Ossewaarde *et al.* (1994b) reported an ELISA with an enhanced specificity for the detection of antibodies to *C. trachomatis*. The system involved the pre-treatment of *C. trachomatis* L2 elementary bodies with either sodium periodate (which oxidises LPS) or Triton X-100 (which extracts LPS) and measured the end-point titres of serum samples from patients with culture proven *C. trachomatis* infection, patients with symptoms suggestive of *C. trachomatis* infection, and patients with *C. psittaci* infection. The same methods were investigated.

Three EIA plates were coated with 10µg/ml of LGV1 antigen, and another three plates with 10µg/ml of *C. pneumoniae* antigen. The plates were left overnight at 4°C in a moist chamber, and were then washed once with PBS-Tween. 50µl of 1% sodium periodate was added to the wells of one set of plates (one coated with LGV1 antigen the other *C. pneumoniae* antigen), 50µl of 1% Triton X-100 was added to the wells of the second set of plates; the final set of plates were not pre-treated. All the plates were then incubated at 37°C for 10min, and washed twice with PBS-Tween.

A selection of serum samples with known MIF titres against both *C. trachomatis* and *C. pneumoniae* were serially diluted in serum diluent. The samples tested included a *C. trachomatis* specific (CT SP) serum (IgG MIF serovar A-C 1:64, D-K 1:128, LGV1-LGV3 1:128); a *Chlamydia* dual species (C DS1) serum containing chlamydial IgG against both *C. trachomatis* and *C. pneumoniae* (IgG MIF serovar A-C 1:256, D-K 1:256, LGV1-LGV3 1:256; *C. pneumoniae* 1:64); *C. pneumoniae* specific (CPN SP) serum (IgG MIF *C. pneumoniae* 1:512); a *Chlamydia* dual species (C DS2) serum containing chlamydial IgG against both *C. pneumoniae* and *C. trachomatis* (IgG MIF *C. pneumoniae* 1:256; serovar A-C 1:16, D-K 1:16, LGV1-LGV3 1:16); and a negative serum with no detectable antibodies against any chlamydial species as determined by MIF.

50µl of each serum dilution was placed into two wells on each plate, and the plate incubated for 1h at 37°C in a moist chamber. Each plate was then washed three times in

PBS-Tween, 50µl of HRP-conjugated anti-human IgG pre-diluted 1:2000 in serum diluent was then added to each well and the plate incubated again at 37°C for 1h. Following incubation the plates were washed three times to remove unbound antibody that could contribute to the generation of a background signal, and excess liquid removed by inverting the plate on tissue paper. 50µl of substrate buffer was placed in each well and the plate left to develop at room temperature for 15min. The enzyme reaction was stopped by the addition of 50µl of 2M H₂SO₄ to each well, and the absorbance was read immediately at 492nm in an ELISA reader.

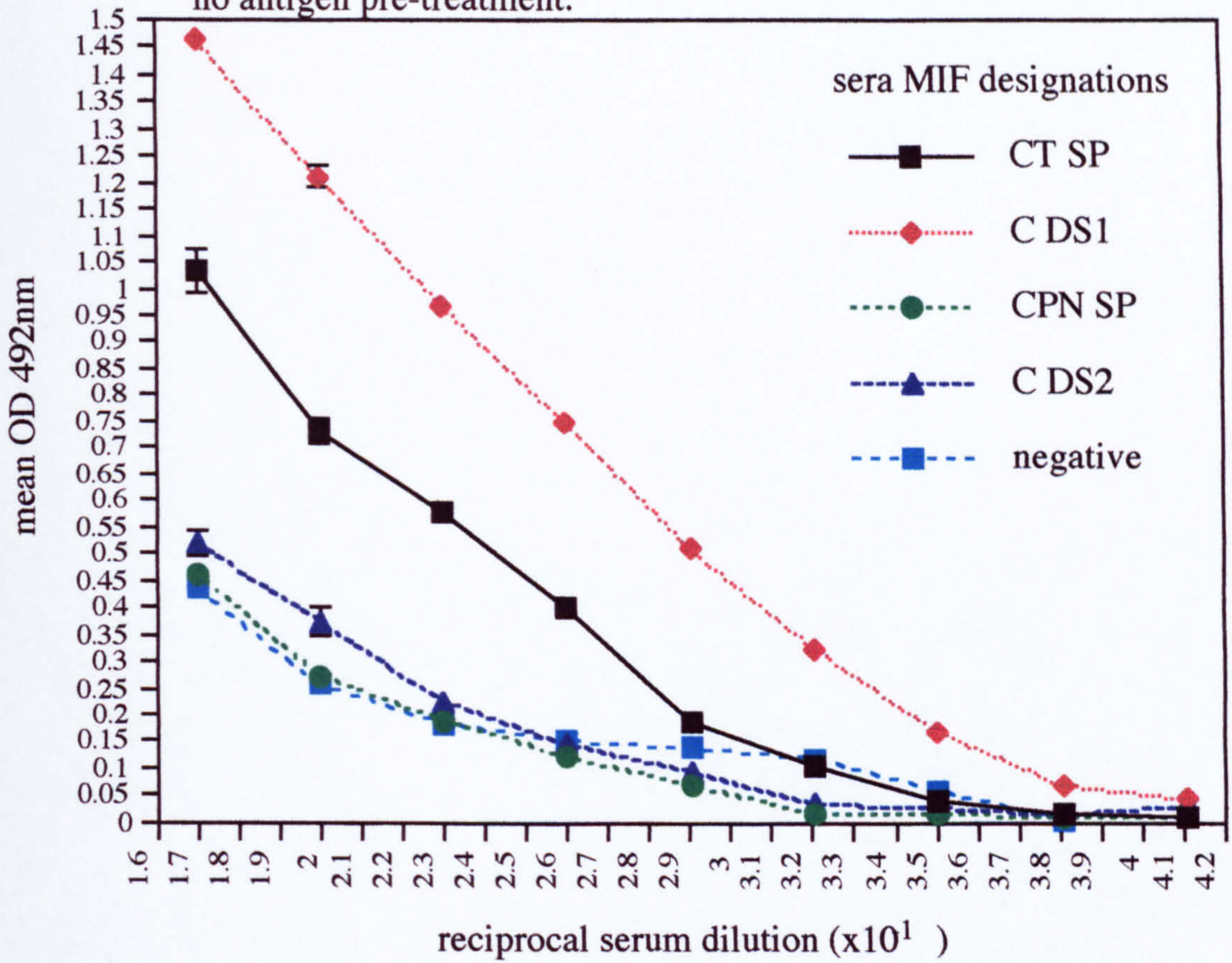
The *C. pneumoniae* and HL cell antigen preparations were used to show the effect of both antigen pre-treatments on the end-point titres of the different test serum samples (data not shown); and to confirm the absence/presence of antibodies to *C. pneumoniae* in the test samples. All the serum samples with detectable levels of anti-*C. pneumoniae* IgG by MIF had detectable IgG by the *C. pneumoniae* ELISA.

The mean OD values for each serum sample against both antigen preparations and all the pre-treatments were calculated and plotted against reciprocal serum dilution ($\times 10^{-1}$) to determine end-point titres (Graphs 15-17). The effect of each pre-treatment on the end-point titres of the different serum samples is shown in Graph 18.

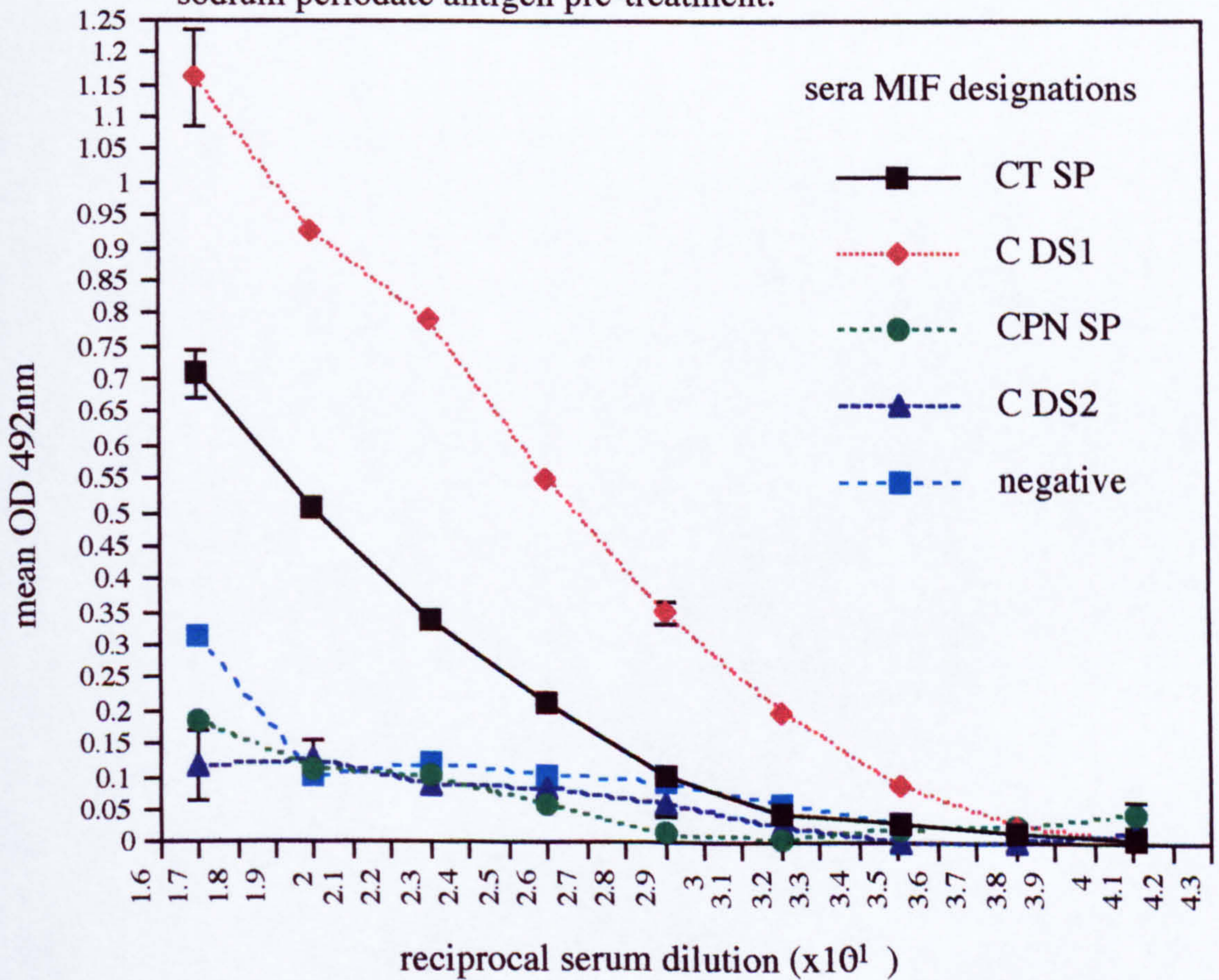
For the untreated LGV1 antigen preparation it can be seen that both the *C. pneumoniae* specific and dual species serum would have given OD values that could have been interpreted as borderline negative/low positive for *C. trachomatis* IgG. This underlines the importance of the removal of genus cross reactive epitopes to give a more 'reliable', species specific serological result.

