

***Chlamydia trachomatis*: quantification,
immunological investigation and co-infection
with HIV-1**

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Declaration

The work presented here is my own, unless otherwise stated or referenced.

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Abstract

Three aspects of *Chlamydia trachomatis* translational research were explored in this thesis. Firstly, as over 75% of patients with LGV are also HIV-1 sero-positive, a cell-culture model was established to investigate whether HIV-1 altered the replication of *C. trachomatis* serovar L2 *in vitro*. Inclusion size was significantly increased in the presence of HIV-1, but there was no significant alteration in chlamydial growth kinetics, infectivity, morphology, or transcription of *16sRNA*, *ompA* or *euo*, suggesting that viral co-infection did not induce chlamydial persistence. It is, therefore, unlikely that the association of HIV-1 and LGV *in vivo* is due to an impact of HIV-1 on chlamydial replication in co-infected cells.

Secondly, as there is no information on the chlamydial load shed by individuals with a rectal *C. trachomatis* infection, a qPCR assay was developed and used to determine the number of *C. trachomatis* organisms per rectal swab in NAAT-positive patients. The geometric mean chlamydial load was 5.0×10^5 organisms per swab (Standard Deviation, 152) and load was associated with proctitis, but not symptoms or HIV-1 infection. Asymptomatic individuals shed as much *C. trachomatis* as patients with rectal symptoms and might maintain transmission in the community.

Finally, an *ex vivo* IFN- γ ELISpot assay was developed to characterise human cellular immune responses to the *C. trachomatis*-specific protein, Pgp3. T-cell epitopes were found along the length of the protein, but the magnitude of the immune responses was low. The Pgp3- induced IFN- γ response correlated with *C. trachomatis* exposure and was dynamic, decreasing after effective treatment. These observations suggest that Pgp3- induced IFN- γ may be useful as a biomarker for current infection, although the sensitivity and specificity of the ELISpot assay need improvement.

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Finally, I would like to thank my friends and family. Without their love and support, I would surely have not made it to submission! But thanks also for reminding me that there is more to life than work, and you all help to make the journey worth it. I'm a very lucky person indeed.

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Table of Contents

Chapter 1	Introduction	Page 13
Chapter 2	Materials and Methods	Page 54
Chapter 3	Co-infection of <i>Chlamydia trachomatis</i> and HIV-1 <i>in vitro</i>	Page 95
Chapter 4	Quantification of rectal <i>Chlamydia trachomatis</i>	Page 148
Chapter 5	Characterisation of <i>ex vivo</i> cellular immune responses to <i>Chlamydia trachomatis</i>	Page 174
Chapter 6	Discussion	Page 208
Chapter 7	References	Page 216
Appendices		Page 243

List of Tables

Chapter 2

Table 2.1 Oligonucleotide primers used in this thesis

Table 2.2 Cell lines used in the investigation

Table 2.3 Patient recruitment for the ELISpot study

Chapter 3

Table 3.1 End-point serial dilutions of pCR[®] 4-TOPO[®] *pmpH*

Table 3.2 *pmpH*-qPCR intra-assay variation

Table 3.3 *pmpH*-qPCR inter-assay variation

Table 3.4 TEM of *C. trachomatis* in the presence and absence of HIV-1

Chapter 4

Table 4.1 Selection of a suitable gene target of amplification

Table 4.2 End point serial dilutions of pCTL11a

Table 4.3 *orf5*-qPCR Intra- assay variation

Table 4.4 *orf5*-qPCR Inter-assay variation

Table 4.5 λ -phage Inhibition Assay

Table 4.6 Mean *C. trachomatis* loads, by patient group

Chapter 5

Table 5.1 Pgp3 overlapping peptide sequences

List of Figures

Chapter 1

Figure 1.1 Phylogenetic tree of the *Chlamydia* genus

Figure 1.2 The life cycle of *Chlamydia*

Figure 1.3 *Chlamydia* morphology

Chapter 3

Figure 3.1 *C. trachomatis* serovar L2 can grow in the absence of cycloheximide

Figure 3.3 Standard curve for the quantification of *C. trachomatis* by qPCR

Figure 3.4 Replication kinetics of *C. trachomatis* in BGMK cells

Figure 3.5 Comparison of *C. trachomatis* yield from different cell-lysis techniques

Figure 3.6 The infectivity of *C. trachomatis* progeny over time

Figure 3.7 The effect of penicillin on chlamydial infectivity, genome copy number, and the transcription of *16srRNA*, *euo*, *ompA* and *omcB*

Figure 3.8 Identification of a cell-line to investigate *C. trachomatis* and HIV-1 co-infection

Figure 3.9 HIV-1_{MN} infection of MAGI P4R5 cells

Figure 3.10 A - Co-infection of HIV-1 infected cells with *C. trachomatis*

Figure 3.10 B - IFM of *C. trachomatis* inclusions in the presence and absence of HIV-1

Figure 3.10 C - Significantly fewer *C. trachomatis* inclusions develop in cells infected with HIV-1, compared to when HIV-1 is absent

Figure 3.10 D- Significantly larger *C. trachomatis* inclusions develop in cells infected with HIV-1, compared to when HIV-1 is absent

Figure 3.10 E -The replication of *C. trachomatis* genomic copies remains unchanged in cells infected with HIV-1 compared to when HIV-1 is absent

Figure 3.10 F- The infectivity of *C. trachomatis* progeny remains unchanged in cells that are infected with HIV-1 compared to when HIV-1 is absent

Figure 3.10 G - The replication of HIV-1 remains unchanged by the addition of *C. trachomatis*

Figure 3.11 A - Co-infection of *C. trachomatis*- infected cells with HIV-1

Figure 3.11 B- IFM of *C. trachomatis* inclusions in the presence and absence of HIV-1

Figure 3.11 C and D - Significantly fewer, but larger, *C. trachomatis* inclusions develop in cells co-infected with HIV-1

Figure 3.11 E and F -The replication of *C. trachomatis* genomic copies and the infectivity of *C. trachomatis* progeny remain unchanged by the presence of HIV-1

Figure 3.11 H – *C. trachomatis* gene transcription in HIV-1 co-infected cultures

Figure 3.11 I - Transmission electron microscopy of *C. trachomatis*- infected cells in the presence and absence of HIV-1

Figure 3.11 J and K - The replication of HIV-1 remains unchanged in cells that are infected with *C. trachomatis*, compared to when the bacteria are absent

Chapter 4

Figure 4.2 Patient recruitment for the qPCR study

Figure 4.1 The *C. trachomatis* plasmid *orf5* standard curve

Figure 4. 2 Patients with proctitis shed significantly more *C. trachomatis* than patients with no evidence of inflammation

Figure 4.3 Patients with rectal symptoms do not shed a significantly different amount of *C. trachomatis* than asymptomatic individuals

Figure 4.4 Patients with LGV have high *C. trachomatis* loads

Figure 4.5 There is no significant difference in the *C. trachomatis* load between HIV-1 sero-positive and sero-negative individuals