The *C. trachomatis* specific serum had a reduced end-point titre after LGV1 antigen pre-treatment with sodium periodate (from \log^{10} 3.0-2.8), but the Triton X-100 pre-treatment had no effect on the end-point titre when compared against the untreated LGV1 antigen. The same effect was observed with the *C. trachomatis* dual species sample and sodium periodate, as you would expect after the oxidation of LPS. The Triton X-100 gave a

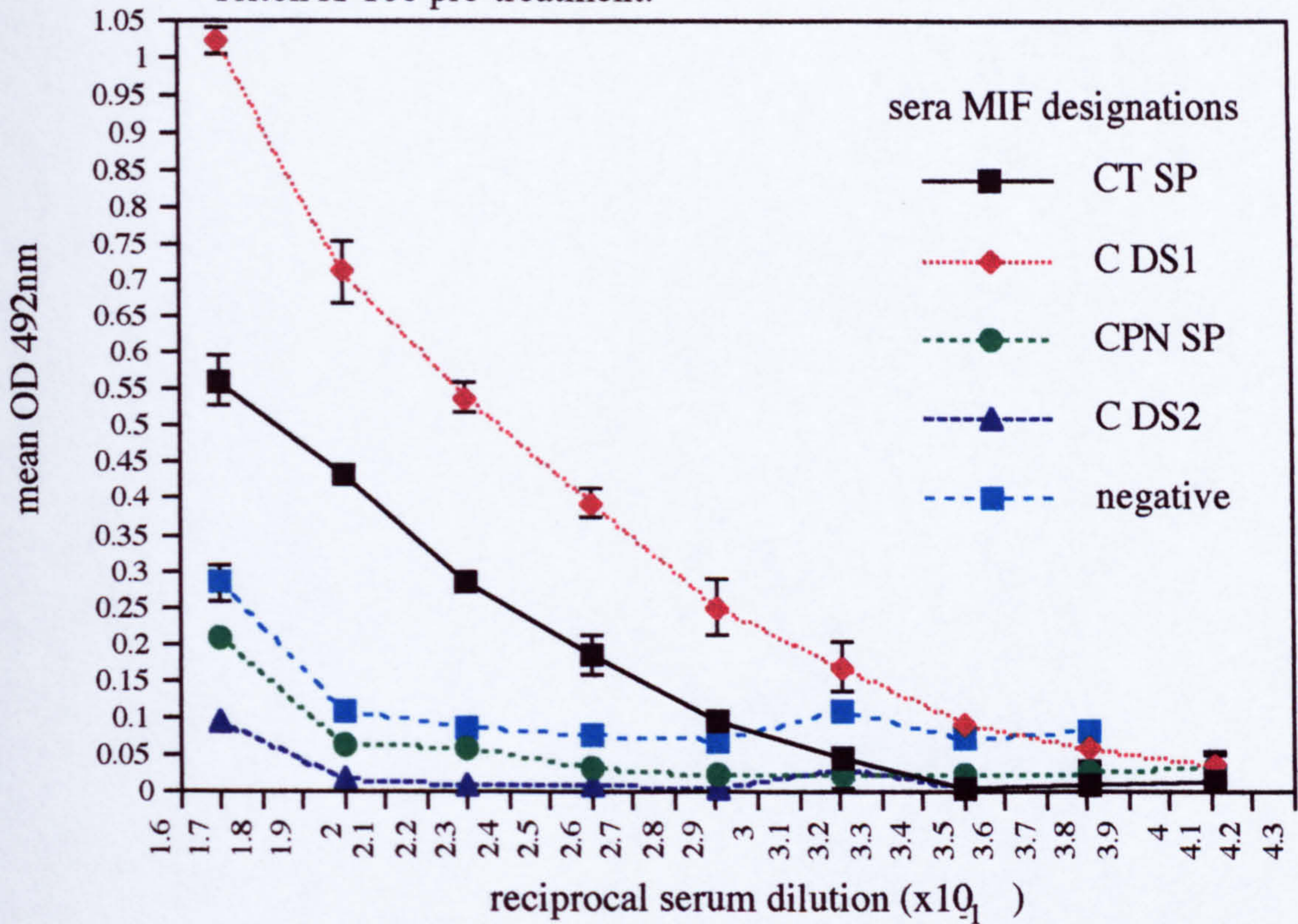
Graph 15. The determination of end point titres of known micro-immunofluorescence (MIF) titre sera against LGV1 antigen with no antigen pre-treatment.



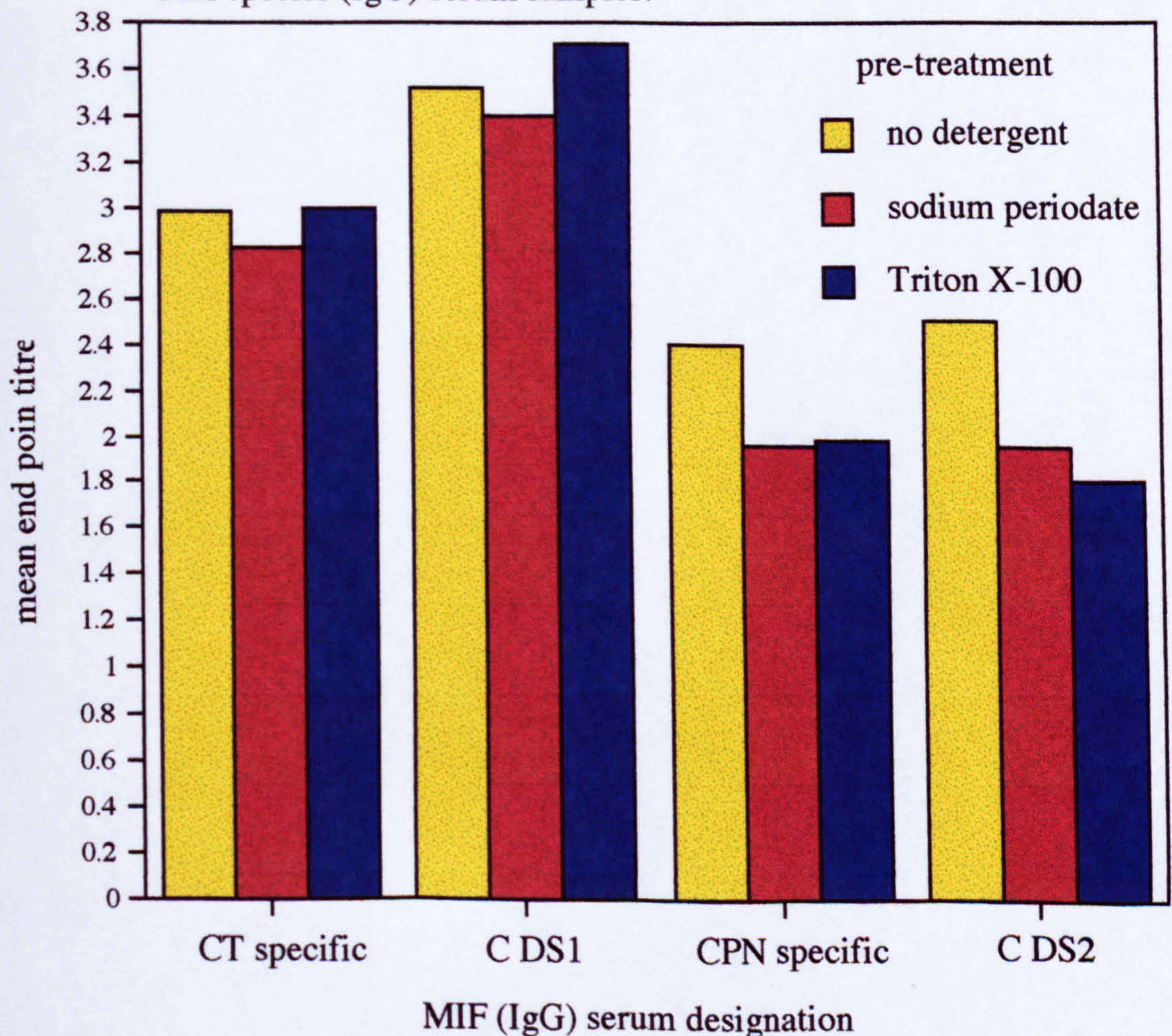
Graph 16. The determination of end point titres of known micro-immunofluorescence (MIF) titre sera against LGV1 antigen with sodium periodate antigen pre-treatment.



Graph 17. The determination of end point titres of known micro-immunofluorescence (MIF) titre sera against LGV1 antigen with Triton X-100 pre-treatment.



Graph 18. The effect of different enzyme-linked immunoassay pre-treatments on the end point titres of *Chlamydia* specific and dual species (IgG) serum samples.



higher end-point titre as compared to the untreated and the sodium periodate treated antigen preparations. In this instance, the Triton X-100 pre-treatment enhanced the effect of having both antibodies against *C. pneumoniae* and *C. trachomatis* in the same serum sample.

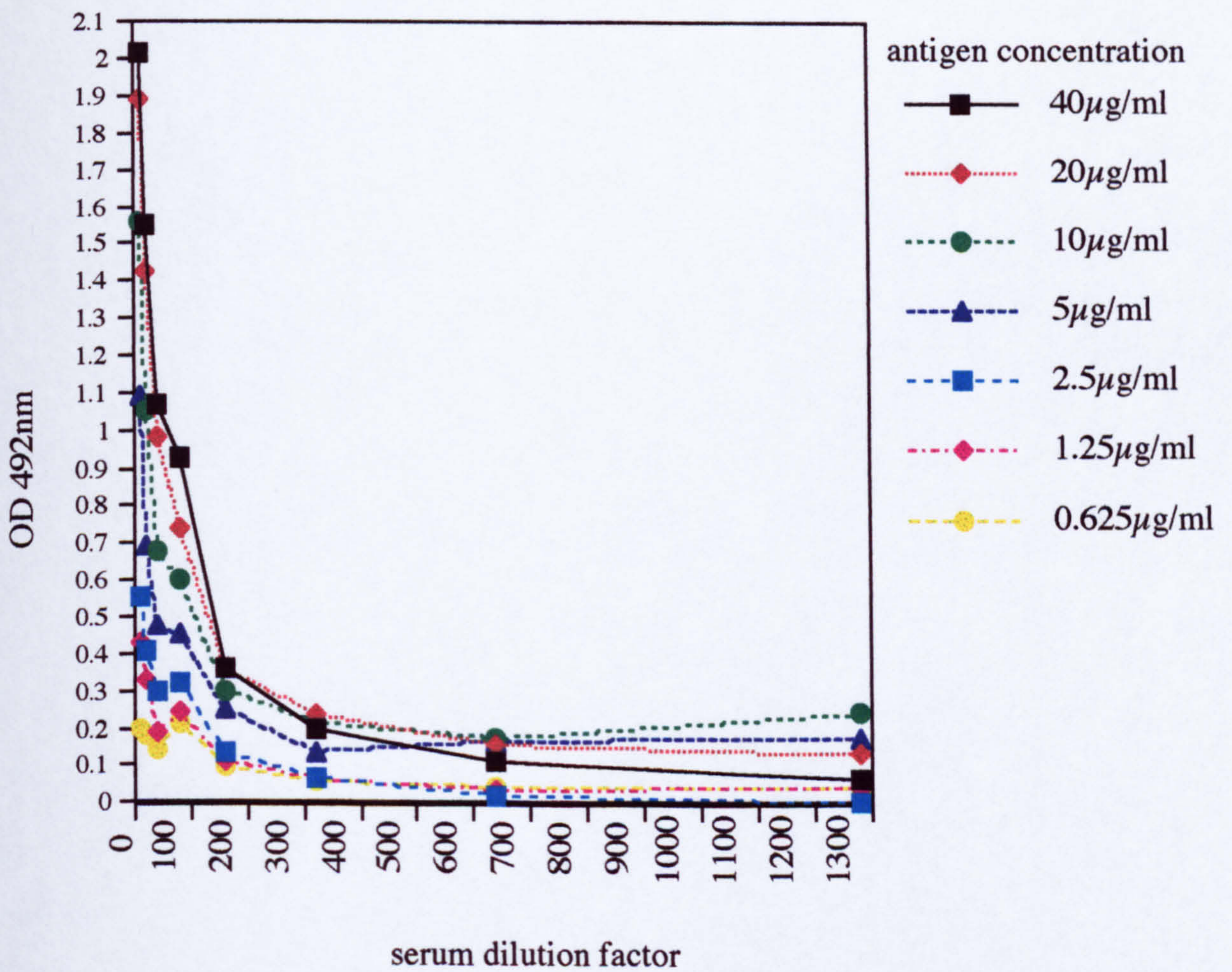
The effect of both pre-treatments on the LGV1 antigen preparation was more marked with the *C. pneumoniae* specific and dual species samples. Both sodium periodate, and Triton X-100 reduced the ELISA OD values to below that of the chlamydia negative sample at all serum dilutions, suggesting that both pre-treatments work equally as well in reducing cross reactivity. Ossewaarde *et al.* (1994b) recommended the use of sodium periodate over Triton X-100, as detergents like Triton X-100 can remove proteins from chlamydial elementary body preparations (Caldwell *et al.*, 1981) which could possibly reduce the sensitivity of the ELISA. For all subsequent ELISA runs, the sodium periodate pre-treatment of antigen was used routinely.

Titration of LGV1 antigen with sodium periodate

Due to the fact that sodium periodate pre-treatment reduced the mean OD and end-point titres of all the test samples examined previously, the antigen was re-titrated against a *C. trachomatis* specific serum (IgG MIF 1:512). The titration method was the same as previously described with a few minor alterations; after the overnight incubation of the antigen at 4°C, the plate was washed once in PBS-Tween and 50µl of 1% sodium periodate was added to each well and the plate incubated at 37°C for 10min. The plate was then washed twice in PBS-Tween before the addition of the diluted serum.

A decreasing OD with increasing serum dilution, and decreasing antigen concentration was observed (Graph 19). For the sodium periodate treated LGV1 antigen preparation a final antigen concentration of 20µg/ml, and a serum dilution of 1:100 was finally decided upon for all further developmental ELISA experiments.

Graph 19. IgG enzyme-linked immunoassay standardization with a *C. trachomatis* specific serum (IgG micro-immunofluorescence titre 1:512) with sodium periodate pre-treatment of LGV1 antigen, at a IgG conjugate dilution of 1:2000.



1:2000 vs. 1:4000 IgG conjugate titration

It was observed that some serum samples showed a small degree of cross reactivity against the McCoy cell antigen preparation, although it must be noted that the OD of cross reactive samples was below that of the negative cut off value. In an attempt to reduce this effect the conjugate dilution was altered from 1:2000 to 1:4000.

A standard ELISA was run using 20µg/ml of both LGV1 and McCoy cell antigen preparations, sodium periodate pre-treatment and a selection of known MIF serum samples diluted 1:100 (one *C. trachomatis* MIF 1:2048, two *C. trachomatis* MIF 1:512, and five chlamydia negative samples), using an IgG conjugate dilution of both 1:2000 and 1:4000 (Graph 20 and Graph 21).

There was little reduction in the degree of cross reactivity between some of the serum samples and the LGV1 and McCoy cell antigen preparations. There was a reduced OD against the LGV1 antigen, at an IgG conjugate dilution 1:4000, when compared to the same serum sample at 1:2000 IgG conjugate dilution. The cross reactivity may simply have been due to the relatively low serum dilution, with this in mind the LGV1 antigen was re-titrated with the IgG conjugate diluted 1:4000.

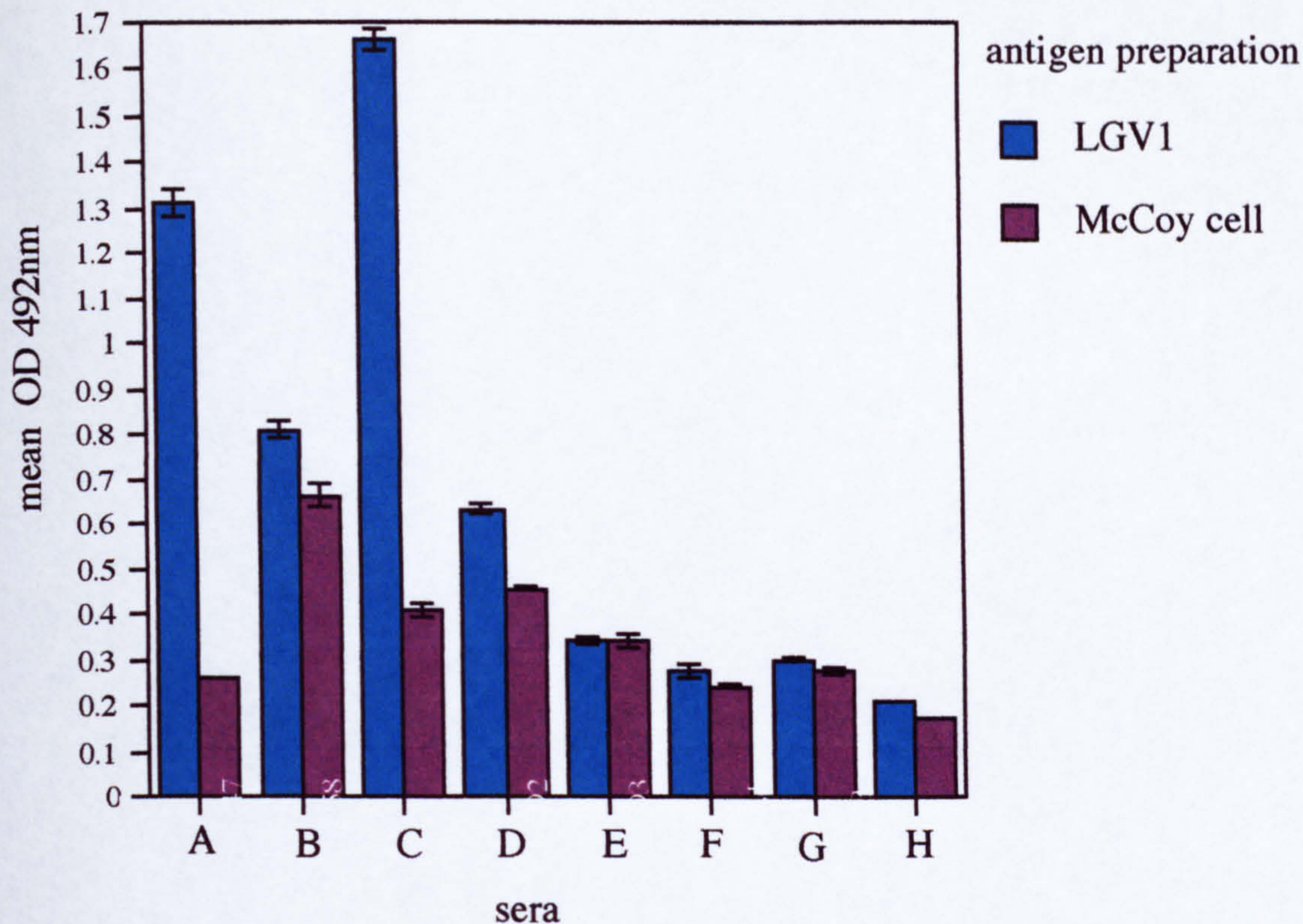
Titration of LGV1 antigen with 1:4000 IgG conjugate dilution

The LGV1 antigen was titrated as formerly described at antigen concentrations ranging from 40µg/ml to 5µg/ml and at serum dilutions from 1:50 to 1:800 (*C. trachomatis* IgG MIF specific 1:512). The results are shown in Graph 22. From the data obtained from this experiment, for all ELISA of actual study samples the antigen concentration was left at 20µg/ml with sodium periodate treatment, and the serum dilution altered to 1:200.