Chapter 5

Figure 5.1 ELISpot study patient recruitment

Figure 5.2 A schematic of the ELISpot Assay

Figure 5.3 Example ELISpot data

Figure 5.4 Pre-coated Vs non-pre-coated plates

Figure 5.5 Pgp3 is a target of the human cellular immune response to *C. trachomatis*

Figure 5.6 Pgp3 contains T-cell epitopes along its entire length

Figure 5.7 Pgp3-induced IFN- γ and *C. trachomatis*-exposure

Figure 5.8 Pgp3 specific IFN- γ responses are dynamic

Figure 5.9 Pgp3-specific IFN- γ responses do not correlate with *Chlamydia* load

Appendices

Appendix 1. Bristol Sexual Health Centre Ethics Approval

Appendix 2. St. Mary's Hospital Ethics Approval for Recruitment of Adults

Appendix 3. St. Mary's Hospital Ethics Approval for Recruitment of Children

List of abbreviations

CT	<i>Chlamydia trachomatis</i>
DFA	Direct Fluorescent Antibody
EB	Elementary Body
ECDC	European Centre for Disease Prevention and Control
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
ELISpot	Enzyme Linked Immunosorbent Spot-forming Assay
EP	Ectopic Pregnancy
FACS	Fluorescence Activated Cell-Sorter
FDA	Food and Drug Administration
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GUM	Genitourinary Medicine
HCV	Hepatitis-C Virus
HIV-1	Human Immunodeficiency Virus-1
HSP60	Heat-Shock Protein 60
HSV-2	Herpes Simplex Virus-2
IB	Intermediate Body
IDO	Indoleamine 2,3-dioxygenase
IFM	Immunofluorescence Microscopy
IFN- γ	Interferon-gamma
IFU	Infectious Forming Unit
iNOS	inducible Nitric Oxide Synthase
LGV	Lymphogranuloma Venereum
MCC	Minimum Chlamydicidal Concentration
MHC	Major Histocompatibility Complex
MIC	Minimum Inhibitory Concentration
MIF	Microimmunofluorescence
MOI	Multiplicity of Infection
MOMP	Major Outer Membrane Protein
MSM	Men who have sex with men
NAAT	Nucleic-acid Amplification Techniques
NCSP	National Chlamydia Screening Programme
nv	new variant
OMP2	Outer Membrane Protein-2
<i>orf1</i>	open reading frame 1
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PID	Pelvic Inflammatory Disease
PMN	Polymorphonuclear cell
PMPD	Polymorphic Membrane Protein D
qPCR	quantitative PCR
RB	Reticulate Body
RFLP	Restriction Fragment Length Polymorphism
rRNA	ribosomal Ribose-nucleic acid
SARA	Sexually Transmitted Reactive Arthritis
SDA	Strand Displacement Assay

SFC	Spot-Forming Cell
SFMCs	Synovial Fluid Monocytic Cells
SG-PERT	SYBR Green I-based product-enhanced reverse transcriptase assay
T3S	Type 3 Secretion
TEM	Transmission Electron Microscopy
TFI	Tubal Factor Infertility
Th	T-helper
TMA	Transcription-Mediated Amplification

Chapter 1

Introduction

1.1. *Chlamydia* Taxonomy

The *Chlamydia* genus is comprised of Gram-negative, obligate intracellular bacteria that are remarkably ubiquitous in nature and are responsible for a great diversity of disease in both animals and humans. The genus belongs to the family *Chlamydiaceae*, which, until recently, was divided into the *Chlamydia* and *Chlamydophila* genera, but is now in the process of reunification into a single genus, *Chlamydia* (Stephens *et al.*, 2009). Over the years the genus has been divided into variable numbers of species. At present, there are nine: *Chlamydia abortus*, *C. pecorum*, *C. felis*, *C. caviae*, *C. suis*, *C. muridarum*, *C. psittaci*, *C. pneumoniae* and *C. trachomatis* (Pantchev *et al.*, 2009). A recently constructed phylogenetic tree is shown in Figure 1.1 (Stephens *et al.*, 2009).

Three *Chlamydia* species infect humans and are of significant public health importance: *C. trachomatis*, *C. pneumoniae* and *C. psittaci*. This thesis is restricted to the study of *C. trachomatis* which is further sub-divided into serovars, based on differences in the major outer membrane protein (MOMP). Serovars A, B, Ba and C cause ocular trachoma, serovars D-K are responsible for genital infection, neonatal conjunctivitis and sexually acquired reactive arthritis (SARA), whereas serovars L1, L2 and L3 are responsible for the sexually transmitted disease, lymphogranuloma venereum (LGV) (Reviewed by Mårdh, 2005).

Figure 1.1 Phylogenetic Tree of the *Chlamydia* genus

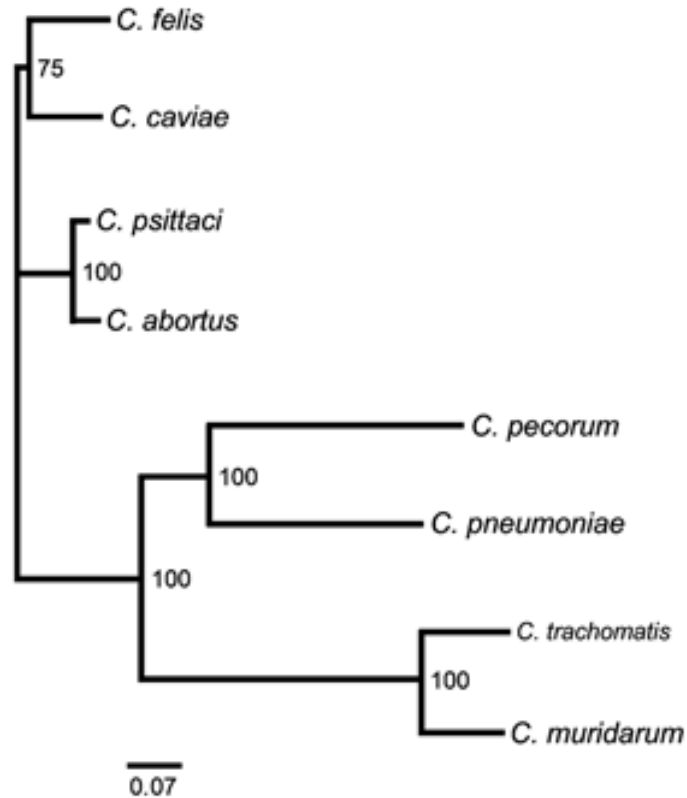


Figure 1.1 The peptide sequences of one hundred and ten conserved proteins from the genomes of *Chlamydia trachomatis*, *C. pneumoniae*, *C. muridarum*, *C. pecorum*, *C. felis*, *C. psittaci*, *C. abortus* and *C. caviae* were identified and aligned. They were then back-translated into nucleotide alignments that were further analysed and used to generate a phylogenetic tree, (Stephens *et al.*, 2009). *C. suis* is absent from the diagram, but is more closely related to *C. trachomatis* and *C. muridarum* than the other species (Bush *et al.*, 2001).

1.2. History

The first documented report of chlamydial disease was in connection with the ocular infection, trachoma. The disease was recognised by the ancient civilisations of China and Egypt thousands of years BC, who describe the application of copper salts around the eyes as a management strategy for the disease. However, the term “trachoma” (rough eye) was not coined until around 60AD, by the Sicilian physician Pedanius Dioscorides (reviewed by Mårdh *et al.*, 1989a). The disease is described in Roman, Greek and Arabic texts and was disseminated by crusaders returning from Palestine and the Napoleonic Egyptian Campaign. Despite this long history, the aetiology of trachoma was not identified until after the Germ Theory of disease in the late 19th Century. Similarly, sexually transmitted diseases have been recorded since the Old Testament, however, gonorrhoea was not distinguished from syphilis and chancroid until 1838 and it was not until the advent of Gram’s staining technique in 1884 that urethral discharges and neonatal ophthalmia could be segregated into gonococcal and non-gonococcal forms (reviewed by Ridgway, 2008).

The first aetiological investigations into trachoma were conducted by Robert Koch in 1883, however, it was not until 1907 that the intracytoplasmic inclusions responsible for the disease were identified in experimentally infected orang-utans by Halberstaedter & von Prowazek (Halberstaedter & Prowazek, 1907; reviewed by Ridgway, 2008). Similar inclusions were subsequently noticed in the conjunctival cells of babies with neonatal conjunctivitis, in urethral epithelial cells and secretions of men with urethritis and in the cervical secretions from mothers of infected new-borns. These observations were consolidated in 1910, when the link between ocular and genital disease was established by Fritsch, Hofstatter and Linder who inoculated monkeys with material from babies with neonatal conjunctivitis, the urethra of their fathers and cervix of their mothers (Fritsch *et al.*, 1910; reviewed by Ridgway, 2008).

Halberstaedter and von Prowazek first named the organisms “*Chlamydozoa*” (mantle animals) after the Greek *chlamys* (cloak), in reference to their cloak-like appearance on staining, however, initially the organisms were thought to be protozoan parasites. When it was discovered that the infection could pass through bacterial filters, the organisms were then considered to be viruses (reviewed by Ridgway, 2008).

In 1930, Bedson and others characterised the aetiology and life-cycle of the infectious agent responsible for psittacosis, a respiratory disease in people acquired from exposure to psittacine birds. The organism was termed “*Bedsonia*” (later re-named *C. psittaci*) and similarities were noticed between its life cycle and those of the agents responsible for trachoma and the newly discovered disease, lymphogranuloma venereum (Bedson & Bland, 1934). However, as both chlamydial and rickettsial organisms are obligate intracellular pathogens that share similarities in their life-cycles, *Chlamydia* were miss-classified as a member of the *Rickettsiae* in the 1930s (reviewed by Ridgway, 2008).

Despite these advances, it was not possible to isolate and cultivate chlamydial organisms from infected patients until, in 1957, T’ang *et al.* successfully recovered *C. trachomatis* from an embryonated hen’s egg (T’ang *et al.*, 1957). This paved the way for yolk-sac culture studies that enabled *Chlamydia* to be isolated from genital material of infected patients and the eyes of an infant with inclusion conjunctivitis (Jones *et al.*, 1959) and confirm the aetiology of non-gonococcal urethritis, associated cervicitis and inclusion conjunctivitis (Dunlop *et al.*, 1964; Dunlop *et al.*, 1966). Yolk-sac culture studies also made it possible *Chlamydia* to be eventually classed as a bacterial infection (Moulder *et al.*, 1966).

In 1965, Gordon & Quan discovered that McCoy cells (mouse fibroblast cells) could be used to isolate *C. trachomatis* if they were irradiated before infection (Gordon & Quan, 1965). Cell culture systems were further simplified in 1977 by the application of cycloheximide to permit chlamydial growth without the need for irradiating the cultures. Cycloheximide inhibits eukaryotic DNA and protein synthesis and enhances the growth of *C. trachomatis* in culture by reducing the cellular metabolism of nutrients in the growth medium (Ripa & Mårdh, 1977). This technique finally permitted, for the first time, routine, large-scale diagnosis of *C. trachomatis* by cell culture methods that superseded yolk-sac culture and enabled a new era of chlamydial molecular and cellular biological research to flourish.

In the 1970s and 1980s, there was an explosion of studies into the molecular biology, protein chemistry, pathogenesis, immunology and epidemiology of chlamydial organisms. However, it was the advent of nucleic acid amplification techniques (NAAT) in the mid 1990s that revolutionised *Chlamydia* detection and paved the way for chlamydial screening (discussed in Section 1.9).

Presently, a hundred years after the first demonstrable link between ocular and genital chlamydial disease, there are still many hurdles to overcome. Clinically, there is no non-invasive way of identifying infected individuals who are not shedding organisms, nor is there a licensed vaccine against chlamydial diseases. In addition, as discussed below, the natural history of *C. trachomatis in vivo* is not fully understood and, as the organism cannot be genetically modified, scientists are forced to use surrogate expression systems to investigate pathogen-host-cell interactions. Given that technological advances in the early 21st Century are occurring at an ever-increasing rate, there may well be breakthroughs in these areas in the decades to come.

1.3. *Chlamydia trachomatis* organisms

C. trachomatis exists in two main forms: An Elementary Body (EB) and a Reticulate Body (RB). The EB is typically round and 200-300nm in diameter with an irregular core of DNA, a plasma membrane, periplasmic space and an outer membrane (reviewed by Ward & Ridgway, 1998). It is the only infectious form and the only one capable of surviving for any period outside the host. In contrast, the RB is larger (approximately 1µm in diameter) and has a much less rigid outer membrane. It is metabolically active and the only form of the organism that replicates, by binary fission (reviewed by Ward & Ridgway, 1998).

Each organism possesses a circular double stranded DNA chromosome, approximately one million base-pairs in length, encoding approximately one thousand proteins (reviewed by Dean *et al.*, 2006) and a plasmid, termed the “cryptic plasmid” (Thomas *et al.*, 1997) that is approximately 7,500 base pairs in length and encodes 8 open reading frames (Black *et al.*, 1989; Comanducci *et al.*, 1988; Comanducci *et al.*, 1990; Hatt *et al.*, 1988; Sriprakash & MacAvoy 1987; Thomas & Clarke 1997). The estimated number of plasmid copies per organism varies between studies and strains. Recently, one study calculated there to be 4 plasmids per EB (+/- 0.8), increasing to 7.6 per organism during the replication cycle (Pickett *et al.*, 2005). Another study found there to be 7.72 (+/- 0.68) copies per bacterium (Michel *et al.*, 2007) and another found between approximately 2 and 6 plasmids per organism in genital tract isolates and L2 (Seth-Smith *et al.*, 2009).