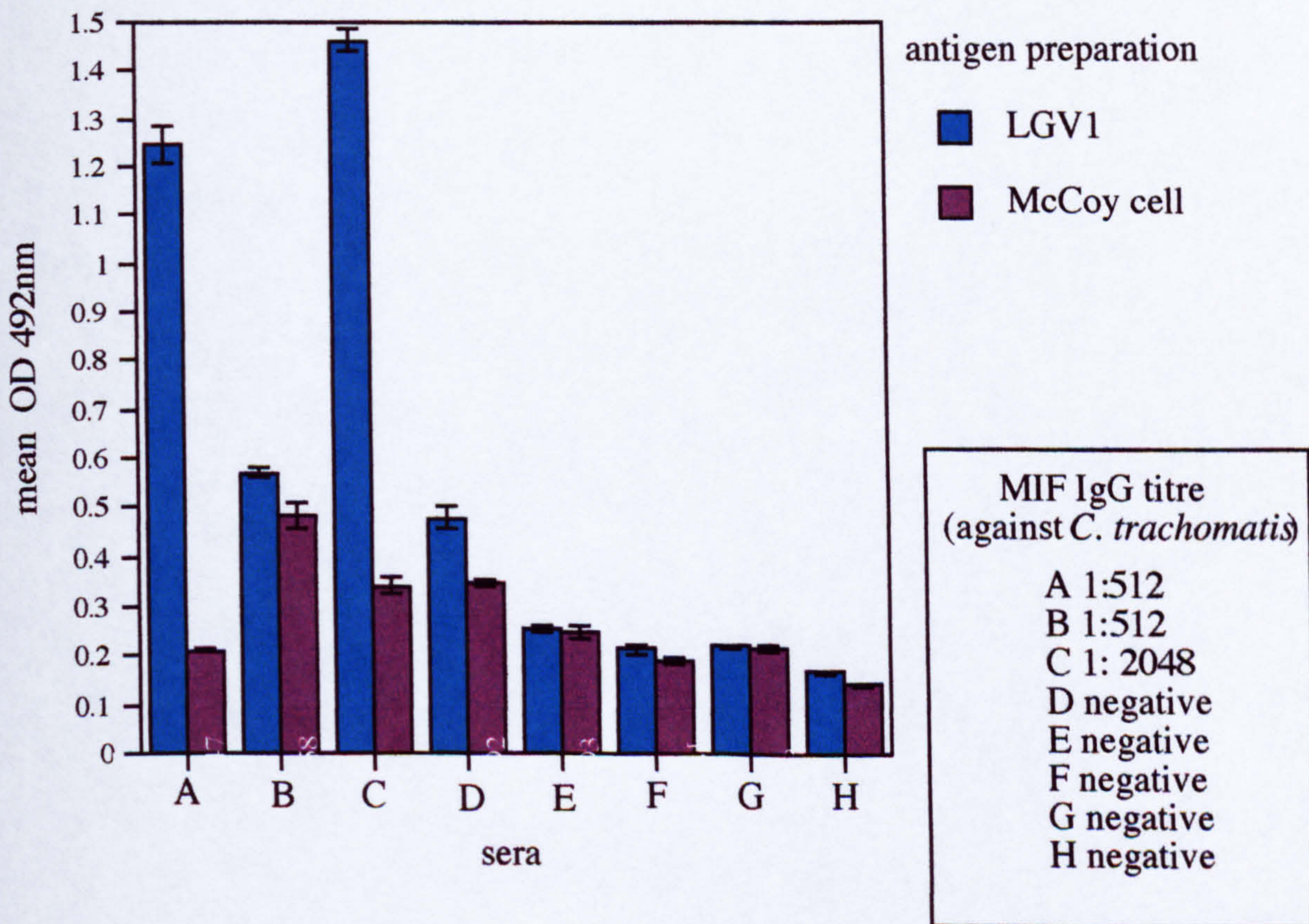
Validation of ELISA with known titre MIF samples

Using the established ELISA method, with a sodium periodate pre-treatment of the antigen, a selection of MIF samples with known IgG titres were tested to see how the mean OD of samples varied with differing levels of *C. trachomatis* IgG.

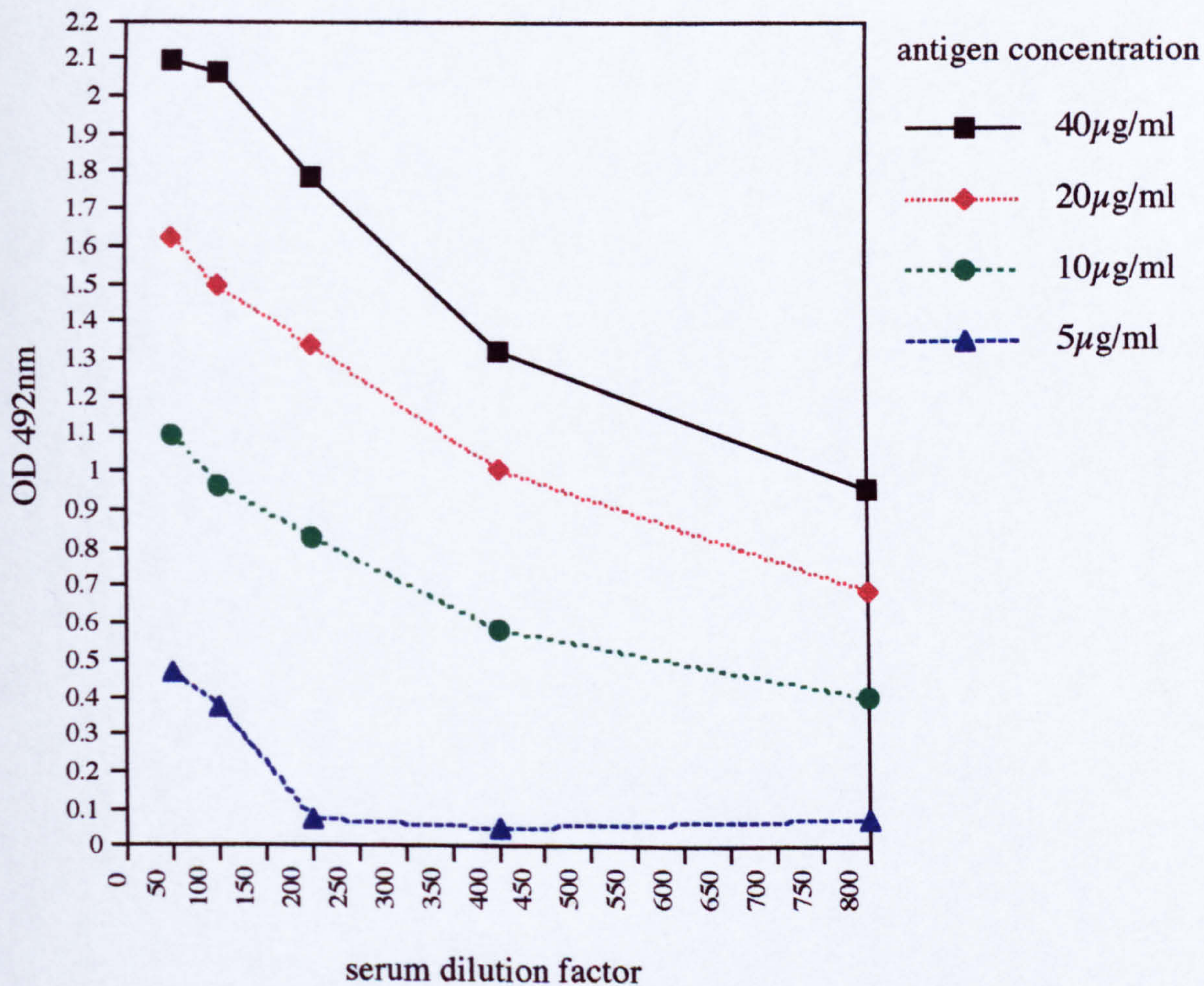
Graph 20. Comparison of mean OD values of a panel of sera (diluted 1:100) against both LGV, and McCoy cell antigen preparations (20 μ g/ml), after sodium periodate treatment at aIgG conjugate dilution of 1:2000.



Graph 21. Comparison of mean OD values of a panel of sera (diluted 1:100) against both LGV1 and McCoy cell antigen preparations (20 μ g/ml), after sodium periodate treatment at an IgG conjugate dilution of 1:4000.



Graph 22. IgG ELISA standardization with a *C. trachomatis* specific serum (IgG micro-immunofluorescence titre 1:512) with sodium periodate antigen pre-treatment, at an IgG conjugate dilution of 1:4000.



The MIF samples (Northern General Hospital) were tested in triplicate at a serum dilution of 1:100 against 20µg/ml of the LGV1, and at an IgG conjugate dilution of 1:4000. The mean ODs of a series of *C. trachomatis* IgG positive samples were compared to the mean OD of two chlamydia negative samples (Graph 23); and the mean OD of a selection of chlamydia negative samples (labelled A through to T) were compared to a high titre *C. trachomatis* positive (IgG MIF 1:512) serum sample (Graph 24).

The high 1:512 titre MIF samples (Graph 23) gave a range of mean OD values (0.7-1.4) and the 1:128 samples ranged from 0.7-0.85. When compared to the appropriate negative controls all the samples would have been considered positive. The negative serum samples (Graph 24) also had a range of OD values (0.058-0.36), and by calculating the mean OD of the negative samples with addition of three times the mean standard deviation (SD), all the negative samples would have been considered negative if tested by ELISA alone.