1.4. *Chlamydia trachomatis* life cycle

C. trachomatis replicate in a biphasic life cycle (Figure 1.2). Initially, the EB binds to host cells and is taken up by receptor-mediated endocytosis into an endocytic vesicle. Shortly after gaining entry into the host cell, the vesicle containing the EB becomes dissociated from the

endocytic pathway and the EB differentiates into a larger, metabolically active RB that replicates by binary fission within the vesicle, termed an “inclusion” which expands to accommodate the growing number of organisms (reviewed by Mårdh, 2005). Chlamydial transmembrane proteins become embedded in the inclusion membrane and are known to interact with host cellular proteins in the cytoplasm. In addition, RBs are attached to the inclusion by means of Type 3 Secretion (T3S) systems that facilitate the translocation of chlamydial proteins directly into the host cell-cytosol (Peters *et al.*, 2007). The *Chlamydia* organisms are, therefore, maintained in an intracellular micro-environment provided by the inclusion, yet are able to communicate with the host-cell cytosol and interact with cellular pathways in a co-ordinated fashion that can be remarkably complex. The bacteria can inhibit apoptosis, down-regulate major histocompatibility complex (MHC) presentation, interact with the NF- κ B pathway, intercept vesicles budding from both the Golgi network and multivesicular bodies, and exclude the fusion of vesicles from the lysosomal pathway (reviewed by Cocchiario & Valdivia, 2009).

Most RBs differentiate back to EBs following replication, so inclusions eventually contain a heterogeneous population of RBs and EBs (Figure 1.3 A), as well as forms that are mid-way through differentiation (sometimes referred to as Intermediate Bodies, or IBs). Organisms are typically released during host-cell lysis and released EBs go on to infect additional cells, propagating the infection (reviewed by Mårdh, 2005), although there have been reports of extrusion of intact inclusions from infected host-cells (Hybiske *et al.*, 2007). The *in vitro* replication of *C. trachomatis* in permissive HeLa cells is typically complete by 48-50 hours post-infection in favourable culture conditions (Villareal *et al.*, 2002; Brunham & Rey-Ladino, 2005), however, in unfavourable *in vitro* growth conditions, the bacteria undergo a reduction in metabolic activity and enter into a state of “persistence”. Chlamydial persistence

has parallels with viral latency (Beatty *et al.*, 1994b) and is characterised by the prolonged intracellular presence of viable organisms that are non-cultivable (reviewed by Beatty *et al.*, 1994b) and display an altered gene transcription profile (Belland *et al.*, 2003; Hogan *et al.*, 2004; Ouellette *et al.*, 2006; Goellner *et al.*, 2006).

Factors that favour chlamydial persistence include the addition of interferon-gamma (IFN- γ) (Beatty *et al.*, 1993; Beatty *et al.*, 1995; Pantoja *et al.*, 2001; Jones *et al.*, 2001) and antibiotics such as penicillin (Matsumoto & Manire, 1970; Lambden *et al.*, 2006) to cell cultures, the depletion of essential amino acids (Coles *et al.*, 1993; Jones *et al.*, 2001) and iron (Raulston, 1997) from infected cells, growth in continuous culture (Hogan *et al.*, 2003; Kutlin *et al.*, 2001), heat-shocking (Kahane *et al.*, 1992), bacteriophage infection (Hsia *et al.*, 2000), growth in monocytes (Koehler *et al.*, 1997) and viral co-infection of *C. trachomatis*-infected cell cultures with herpes simplex virus type 2 (HSV-2) (Deka *et al.* 2006, Deka *et al.* 2007; Vanover *et al.*, 2008). Reactivation of the lytic cycle occurs upon removal of the persistence-inducing factor (reviewed by Beatty *et al.*, 1994b; Hogan *et al.*, 2004).

Figure 1.2 The Life cycle of *Chlamydia*

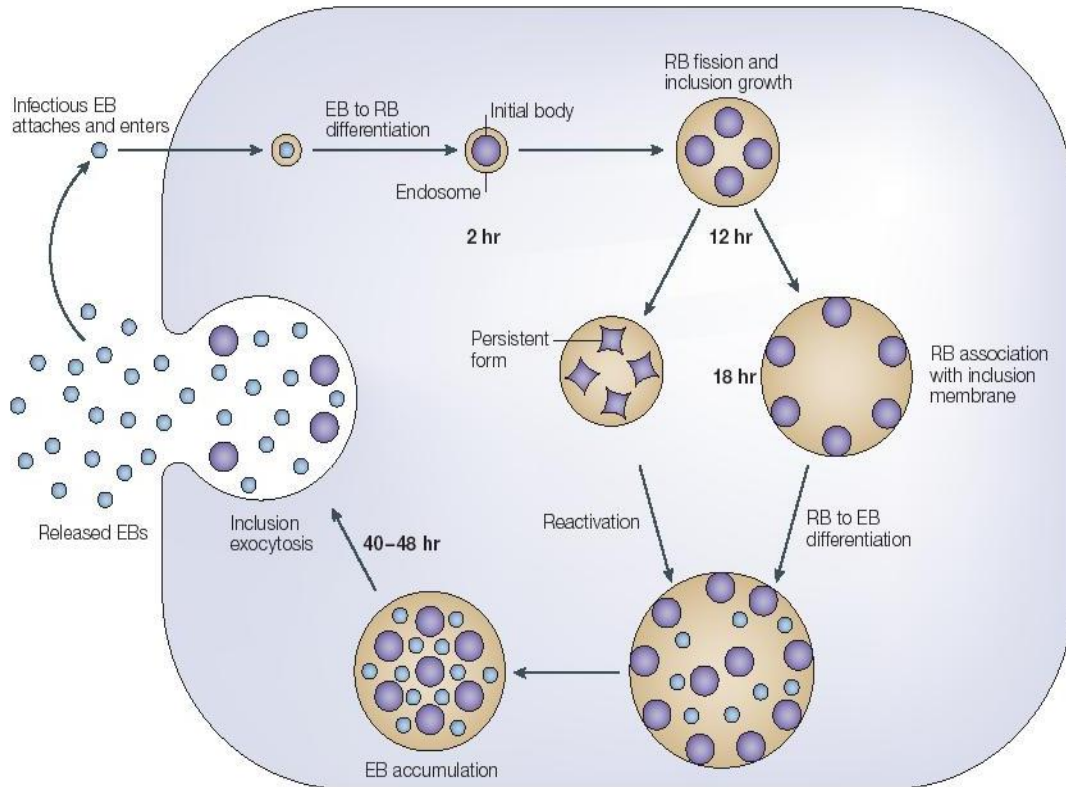


Figure 1.2 (adapted from Brunham & Rey-Ladino, 2005) shows the life-cycle of *C. trachomatis* in an infected epithelial cell. See text for details. EBs develop into RBs and replicate within the inclusion. The RBs differentiate to EBs and are released during host-cell lysis at approximately 40-48hours post-infection in favourable culture conditions. In unfavourable growth conditions, intracellular RBs persist, and fail to differentiate back to EBs until the persistence-inducing factor has been removed.

Figure 1.3 *Chlamydia* morphology

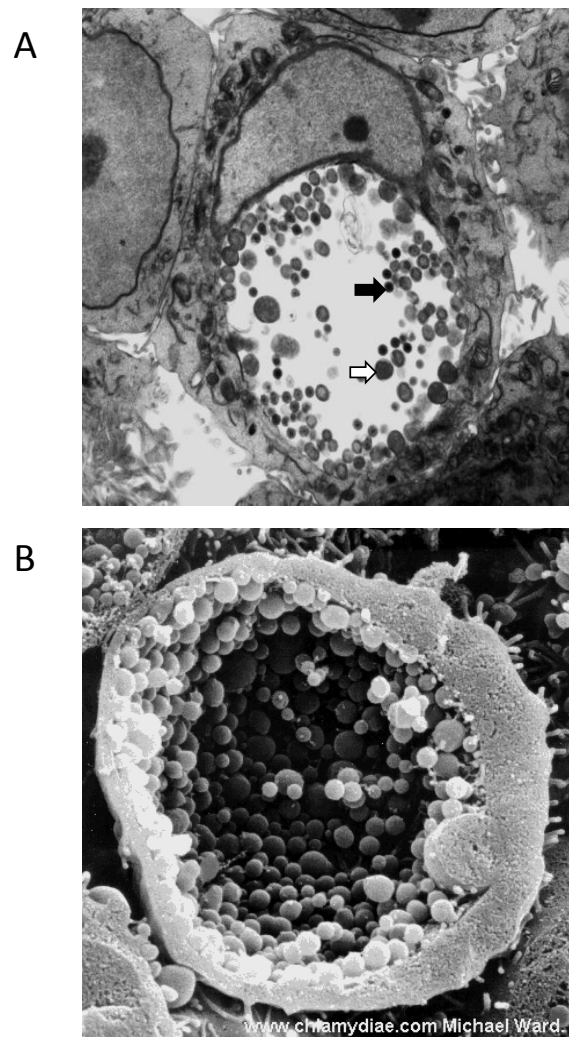


Figure 1.3 A. Transmission electron micrograph of a *C. trachomatis* serovar L2 inclusion in a HeLa epithelial cell 36 hours post-infection showing RBs (white arrow) and EBs (black arrow). **B.** A scanning electron micrograph of a *C. psittaci* inclusion showing intracellular organisms (Picture by Michael Ward and available from www.chlamydiae.com).

1.5. *C. trachomatis* persistence *in vivo*

The phenomenon of chlamydial persistence may have significant clinical consequences. Antibiotic sensitivity testing *in vitro* has shown that doxycycline is more effective against lytic organisms than persistent ones whereas azithromycin is more efficacious against persistent organisms than lytic (Reveneau *et al.*, 2005). As both drugs are used in clinical practice (discussed in Section 1.9), it would be of benefit to know the phenotype of the bacteria *in vivo*. Yet, despite this importance, studying *Chlamydia* persistence *in vivo* has proved to be difficult, as invasive techniques are required in order to obtain tissue biopsies. Such techniques may be damaging and could lead to fibrosis with adverse sequelae such as urethral stricture and impaired fertility. Moreover, as the majority of patients can be managed without invasive procedures, ethical questions are raised regarding the benefit of risking complications in order to obtain samples.

Typically, investigators either look for the presence of aberrant bodies in electron micrographs of biopsy samples, or for evidence of a prolonged or continual chlamydial infection in animal models. Morphologically aberrant chlamydial organisms have been detected in both murine models of *C. trachomatis* cervical infection (Phillips *et al.*, 1998) and pigs infected with *C. suis* (Pospischil *et al.*, 2008), while the prolonged presence of chlamydial DNA and antigen have been identified in the genital tracts of both sheep and macaques experimentally infected with *C. psitacci* (now *C. abortus*) and *C. trachomatis*, respectively (Papp *et al.*, 1996; Patton *et al.*, 1997). In addition, high levels of chlamydial RNA were identified 7-16 weeks post infection in a primate model of ocular trachoma when culture and direct fluorescent antibody assays were negative (Cheema *et al.*, 1991). These data suggest that chlamydial persistence can occur *in vivo* animal models of infection.

Human studies have yielded conflicting results. Some studies identified the prolonged presence of *Chlamydia* DNA in untreated patients (Joyner *et al.*, 2002; Carter *et al.*, 2009), as well as chlamydial DNA and antigen in women who were treated with antibiotics for a prior tubal factor infertility (Kiviat *et al.*, 1986; Campbell *et al.*, 1993; Patton *et al.*, 1994). In addition, out of 7 women who became infected with *C. trachomatis* of the same serovar on more than 3 separate occasions over the course of 2-5 years, 4 were found to have identical MOMP genotypes, with another 2 found to have only 1 or 2 amino-acid changes (Dean *et al.*, 2000). These data suggest that a prolonged chlamydial infection can occur in humans, however, the genetic and immunogenic material detected may be remnants of a previous infection and may not reflect a current infection. One means of addressing this problem is to identify unprocessed chlamydial ribosomal (r) RNA transcripts in patient samples. The presence of such transcripts signifies that *de novo* RNA transcription must be taking place, indicating that organisms are viable. Unprocessed chlamydial rRNA transcripts have been detected in the synovial tissue from the joints of people with sexually acquired reactive arthritis (SARA) (Beutler *et al.*, 1994; Gérard *et al.*, 1998). These continued to be detected, even when culture assays were negative (Beutler *et al.*, 1997), indicating that organisms were viable but non-cultivable, two hallmarks of persistence.

These studies strongly suggest that chlamydial persistence occurs *in vivo*, however, while these observations are consistent with the results of *in vitro* investigations, there has been no study *in vivo* that identifies intracellular aberrant bodies that are viable, non-infectious and have an altered gene transcriptional profile. Moreover, establishing definitively that a patient has a prolonged or continued infection in spite of treatment is also problematic, as it is difficult to control for re-exposure to untreated partners. Additional studies have found no evidence that *C. trachomatis* persists in the fallopian tubes of infected patients. Bjartling *et*

al., 2007 failed to find *C. trachomatis* DNA in 55 tubal samples from women with ectopic pregnancies, and Dietrich *et al.*, 2008 found *C. trachomatis* DNA in only 2 out of 202 infertile women with swabs taken at multiple anatomic sites in the reproductive tract.

1.6. Host responses to *Chlamydia*

Innate immune responses

In contrast to the prolonged chlamydial infections described above, patients infected with *C. trachomatis* can spontaneously clear their infection in the absence of antibiotic therapy (Parks *et al.*, 1997; Joyner *et al.*, 2002; Molano *et al.*, 2005). These observations suggest people can develop a protective immunity against *C. trachomatis*, however, re-infection is common.

The first line of protection against mucosal pathogens is the epithelium. Infection of epithelial cells with *C. trachomatis in vitro* induces the secretion of an array of pro-inflammatory cytokines (Rasmussen *et al.*, 1997) which have also been documented in murine and guinea-pig models of genital tract infection (Belay *et al.*, 2002; Darville *et al.*, 1995; Darville *et al.*, 1997), coinciding with the infiltration of inflammatory cells into the infected area. The initial inflammatory infiltrate is largely comprised of neutrophils and macrophages, which play central roles in the host innate immune response to infection (reviewed by Darville, 2006). Dendritic cells are also recruited to the site of chlamydial infection (Zhang *et al.*, 1999) and carry antigens to peripheral lymph nodes, where antigen is presented to naive T-cells (Neutra *et al.*, 1996) to activate acquired immune responses.