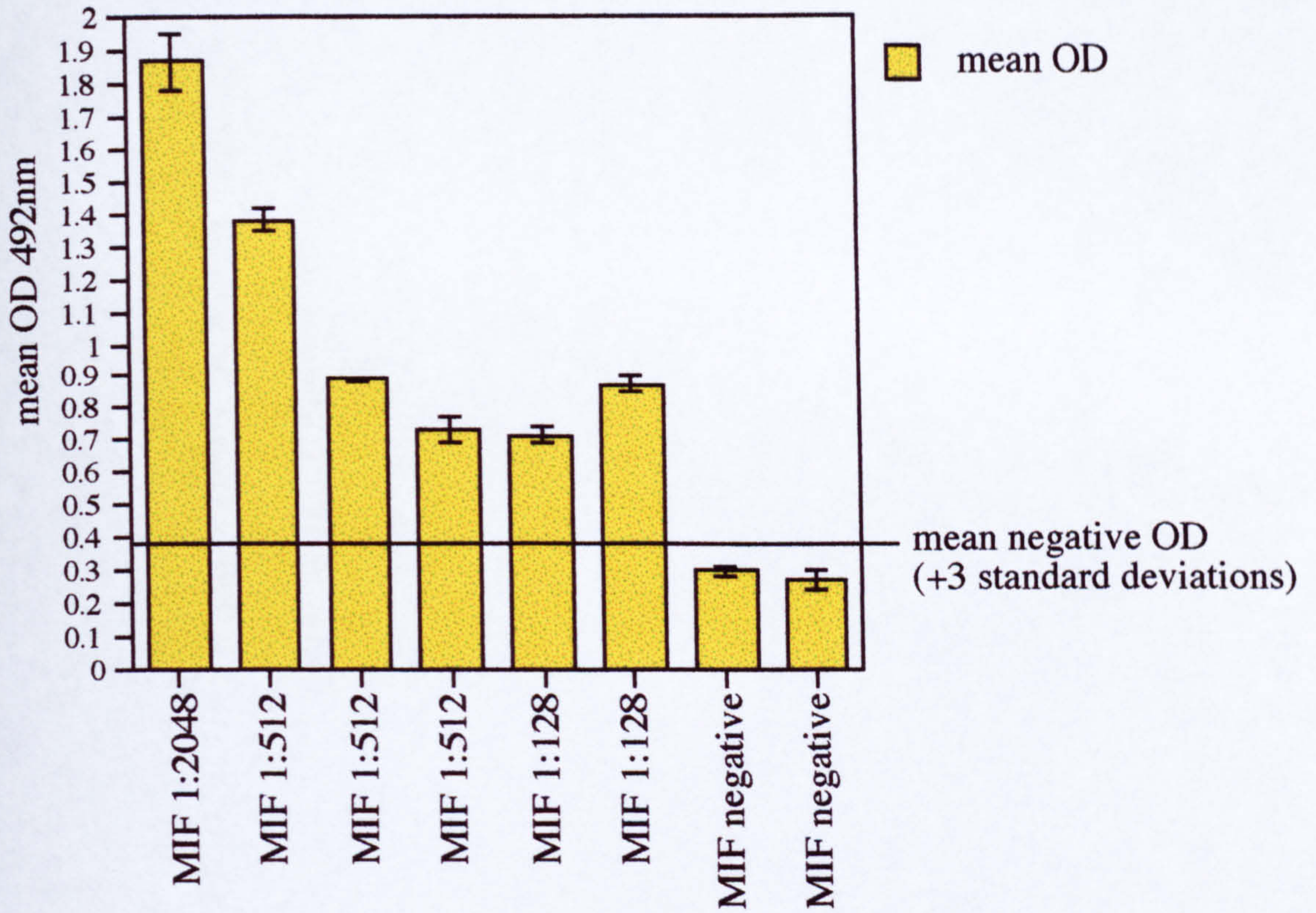
This experiment highlighted one of the problems associated with MIF, the subjectivity of the reader. It is highly likely that the serum samples tested were tested by different operators, which could give variability in MIF titres accounting for the range of optical densities seen by ELISA. Generally speaking, the designated MIF titres of the samples were reflected in the mean optical densities after ELISA, in that negative MIF sera OD values fell below the negative cut off value; and positive MIF samples gave OD results that ranged above the negative experimental cut off. A similar study by Ben-Ahmeida *et al.* (1990) using a panel of human sera, determined the presence of IgG antibody against chlamydia by three serological assays; CFT, IF, and ELISA. This study showed that there was a strong correlation between IF titre and ELISA readings.

6.1.2 IgM ELISA development

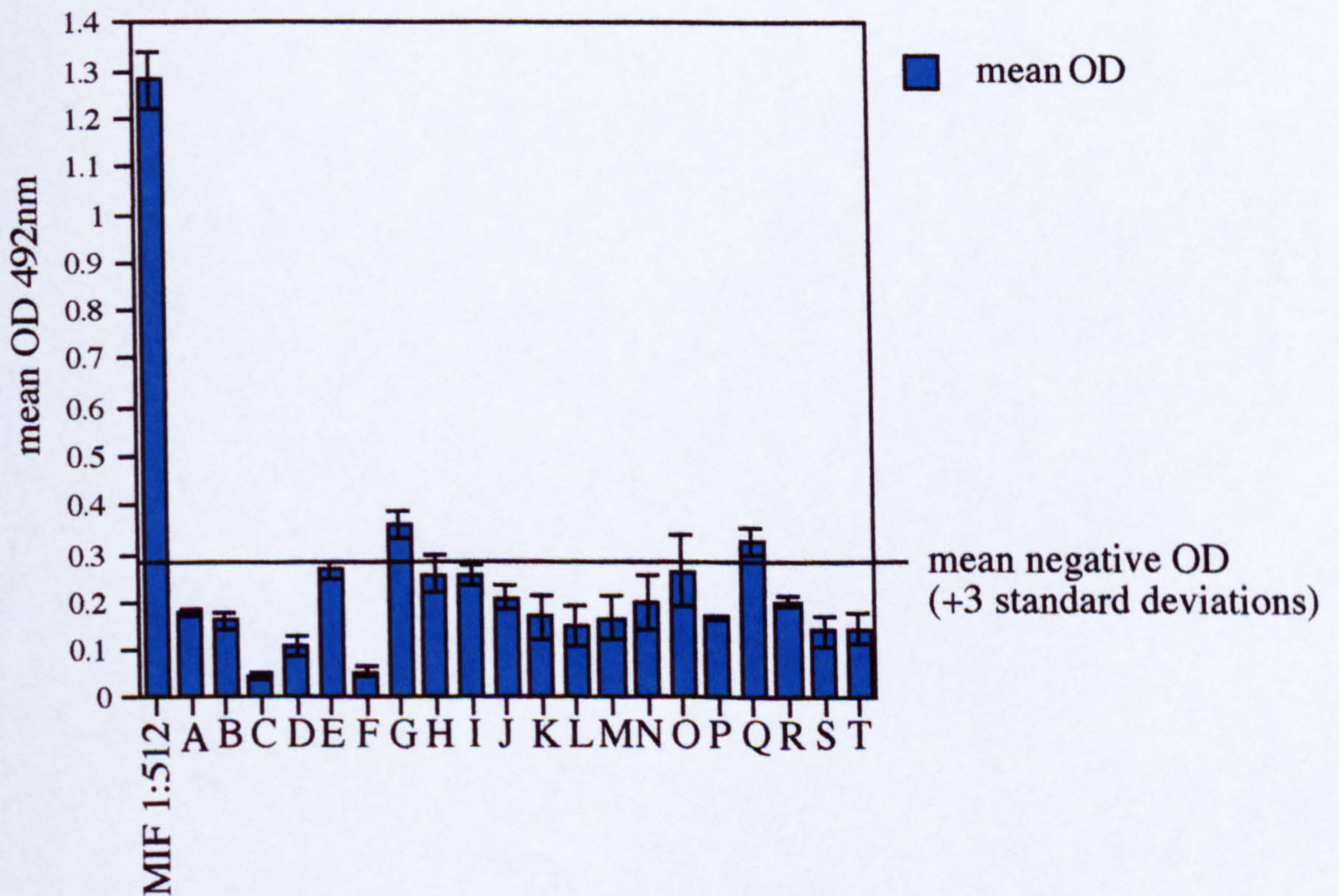
IgM titration

As previously described, the IgM conjugate was titrated against the LGV1 antigen preparation, after sodium periodate treatment. Briefly, the LGV1 antigen was diluted to

Graph 23. Comparison of different micro-immunofluorescence (MIF) titre sera (IgG) by IgG enzyme-linked immunoassay (with sodium periodate antigen pre-treatment).



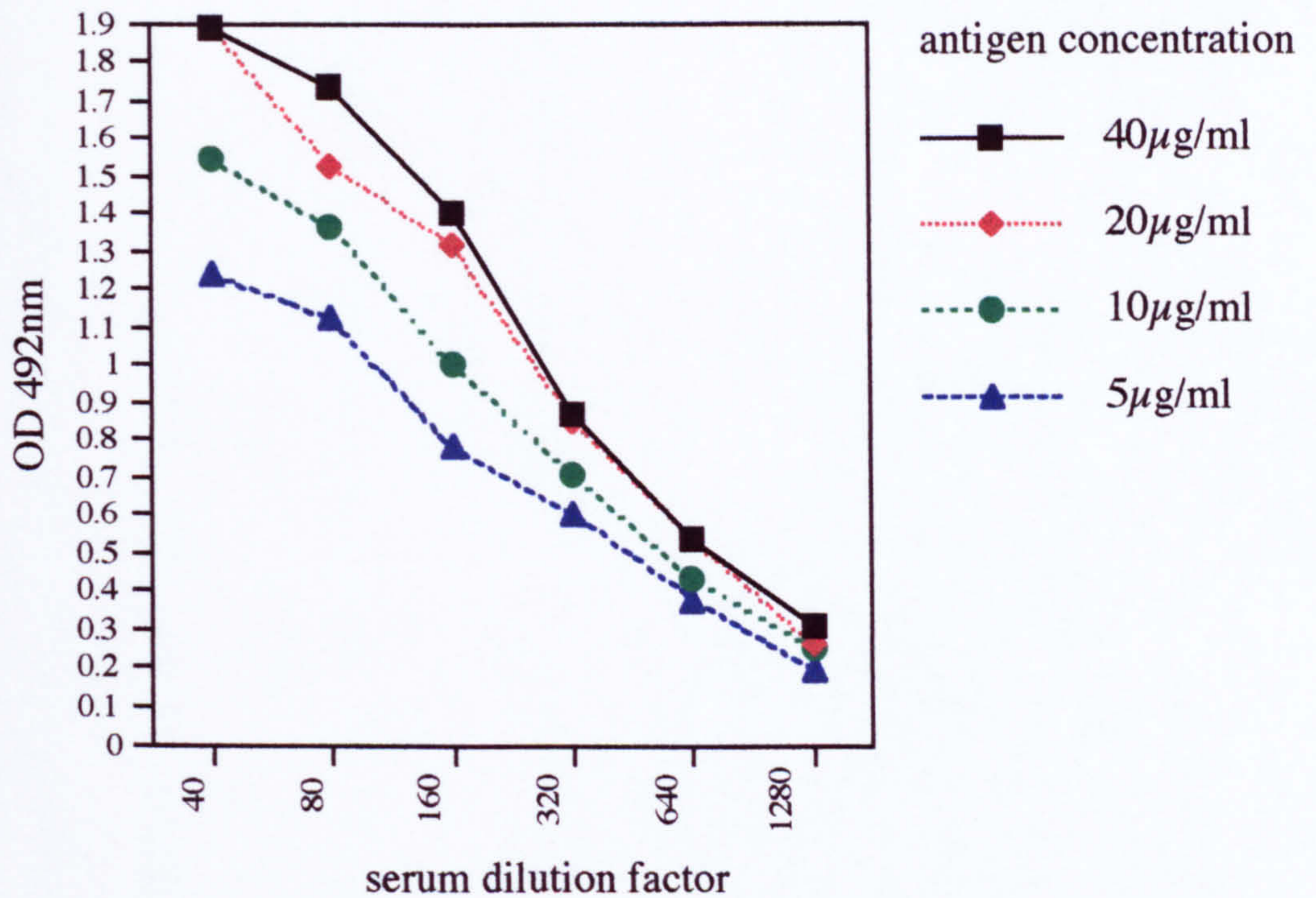
Graph 24. Comparison of the mean OD by IgG enzyme-linked immunoassay (with sodium periodate antigen pre-treatment) of twenty serum samples negative for IgG against *C. trachomatis* by micro-immunofluorescence.



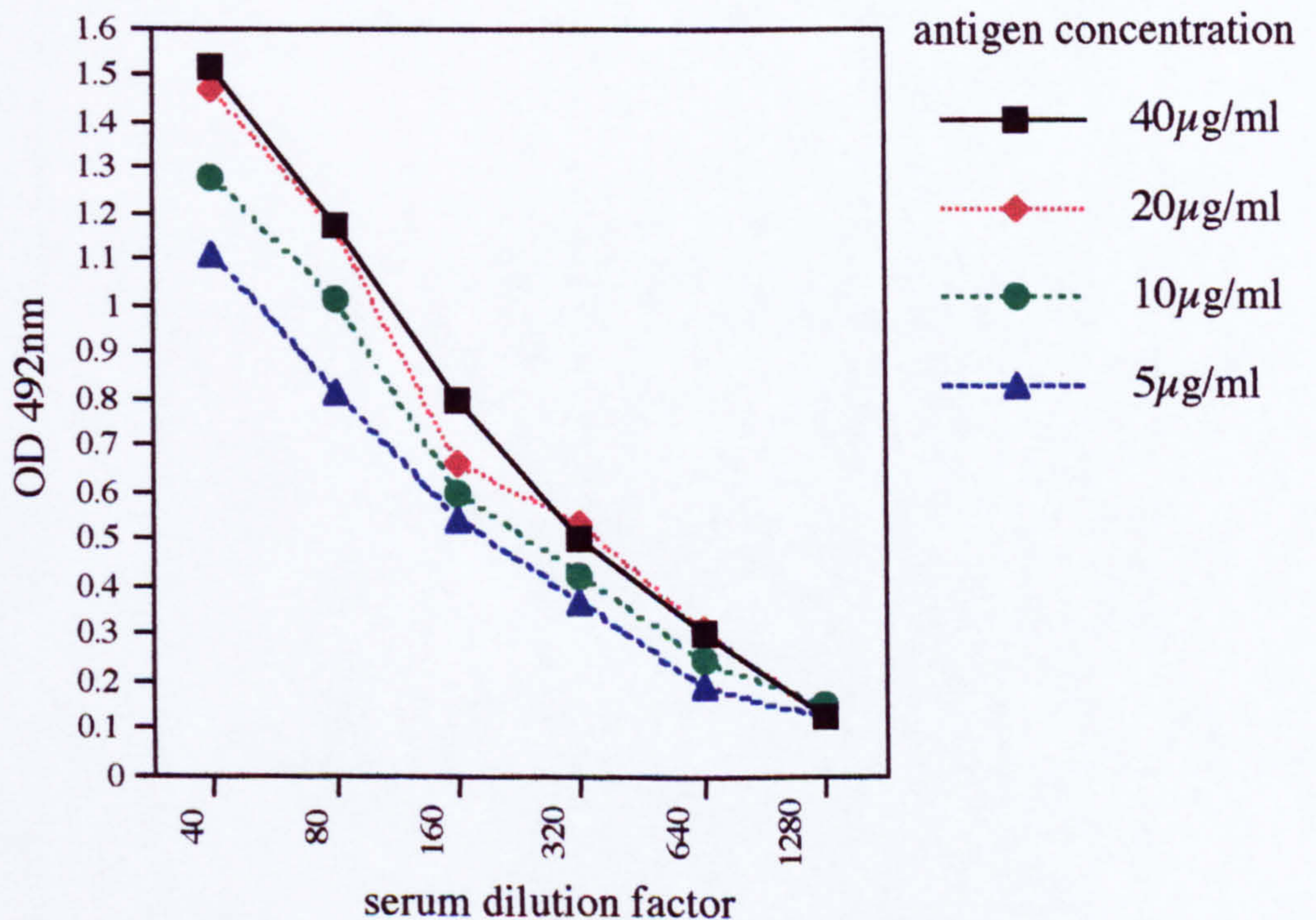
give a range of concentrations (40µg/ml to 5µg/ml). Two serum samples were tested, a low IgM MIF *C. trachomatis* specific serum (IgM MIF 1:20), and a high IgM MIF *C. trachomatis* specific serum (IgM MIF>1:40). Both sera were diluted from 1:40 to 1:1280, and titrated against the LGV1 antigen at all antigen concentrations. The basic ELISA methodology was the same as previously stated for IgG ELISA, except the substrate buffer was left to develop at room temperature for 35min.

The results for both samples are shown in Graph 25, and Graph 26. From the data obtained in this experiment the optimal antigen concentration for IgM ELISA was 20µg/ml, with a conjugate dilution of 1:2000, and a serum dilution of 1:200.

Graph 25. IgM standardization with sodium periodate LGV1 treatment with a *C. trachomatis* specific serum (IgM micro-immunofluorescence titre >1:40), at an IgM conjugate dilution of 1:2000.



Graph 26. IgM standardization with sodium periodate LGV1 treatment with a *C. trachomatis* specific serum (IgM micro-immunofluorescence titre >1:20), at an IgM conjugate dilution of 1:2000.



6.2 Appendix 2. Appendices keys

6.2.1 Key for Appendices 3 and 4.

Patient details	? - age not given	† - no significant clinical details	EP* - samples taken at the time of surgery for EP
Histology	mild acute inflammation - neutrophils predominated	mild chronic inflammation - lymphocytes and macrophages predominated	
Serology	Y - serum available	none - serum not available	+ - anti-chlamydial antibody detected - - anti-chlamydial antibody not detected
Tissue samples	E - endometrium	O - ovary	F - Fallopian tube
	+ve - indicated sample positive for chlamydial DNA		N/A - not applicable
	AS-ve - all tissue samples tested were negative for chlamydial DNA		-ve - indicated sample negative for chlamydial DNA serovar - indicates the <i>C. trachomatis</i> serovar as determined by MOMP PCR and restriction enzyme digestion.
TNF gene polymorphisms	+/+ - normal/normal	+/- - normal/polymorphism	-/- - polymorphism/polymorphism

6.2.2 Key for Appendix 5.

+ - anti-chlamydial IgG detected	- - anti-chlamydial IgG not detected
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6.3 Appendix 3. Individual patient results for Sheffield/Bristol.

6.3.1 Group: Controls

Patient code	age	samples for PCR	samples for ISH	serum	PCR/Southern	ISH	TNF α	TNF β	IgG	IgM	clinical details/history and histological findings
JHW05	36	EOF	EOF	Y	AS -ve	AS -ve	+/+	+/+	-	+	O F and pelvis normal at peration.
JHW06	38	EOF	EOF	Y	AS -ve	AS -ve	+/-	+/+	-	-	Hysterectomy. EOF normal at time of operation.
JHW07	34	EOF	E	Y	F +ve (serovar F)	AS -ve	+/+	+/-	-	-	EOF normal at time of operation.
JHW08	57	EOF	EOF	Y	AS -ve	AS -ve	+/+	+/+	-	-	EOF normal at time of operation. On HRT.
JHW09	33	EOF	N/A	Y	AS -ve	N/A	+/+	+/+	-	-	EOF normal at time of operation.
JHW10	35	EOF	EO	Y	AS -ve	AS -ve	+/+	+/+	-	-	O F normal, dysfunctional uterine bleeding.
JHW11	?	EOF	EF	Y	AS -ve	AS -ve	+/+	+/-	-	-	No information available.
JHW12	39	EOF	EF	Y	AS -ve	AS -ve	+/-	+/-	-	-	Bulky uterus, O F and pelvis normal.
JHW13	38	EOF	EOF	Y	AS -ve	AS -ve	+/-	+/-	-	-	O F normal at time of operation. E-possible chronic inflammation.
JHW15	46	EOF	EOF	Y	AS -ve	AS -ve	+/+	+/+	-	-	normal pelvic anatomy. Previous PID and STD.
JHW16	49	EOF	EOF	Y	AS -ve	AS -ve	+/+	+/+	-	-	No information available.
JHW17	43	EOF	E	Y	AS -ve	AS -ve	+/+	+/-	+	-	possible fibroids.
JHW18	?	EOF	OF	Y	AS -ve	AS -ve	+/-	+/-	-	+	chronic pelvic pain, no infertility.
JHW20	?	EOF	EOF	Y	AS -ve	F+ve	+/+	+/-	+	-	F- chronic inflammation. E- focal cysts

Patient code	age	samples for PCR	samples for ISH	serum	PCR/Southern	ISH	TNF α	TNF β	IgG	IgM	clinical details/history and histological findings
JHW21	40	EOF	EF	Y	AS -ve	AS -ve	+/+	+/+	-	-	Hysterectomy.
JHW25	45	EOF	EOF	Y	F +ve	AS -ve	+/+	+/-	-	-	No information available. E- focal aggregates.
JHW28	39	EOF	EOF	Y	AS -ve	AS -ve	+/+	+/+	-	-	O F normal at time of operation.
JHW30	44	EOF	EOF	Y	AS -ve	AS -ve	+/+	+/+	-	-	O F normal at time of operation.
BR05	34	OF	OF	Y	AS -ve	AS -ve	+/+	+/+	+	-	F- mild chronic inflammation/ mild acute inflammation.
BR12	39	EOF	EO	Y	O +ve	AS -ve	+/+	+/-	-	-	F- mild chronic inflammation/ mild acute inflammation. Lymphoid aggregates.