Acquired immune responses

Murine models have clearly demonstrated that T cells are critical to the resolution of infection and resistance to re-infection (reviewed by Brunham & Rey-Ladino 2005; Rank, 2006). Nude mice cannot control chlamydial infection (Rank *et al.*, 1985), whereas adoptive transfer of *Chlamydia*-specific T-cell lines restores this ability (Ramsey *et al.*, 1991). There are broadly two main types of T cell, CD4⁺ and CD8⁺. Protection is thought to be mediated by CD4⁺ cells, as mice deficient in either *Chlamydia*-specific CD4⁺ cells, or components of the MHC II- CD4 antigen presentation and effector pathways show a marked inability to control chlamydial infection (Morrison *et al.*, 1995; Morrison *et al.*, 2000; Perry *et al.*, 1997; Wang *et al.*, 1999; Johansson *et al.*, 1997a). There are two main subsets of CD4⁺ T-cells, T-helper (Th)1 and Th2. The Th1 subset secrete IFN- γ and adoptive transfer of these cells protects nude mice against infection with *C. muridarum*, whereas adoptive transfer of the Th2 subset does not (Hawkins *et al.*, 2002), indicating that Th1 cells are protective.

The role of CD8⁺ T-cells in infection is less clear. Mice deficient in components of the MHC class I-CD8 presentation pathways, or certain CD8⁺ T-cell effector responses continue to clear chlamydial infection (Morrison *et al.*, 1995; Morrison *et al.*, 2000; Perry *et al.*, 1999), indicating they are not necessary for clearance of the organism. However, there is a growing amount of evidence that CD8⁺ T cells do play some role in controlling infection. Firstly, it is known that CD8⁺ T cells are elicited during a chlamydial infection (Magee *et al.*, 1995; Igietseme *et al.*, 1994). Secondly, splenic CD8⁺ T cells extracted from infected mice lyse *Chlamydia*-infected cells (Beatty *et al.*, 1994a). Thirdly, adoptive transfer of *Chlamydia*-specific CD8⁺ T cells provide some protection to infected mice (Igietseme *et al.*, 1994; Starnbach *et al.*, 1994) and, lastly, depletion of CD8⁺ T cells in immune mice abrogates protection upon challenge with *C. psittaci* (Buzoni-Gatel *et al.*, 1992).

The role of B cells in protective anti-chlamydial immunity is also uncertain as, although mice lacking B cells do not show a markedly altered course of *C. muridarum* genital infection (Ramsey *et al.*, 1988), mice lacking T-cells still successfully resolve infection, indicating they may play some role in protection (Morrison *et al.*, 2000; Morrison *et al.*, 2001). It should also be noted that studies using the guinea pig model of infection have shown that both cell-mediated and antibody-mediated responses are necessary for resolution of infection and immunity to re-infection (reviewed by Rank, 2006). Currently, it remains unknown which animal model most closely approximates human infection with regard to the relative contribution of humoral and cellular immunity, which highlights the limitation of animal models. In fact, data from mouse models using *C. muridarum* cannot be directly extrapolated to *C. trachomatis* infection because of fundamental differences in the biology of the organisms: Firstly, a *C. trachomatis* infection can last months in humans, whereas a *C. muridarum* infection is typically resolved after around 4 weeks (Golden *et al.*, 2000; Joyner *et al.*, 2002; Parks *et al.*, 1997). *C. trachomatis* is also capable of evading the IFN- γ response mounted by the host (discussed below), whereas *C. muridarum* does not (Caldwell *et al.*, 2003). Finally, *C. trachomatis* shows substantial allelic variation of the major outer membrane protein, MOMP, whereas *C. muridarum* has a single allele (Read *et al.*, 2000).

Research has, therefore been conducted using serum, peripheral blood mononuclear cells (PBMCs) and synovial fluid mononuclear cells (SFMCs) from human patients infected with *C. trachomatis*, or from patients with SARA (discussed in section 1.7).

Central to the success of the immune response in clearing chlamydial infection is the action of IFN- γ . This cytokine not only induces nitric oxide synthase (iNOS) to enhance the

production of nitric oxide (NO) that inhibits chlamydial growth (Ramsey *et al.*, 2001a and b), but also upregulates the expression of the enzyme indoleamine-2,3-dioxygenase (IDO) *in vitro* (Byrne *et al.*, 1986a), which catalyses the initial step in tryptophan degradation (Shimizu *et al.*, 1978). This IFN- γ -induced tryptophan degradation inhibits chlamydial replication (Byrne *et al.*, 1986b) as tryptophan is an essential amino-acid for the bacteria. Genital serovars have evolved, however, to synthesise tryptophan in the presence of IFN- γ from indole, a substrate provided by other flora of the female lower genital tract (Caldwell *et al.*, 2003; Fehlner-Gardiner *et al.*, 2002). This phenomenon probably allows certain genital strains to escape IFN- γ induced tryptophan starvation.

1.7 *Chlamydia* antigens

Fuelled by the search for a sub-unit *Chlamydia* vaccine, much work has aimed at identifying chlamydial antigens that are recognised by the immune responses described above. Murine studies have identified Cap-1 (Fling *et al.*, 2001), CrpA (Starnbach *et al.*, 2003), and NrdB (Barker *et al.*, 2008) as targets of the cellular immune response during a *Chlamydia* infection, however, the immunodominance of these proteins has not been validated in human subjects.

In contrast, the following *Chlamydia* antigens have been identified as targets of the cellular response in humans: MOMP (Ortiz *et al.*, 1996; Ortiz *et al.*, 2000; Kim *et al.*, 1999; Kim *et al.*, 2000), the outer membrane protein 2 (OMP2) (Goodall *et al.*, 2001a; Gervassi *et al.*, 2004), heat-shock protein 60 (HSP60) (Deane *et al.*, 1997; Kinnunen *et al.*, 2003), polymorphic membrane protein D (PMPD), Enolase, CT579 (Goodall *et al.*, 2001b), CT043 (Meoni *et al.*, 2009), CT511, CT521 (Olsen *et al.*, 2006), CT583, CT603, and CT610 (Olsen *et al.*, 2007). However, following sequence homology searches of the SwissProt and translated GenBank databases of all known protein sequences, these proteins were found to

possess homologues in the common respiratory pathogen, *C. pneumoniae*. This is consistent with the fact that 80% of the *C. pneumoniae* coding sequences contain orthologues in *C. trachomatis* (Kalman *et al.*, 1999). Conversely, out of the *C. trachomatis* proteins known to be absent from *C. pneumoniae*, T-cell antigenicity has been characterised in none.

The majority of studies that identify chlamydial antigens in human subjects have relied on the *in vitro* expansion of T-cell clones from PBMCs from patients with genital tract *C. trachomatis* infection (Ortiz *et al.*, 1996; Ortiz *et al.*, 2000; Kim *et al.*, 1999; Kim *et al.*, 2000; Goodall *et al.*, 2001a; Gervassi *et al.*, 2004; Olsen *et al.*, 2006; Olsen *et al.*, 2007), synovial fluid monocytic cells (SFMCs) from patients with reactive arthritis (Goodall *et al.*, 2001a & b; Deane *et al.*, 1997) or T-cell lines generated from fallopian tube and endometrial biopsies (Kinnunen *et al.*, 2003). Only one study has investigated human cellular immune responses to a chlamydial protein in the absence of *in vitro* clonal expansion (Meoni *et al.*, 2009), however, the protein identified as containing T-cell epitopes (CT043) shares 95% identity with a *C. pneumoniae* protein (Cpn0387). There have been, therefore, no studies that investigate human *ex vivo* cellular immune responses to a *C. trachomatis*-specific antigen.