6.3.2 Group: Ectopic pregnancy

Patient code	age	samples for PCR	samples for ISH	serum	PCR/Southern	ISH	TNF α	TNF β	IgG	IgM	clinical details/history and histological findings
JHW19	29	EOF	EO	Y	F +ve (serovar E)	AS -ve	+/+	+/-	+	-	EP* 2x previous EP & 6yr infertility. Previous STP and PID. Adhesions in pelvis.
JHW22	40	EOF	EOF	Y	AS -ve	AS -ve	+/-	+/+	+	-	EP* 1x previous EP. 6yr infertility, previous PID, endometriosis. E-chronic inflammation.
JHW23	31	EOF	EOF	Y	AS -ve	AS -ve	+/+	+/-	+	+	EP* 1x previous EP, 4yr infertility, previous PID.
JHW24	24	EOF	EO	Y	O +ve	AS -ve	+/+	+/-	-	-	EP* Vaginal bleeding and abdominal pain.
JHW26	29	EOF	EO	Y	F +ve (serovar F)	AS -ve	+/+	+/-	+	-	EP* †
JHW29	31	EOF	EOF	Y	AS -ve	AS -ve	+/+	+/-	+	+	EP* †
JHW32	?	none	EF	none	N/A	AS -ve	N/A	N/A	N/A	N/A	EP* F-mild acute/mild chronic inflammation.
JHW33	25	none	EF	none	N/A	AS -ve	N/A	N/A	N/A	N/A	EP* 1x previous EP. F-mild acute/mild chronic inflammation.
JHW35	38	EOF	EOF	Y	EF +ve	EF +ve	+/+	+/-	-	-	EP* 1x previous EP. Previous IVF.
JHW36	24	EOF	EOF	Y	EO +ve	E +ve	+/+	-/-	-	-	EP* †
JHW37	34	EOF	EOF	Y	AS -ve	AS -ve	-/-	-/-	+	-	EP* possible evidence of PID.

6.3.3 Group: Tubal Factor Infertility

Patient code	age	samples for PCR	samples for ISH	serum	PCR/Southern	ISH	TNF α	TNF β	IgG	IgM	clinical details/history and histological findings
JHW14	24	EOF	EOF	Y	AS -ve	F+ ve	+/+	+/+	-	-	3yr infertility, previous left salpingitis, severe right peritubo-periovarian-tuboovarian disease. Previous PID and STD.
JHW27	27	EOF	EOF	Y	AS -ve	AS -ve	+/+	+/-	-	-	left hydrosalpinx, 8 yr infertility, previous PID and adhesions in Pouch of Douglas/ovaries
JHW34	31	EF	EF	Y	AS -ve	AS -ve	-/-	-/-	+	-	Primary infertility, bilateral hydrosalpinx. Chlamydial serology 1:128 (IgG). F-mild acute/mild chronic inflammation. Thickened tube, lack of mucosal folds and ciliated epithelia.
BR01	30	EOF	EF	none	F +ve	F +ve	+/-	+/-	N/A	N/A	Primary infertility.
BR02	31	OF	EOF	Y	AS -ve	AS -ve	+/-	+/-	+	+	No information available.
BR03	36	EOF	EO	Y	E +ve	E +ve	+/+	+/+	+	-	Secondary infertility. 1x miscarriage.
BR04	32	EOF	EOF	Y	EF +ve	EF +ve	+/+	+/+	+	-	Secondary infertility.
BR06	24	EOF	EOF	Y	F +ve	F +ve	+/-	-/-	-	-	Secondary infertility. 1x miscarriage.
BR07	29	OF	OF	Y	O +ve	AS -ve	+/+	+/+	+	-	Secondary infertility. Salpingitis isthmic nodosa.
BR08	28	EOF	EO	Y	EOF +ve	AS -ve	+/-	+/-	+	-	Primary infertility.
BR09	30	EOF	EO	Y	EOF +ve	AS -ve	+/+	+/-	+	-	Primary infertility. EP following tubal surgery. No ciliated epithelial layer on Fallopian tube.

Patient code	age	samples for PCR	samples for ISH	serum	PCR/Southern	ISH	TNF α	TNF β	IgG	IgM	clinical details/history
BR10	35	EO	EOF	Y	EO +ve	AS -ve	+/-	+/-	+	-	Secondary infertility. F-mild acute/mild chronic inflammation.
BR11	33	EO	EOF	none	EO +ve	AS -ve	+/-	+/-	N/A	N/A	Secondary infertility.
BR13	26	EOF	EOF	Y	E +ve (serovar F)	F +ve	+/+	+/+	+	-	Secondary infertility. 1x miscarriage.

6.4 Appendix 4. Individual patients results for Trinidad.

6.4.1 Group: Controls

Patient code	age	samples for PCR	samples for ISH	serum	PCR/Southern	ISH	TNF α	TNF β	IgG	IgM	clinical details/history and histological findings
M001	33	EOF	EOF	Y	AS -ve	As -ve	+/+	+/-	+ve	-ve	1x still birth.
M003	24	EOF	EOF	Y	AS -ve	AS -ve	+/+	+/+	+ve	-ve	†
M005	34	EOF	EOF	Y	AS -ve	AS -ve	+/+	+/-	-ve	-ve	†
M006	32	EOF	EF	Y	O +ve	AS -ve	+/+	+/-	-ve	-ve	†
M008	24	EOF	EOF	Y	AS -ve	AS -ve	+/+	+/-	-ve	-ve	†
M009	42	EOF	EOF	Y	AS -ve	AS -ve	+/+	+/-	+ve	-ve	†
M023	29	EOF	EOF	Y	O +ve	AS -ve	+/+	+/-	-ve	+ve	†
M027	32	EOF	EO	Y	AS -ve	AS -ve	+/+	+/+	+ve	-ve	Previous PID.
M028	33	EO	EO	Y	AS -ve	AS -ve	+/+	+/-	+ve	-ve	1x induced abortion.
M033	37	EF	EOF	none	AS -ve	AS -ve	+/+	+/+	N/A	N/A	1x induced abortion.
M035	22	EF	F	Y	AS -ve	AS -ve	+/+	+/-	-ve	-ve	1x induced abortion.
M036	25	EOF	EOF	Y	AS -ve	AS -ve	+/+	+/+	-ve	-ve	†
M038	36	EOF	EOF	none	AS -ve	AS -ve	Failed	Failed	N/A	N/A	†
MM103	39	EOF	none	Y	EF +ve (serovar E)	N/A	+/+	+/+	+ve	-ve	1x induced abortion.
MM104	39	EOF	EF	Y	AS -ve	AS -ve	+/+	+/+	-	-	†
MM109	23	EOF	N/A	Y	OF +ve	N/A	+/-	+/-	-ve	-ve	†
MM113	29	OF	N/A	Y	AS -ve	N/A	+/+	+/+	-ve	-ve	1x induced abortion.

Patient code	age	samples for PCR	samples for ISH	serum	PCR/Southern	ISH	TNF α	TNF β	IgG	IgM	clinical details/history and histological findings
MM114	33	OF	N/A	Y	AS -ve	N/A	+/+	-/-	-ve	-ve	3x induced abortions.
MM125	29	OF	N/A	Y	OF +ve	N/A	+/+	+/+	-ve	+ve	†
MM126	34	OF	N/A	Y	AS -ve	N/A	+/+	+/+	-ve	-ve	3x induced abortions.
MM132	44	OF	N/A	Y	F +ve	N/A	+/+	+/+	-ve	-ve	2x induced abortions.
MM133	31	OF	N/A	Y	O +ve	N/A	+/-	+/-	+ve	-ve	†
MM134	31	OF	N/A	Y	F +ve	N/A	+/+	+/-	-ve	+ve	2x induced abortions.
MM135	32	OF	F	Y	AS -ve	AS -ve	+/+	+/-	-ve	-ve	†
MM137	32	OF	N/A	Y	AS -ve	N/A	+/+	+/-	+ve	-ve	1x induced abortion.
MM140	32	OF	N/A	Y	OF +ve	N/A	+/+	+/+	-ve	-ve	†
MM150	32	OF	OF	Y	OF +ve	F +ve	+/+	+/+	-ve	-ve	†
MM151	35	OF	N/A	Y	AS -ve	N/A	+/+	+/+	-ve	-ve	†
MM152	26	OF	N/A	Y	O +ve	N/A	+/-	-/-	+ve	-ve	†
P001	?	EOF	EOF	Y	O +ve	F +ve	+/+	+/-	+ve	-ve	†

6.4.2 Group: Miscarriage

Patient code	age	samples for PCR	samples for ISH	serum	PCR/Southern	ISH	TNF α	TNF β	IgG	IgM	clinical details/history and histological findings
M002	25	EOF	EF	Y	AS -ve	AS -ve	Failed	+/-	-ve	-ve	1x miscarriage.
M004	?	EOF	EOF	Y	OF +ve	F +ve	+/+	+/-	+ve	-ve	3x miscarriages.
M010	41	EOF	EOF	Y	E +ve	AS -ve	+/+	+/+	+ve	-ve	1x miscarriage. 1x induced abortion.
M012	34	EOF	EOF	Y	O +ve	E +ve	+/+	+/+	+ve	-ve	1x miscarriage.
M013	32	EOF	F	Y	EO +ve	AS -ve	+/+	+/+	+ve	+ve	1x miscarriage.
MM102	36	OF	N/A	Y	AS -ve	N/A	+/+	+/-	-ve	-ve	4x miscarriages. Previous STD. O - mild acute inflammation.
MM111	29	EOF	N/A	Y	AS -ve	N/A	+/-	-/-	-ve	+ve	1x miscarriage.
MM112	35	EF	N/A	Y	E +ve (serovar E)	N/A	+/+	-/-	-ve	-ve	3x miscarriages. Previous PID. Pelvic adhesions, fimbrial tubal blockage.
MM118	30	OF	N/A	Y	OF +ve	N/A	+/+	+/+	-ve	-ve	1x miscarriage. 1x induced abortion. Previous PID.
MM127	39	OF	N/A	Y	OF +ve	N/A	+/+	+/+	-ve	-ve	1x miscarriage.
MM131	36	OF	N/A	Y	OF +ve	N/A	+/+	+/+	-ve	-ve	1x miscarriage.
MM136	29	OF	N/A	Y	O +ve	N/A	+/+	+/+	-ve	-ve	1x miscarriage. Previous PID.
MM138	32	OF	N/A	Y	OF +ve	N/A	+/+	+/+	+ve	-ve	1x miscarriage. Previous PID.
MM141	35	OF	OF	Y	AS -ve	AS -ve	+/+	+/+	-ve	-ve	1x miscarriage.
MM142	30	OF	N/A	Y	AS -ve	N/A	+/-	-/-	-ve	-ve	4x miscarriages. Previous PID.
MM144	32	OF	N/A	Y	AS -ve	N/A	+/+	-/-	+ve	-ve	1x miscarriage. 2x induced abortions.

Patient code	age	samples for PCR	samples for ISH	serum	PCR/Southern	ISH	TNF α	TNF β	IgG	IgM	clinical details/history and histological findings
MM145	38	OF	N/A	Y	F +ve	N/A	+/-	+/-	+ve	-ve	1x miscarriage.
MM146	33	OF	N/A	none	AS -ve	N/A	-/-	-/-	N/A	N/A	5x miscarriages.
MM147	39	OF	N/A	none	F +ve	N/A	+/+	+/-	N/A	N/A	1x miscarriage.
MM149	30	OF	F	Y	O +ve	AS -ve	+/+	+/+	-ve	-ve	1x miscarriage.
P012	24	EOF	EOF	none	AS -ve	AS -ve	+/+	+/+	N/A	N/A	2x miscarriages.

6.4.3 Group: Ectopic pregnancy

Patient code	age	samples for PCR	samples for ISH	serum	PCR/Southern	ISH	TNF α	TNF β	IgG	IgM	clinical details/history and histological findings
M007	33	EOF	EF	Y	AS -ve	E+ve	+/+	+/+	-ve	-ve	1x previous EP
M011	?	EOF	EOF	Y	OF +ve	AS -ve	+/+	+/+	+ve	-ve	EP* †
M030	31	F	F	Y	AS -ve	F +ve	+/+	+/+	+ve	-ve	EP* Previous PID. 1x miscarriage. F- mild acute/mild chronic inflammation.
M031	16	F	N/A	none	AS -ve	N/A	+/+	Failed	N/A	N/A	EP* No information available.
M032	27	F	N/A	none	F +ve	N/A	+/+	+/-	N/A	N/A	EP* 1x miscarriage. F- mild acute/mild chronic inflammation.
M039	20	F	N/A	none	AS -ve	N/A	+/+	+/+	N/A	N/A	EP* Previous PID. F- mild/moderate inflammation. O- mild acute inflammation in stroma.
MM100	33	none	OF	Y	N/A	AS -ve	N/A	N/A	+ve	-ve	EP* Fimbrial tubal blockage, pelvic adhesions.
MM101	35	none	OF	Y	N/A	AS -ve	N/A	N/A	-ve	-ve	EP* F- mild focal chronic inflammation. 1x induced abortion.
MM124	27	F	N/A	Y	F +ve (serovar F)	N/A	+/+	+/+	-ve	-ve	EP* 1x miscarriage. Previous PID.
MM129	36	OF	N/A	Y	OF +ve	N/A	+/+	+/+	+ve	-ve	EP* 1x induced abortion. Previous PID.
MM143	35	F	F	Y	AS -ve	AS -ve	+/+	+/+	-ve	-ve	EP* 5yr primary infertility. 2x miscarriage. Endometriosis.
P002	36	EOF	EOF	Y	F +ve	AS -ve	+/+	+/+	+ve	+ve	EP* No information available.

Patient ID	age	samples for PCR	samples for ISH	serum	PCR/Southern	ISH	TNF α	TNF β	IgG	IgM	clinical details/history and histological findings
P004	32	EOF	EOF	Y	AS -ve	F +ve	+/+	+/-	+ve	+ve	EP* 1x previous EP
P005	40	OF	OF	Y	O +ve	AS -ve	+/+	+/+	-ve	+ve	EP* Fibroids. Previous PID and STD. 1x induced abortion.
P006	32	OF	OF	Y	AS -ve	F+ve	+/+	+/+	+ve	+ve	EP* No information available.
P013	25	EOF	EOF	Y	AS -ve	E +ve	+/-	-/-	-ve	+ve	EP* 1x miscarriage. F- mild chronic inflammation and adhesions.
P014	?	EOF	EOF	none	AS -ve	AS -ve	+/+	-/-	N/A	N/A	EP* No information available.
P015	19	EOF	N/A	none	EOF +ve	N/A	+/+	+/+	N/A	N/A	EP* No information available.
P016	24	EOF	N/A	none	EOF +ve	N/A	+/+	-/-	N/A	N/A	EP* Previous PID.
P017	?	EOF	EOF	none	AS -ve	AS -ve	+/-	-/-	N/A	N/A	EP* No information available. E-moderate chronic/ acute inflammation. F-moderate acute inflammation.
P018	31	E	N/A	none	E +ve	N/A	+/+	+/+	N/A	N/A	EP* No information available.
P019	38	EF	EOF	none	EF +ve	AS -ve	+/+	-/-	N/A	N/A	EP* 1x miscarriage. 1x induced abortion.
P020	?	EOF	N/A	none	E +ve	N/A	+/+	+/-	N/A	N/A	EP* No information available.
P021	36	OF	OF	none	OF +ve	AS -ve	+/-	+/-	N/A	N/A	EP* No information available.
PM100	28	EOF	N/A	Y	OF +ve	N/A	+/+	+/+	+ve	-ve	EP* Pelvic adhesions.
PM101	35	none	EF	Y	N/A	EF +ve	N/A	N/A	+ve	-ve	EP* 2x induced abortions. F- moderate chronic inflammation mainly serosal.
PM102	25	EOF	N/A	Y	EO +ve	N/A	+/-	-/-	+ve	-ve	EP* 2x miscarriages. Previous PID.

Patient ID	age	samples for PCR	samples for ISH	serum	PCR/Southern	ISH	TNF α	TNF β	IgG	IgM	clinical details/history and histological findings
PM103	32	EOF	EOF	Y	O +ve	F +ve	+/+	+/-	-ve	+ve	EP* 1x miscarriage.
PM104	24	EOF	N/A	Y	O F +ve	N/A	+/+	+/-	+ve	-ve	EP* 1x miscarriage. Pelvic adhesions.
PM105	33	EOF	N/A	Y	E O F +ve (serovar E)	N/A	+/+	+/-	-ve	+ve	EP* 1x miscarriage. Previous PID.
PM106	20	EOF	N/A	Y	O F +ve	N/A	+/+	+/-	+ve	+ve	EP* 1x miscarriage. Previous PID.
PM107	34	EOF	N/A	Y	E O F +ve	N/A	+/+	-/-	+ve	-ve	EP* 2x miscarriages.

6.4.4 Group: Tubal Factor Infertility

Patient code	age	samples for PCR	samples for ISH	serum	PCR/Southern	ISH	TNF α	TNF β	IgG	IgM	clinical details/history and histological findings
P003	30	EOF	EOF	Y	AS -ve	AS -ve	+/-	+/-	+ve	-ve	9 yrs infertility. 1x still birth.
PM108	32	EOF	N/S	Y	F +ve	N/A	+/+	+/+	+ve	-ve	1x miscarriage.

6.5 Appendix 5. Comparison of ELISA (IgG) and IF/CFT (Bristol PHLS) results.

blind code	code	ELISA	IF/CFT
1	N1	-	-
2	N1	-	-
3	BR02	+	+
4	BR03	+	+
5	BR04	+	+
6	BR05	+	+
7	BR06	+	-
8	BR07	+	+
9	BR08	+	+
10	BR09	+	+
11	BR10	+	+
12	BR12	-	-
13	BR13	+	+
14	JHW08	-	-
15	JHW34	+	-
16	JHW30	-	-
17	JHW05	-	-
18	JHW27	-	-
19	JHW04	-	+
20	JHW16	-	-

blind code	code	ELISA	IF/CFT
21	JHW07	-	-
22	JHW09	-	-
23	JHW25	-	-
24	JHW13	-	-
25	JHW18	-	-
26	JHW06	-	-
27	JHW10	-	-
28	JHW31	-	-
29	JHW10	-	-
30	JHW11	-	-
31	JHW35	-	-
32	JHW28	-	-
33	JHW37	+	+
34	JHW24	-	-
35	JHW26	+	-
36	JHW15	-	-
37	JHW29	+	+
38	JHW20	+	-
39	JHW14	-	+
40	JHW02	-	-

blind code	code	ELISA	IF/CFT
41	JHW22	+	+
42	JHW17	+	+
43	JHW01	-	-
44	JHW03	-	-
45	JHW29	+	+
46	JHW36	-	-
47	JHW21	-	-
48	JHW23	+	-
49	JHW19	+	+
50	JHW12	-	-
51	JHW05	-	-
52	JHW31	-	-
53	JHW34	+	-
54	JHW05	-	-
55	BR07	+	+
56	JHW02	-	-
57	BR08	+	-
58	JHW31	-	-
59	JHW02	-	-
60	JHW34	+	-

Chapter 7.0
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