Several *C. trachomatis*-specific antigens contain B-cell epitopes: CT089, CT147, CT226, CT681, CT694, CT795, CPAF (Sharma *et al.*, 2006), Pgp3 (Comanducci *et al.*, 1994; Ratti *et al.*, 1995; Bas *et al.*, 2001; Bas *et al.*, 2002; Ghaem-Maghani *et al.*, 2003; Donati *et al.*, 2003) and IncA (Hackstadt *et al.*, 1999; Suchland *et al.*, 2000). The Pgp3 protein, (PubMed Accession number: YP_001569038) is encoded by open reading frame 5 (*orf 5*) of the *Chlamydia* plasmid (Hatt *et al.*, 1988) (PubMed Accession number: NC_010029). While the function of the protein remains unknown, it is located either within the inclusion membrane (Comanducci *et al.*, 1994), or secreted into the host cell cytosol (Li *et al.*, 2008b). It is found

in animal *Chlamydia* infections and *C. psittaci*, but is absent from human *C. pneumoniae* strains, as they do not contain the plasmid (Campbell *et al.*, 1987; Lusher *et al.*, 1989). In human *C. trachomatis* isolates, it is highly conserved (<1% divergence) between strains (Black *et al.*, 1989; Comanducci *et al.*, 1990; Hatt *et al.*, 1988; Seth-Smith *et al.*, 2009) and is not only a target of the humoral response (Comanducci *et al.*, 1994; Ratti *et al.*, 1995; Bas *et al.*, 2001; Bas *et al.*, 2002; Ghaem-Maghani *et al.*, 2003; Donati *et al.*, 2008; Li *et al.*, 2008a), but mice immunised with DNA encoding Pgp3 showed a robust IFN- γ response (Li *et al.*, 2008c) and a reduction in the spread of infection (Donati *et al.*, 2003). Moreover, work in this laboratory has developed an “in-house” ELISA based on the Pgp3 protein for the detection of *C. trachomatis* antibodies in patient serum (Wills *et al.*, 2009).

1.8 *Chlamydia* pathogenesis

It is important to note that as well as being protective, host cell responses may contribute to the pathology caused during a chlamydial infection. *Chlamydia* organisms are known to survive within neutrophils (van Zandbergen *et al.*, 2004), dendritic cells (Matyszak *et al.*, 2002) and macrophages (Numazaki *et al.*, 1995; Bianchi *et al.*, 1997), the latter being responsible for disseminating the infection to more distant sites, such as the joints of those infected with SARA (Moazed *et al.*, 1998). In addition, serovars responsible for LGV are capable of breaching the epithelial basolateral surface (Dessus-Babus *et al.*, 2008) to infect the submucosa and facilitate invasion of the lymphatic system where further pathology occurs. In turn, lymphatic pathology reduces or abolishes lymph drainage from the mucosa, leading to congestion and additional mucosal pathology (Richardson & Goldmeier 2007).

It is known from *in vitro* studies that chlamydial persistence is induced by the addition of IFN- γ to infected cell-cultures, and by the infection of macrophages (Koehler *et al.*, 1997).

As macrophage infiltration and IFN- γ are central to the anti- chlamydial immune response *in vivo*, it is plausible to consider that chlamydial organisms may undergo persistence as a result of the host response to infection.

The ramifications of *in vivo* chlamydial persistence are controversial. The so-called “cellular hypothesis” suggests that the continued intracellular presence of chlamydial organisms in epithelial cells causes the continued secretion of pro-inflammatory cytokines that directly lead to chronic inflammatory cellular responses and tissue damage (Stephens, 2003). This is supported by observations that infected epithelial cells *in vitro* secrete large amounts of proinflammatory chemokines (such as IL-8, granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-1a and IL-6) (Rasmussen *et al.*, 1997). Proinflammatory chemokines have also been detected in murine models of chlamydial infection (Darville *et al.*, 2001), the tears from children with trachoma, and endocervical secretions obtained from women infected with *Chlamydia* (Stephens, 2003). Moreover, the proinflammatory chemokine response is sustained even during an *in vitro* model of chlamydial persistence (Rasmussen *et al.*, 1997).

However, an alternative model of pathogenesis, “immunological hypothesis” suggests that infiltrating immune cells may be the cause of tissue damage by means of effector functions (Brunham *et al.*, 1994). The immunological hypothesis is supported by the following evidence: firstly, protective CD4⁺ Th1 cells preferentially home to the infected tissue, where they can cause tissue damage as well as play an anti-chlamydial role (Johnson *et al.*, 2004; Rank *et al.*, 2000; Van Voorhis *et al.*, 1997). Secondly, Th2 cells that are generated in response to infection with *Chlamydia* may downregulate the protective Th1 immune responses, promoting pathology (Wang *et al.*, 1999; Holland *et al.*, 1996). Thirdly,

Chlamydia-derived antigens (such as HSP60) may be recognised by autoreactive T and B cells through molecular mimicry (Yi *et al.*, 1997; Peeling *et al.*, 1997; Lichtenwalner *et al.*, 2004) and, finally, *C. trachomatis*-specific CD4⁺ and CD8⁺ T-cell epitopes are often identified in *C. trachomatis*-associated chronic infections, such as reactive arthritis (Hassell *et al.*, 1993; Goodall *et al.*, 2001b). In addition, IL-10-deficient mice are more resistant to *C. muridarum* infection and have a shorter duration of infection than wild-type mice (Yang *et al.*, 1999; Igiestseme *et al.*, 2000) and T cells that are reactive to chlamydial HSP60 and produce IL-10 have been found in infertile women (Kinnunen *et al.*, 2003). These cells may be involved in the suppression of *C. trachomatis*-specific responses, which could contribute to the ability of the organism to persist. This, however, warrants further investigation.

1.9 *Chlamydia* Disease, Detection, Treatment and Control

The disease burden, clinical features, detection, treatment, case management and control of genital and rectal infection with *C. trachomatis* serovars D-K and LGV will be discussed.

***C. trachomatis* Serovar D-K Infection**

Disease Burden

C. trachomatis is the most frequently reported sexually transmitted infection and reportable disease in Europe. Between 5 and 10% of sexually active young people are estimated to be infected, with the number of cases diagnosed continuing to increase and the true incidence of infection likely under-estimated (reviewed by ECDC, 2009). In fact, one in 14 people under the age of 25 years screened as part of the National Chlamydia Screening Programme

(NCSP) in the UK tested positive for *Chlamydia* (NCSP website). Rectal infection is detected in both men and women who practice receptive anal intercourse, with a prevalence of between 6.5% and 7.9% in MSM (Benn *et al.*, 2007; Manavi *et al.*, 2004; Kent *et al.*, 2005; Annan *et al.*, 2009; Ota *et al.*, 2009).

Although the majority of cases are asymptomatic, *C. trachomatis* is a major public health concern due to complications caused by untreated infection, which can facilitate HIV transmission and can cause poor reproductive outcome in women that can be expensive to manage (ECDC, 2009).

Clinical features

Approximately 70% of genital serovar D-K infections in women and 50% of infections in men are asymptomatic (Horner & Boag 2006). The remaining symptomatic infections may manifest in post-coital or inter-menstrual bleeding, lower abdominal pain, purulent vaginal discharge, mucopurulent cervicitis and dysuria in women and urethral discharge and dysuria in men (Horner & Boag 2006). The severity of these symptoms is variable and may go unnoticed by the patient. Rectally acquired *C. trachomatis* serovar D-K infection may result in proctitis causing rectal discomfort, bleeding or a mucopurulent discharge, however, infection is usually asymptomatic, with over 80% of men who have sex with men (MSM) with a rectal D-K infection presenting with no symptoms or clinical signs (Anan *et al.*, 2009; Kent *et al.*, 2003; Ward *et al.*, 2009).

In the absence of treatment, an estimated 10-40% of infected women will develop pelvic inflammatory disease (PID) which, although usually asymptomatic or associated with mild symptoms, can result in complications such as tubal factor infertility (TFI), ectopic pregnancy

(EP) or chronic pelvic pain (Paavonen *et al.*, 1999; Simms *et al.*, 2000; Hu *et al.*, 2004; Horner & Boag, 2006; Risser *et al.*, 2007; Simms & Horner, 2008). Genital *C. trachomatis* infection may also lead to epididymo-orchitis in men, conjunctivitis and sexually acquired reactive arthritis (SARA) in both sexes, or conjunctivitis and pneumonia in neonates (Horner & Boag, 2006).

Detection of active *C. trachomatis*

As previously mentioned (Section 1.2), the isolation of *C. trachomatis* from infected patients was initially conducted using embryonated hens eggs (T'ang *et al.*, 1957). This was eventually replaced by cell culture systems using irradiated McCoy cells (Gordon & Quan 1965) or cycloheximide treated cells (Ripa and Mårdh 1977). Cell culture, although highly specific and applicable to all specimen types, has a low sensitivity (60-80%), requires technical expertise, is expensive and is not suitable for high-throughput systems (ECDC, 2009).

Enzyme Immunoassays (EIAs) and direct fluorescent antibody (DFA) assays that detect chlamydial antigen in patient samples were subsequently developed. These were cheaper and easier to perform than cell-culture, however, EIAs have a poor specificity for *C. trachomatis* detection, and DFA requires laboratory staff to be trained in fluorescence microscopy (CDC, 2002). These techniques have been replaced by nucleic acid amplification techniques (NAATs), which are now the accepted “gold standard” of care.

The NAATs amplify chlamydial DNA and are highly sensitive and specific (Horner & Boag 2006). Commercially available NAATs rely either on polymerase chain reaction (PCR), strand displacement amplification (SDA) or transcription-mediated amplification (TMA).

The Cobas Amplicor CT/NG test (Roche, CH), the Abbott RealTime *Chlamydia trachomatis* (CT) assay (Abbot Laboratories, USA) and the artus *C. trachomatis* PCR Kit (Qiagen, DE) rely on PCR, whereas the BD Probetec ET assay (Becton Dickenson, USA) relies on SDA and the Aptima Combo 2 (Genprobe, USA) relies on TMA.

In 2006, a new variant (nv) of *C. trachomatis* serovar E was discovered in Sweden (Ripa & Nilsson 2006; Ripa & Nilsson 2007). This variant spread across the country and now has a reported prevalence of 17% in Sweden (Lagergård et al., 2010), but as of 2007 had not been detected in the UK (Alexander & Ison 2008). This nv *C. trachomatis* contains a 377 base-pair deletion in open reading frame 1 (*orf1*) of the cryptic plasmid (Seth-Smith *et al.*, 2009) that was the target of two NAATs in use at the time it was first identified, the Abbot m2000 (Abbot Laboratories, USA) and the Cobas Amplicor (Roche, CH). Consequently, they gave false-negative results. These two systems have since been updated to target alternative regions of the cryptic plasmid and/or chromosome in order to detect all variants of *C. trachomatis*.

Currently, the Cobas Amplicor (Roche, CH) and the artus *C. trachomatis* PCR Kit (Qiagen, DE) target both a region of the cryptic plasmid and a region of *ompA*, whereas the new formulation Abbott RealTime *Chlamydia trachomatis* (CT) assay targets multiple regions of the cryptic plasmid, the Aptima Combo 2 (Genprobe, USA) targets chlamydial 23S rRNA and the BD Probetec ET assay targets another region of the cryptic plasmid that is present in both the nv *C. trachomatis* and classical strains.

The cryptic plasmid is a common target for the amplification of chlamydial DNA by the NAATs. By targeting this region instead of the chromosome, the sensitivity can be improved as the plasmid is present in multiple copies per organism (Mahony *et al.*, 1993). It is worth

noting that there have been reports of plasmid-less *C. trachomatis* isolates (Magbanua *et al.*, 2007; Farencena *et al.*, 1997; An *et al.*, 1994), however, these strains are not thought to be widely circulating in the community (Alexander & Ison 2007).

For the detection of female lower genital tract infection, cervical or vulvovaginal swabs are the specimens of choice. Studies indicate that the sensitivity is similar when either swab is used (Horner & Boag, 2006), however, variable sensitivities have been reported when ‘first-catch’ urine samples are utilised (Horner & Boag, 2006; Schachter *et al.*, 2003; Jensen *et al.*, 1997, Wiggins *et al.*, 2009). This probably reflects differences in the chlamydial load, which is lower in first-void urine specimens than the swabs (Michel *et al.*, 2007; Wiggins *et al.*, 2009).

At present, none of the NAAT tests developed have been approved by the Federal Drug Administration (FDA) for use in the detection of rectal and pharyngeal *C. trachomatis* infection, despite a large amount of evidence demonstrating a high sensitivity and specificity of NAAT tests in the detection of rectal *C. trachomatis* (Alexander, 2009).

Anti-chlamydial antibodies in patient serum can also be detected using serological assays such as the Microimmunofluorescence (MIF) and enzyme-linked immunoadsorbant (ELISA) assays. A number of serological tests are available, but they suffer from variable sensitivity and cross-reactivity with *C. pneumoniae* (Johnson & Horner, 2008) and are not recommended for chlamydial diagnosis in the UK (Horner & Boag 2006). Moreover, serological testing cannot distinguish between current and past infection.

Many serological assays have not been rigorously evaluated for sensitivity and specificity against well characterised sera from exposed patients compared to those who have not been exposed. In order to address this, the sensitivity and specificity of three commercial serological assays derived from MOMP were recently compared against an in-house ELISA based on the Pgp3 protein (Wills *et al.*, 2009). The pELISA plus (Medac), the SeroCT-IgG ELISA (Savyon), and the IgG enzyme immunoassay (Ani Labsystems) were found to be less sensitive than the Pgp3-ELISA (Pgp3, 59%; Ani Labsystems, 49.2%; Savyon, 47.2%; Medac, 44.4%), though the specificities were comparable (>95%) (Wills *et al.*, 2009).

Treatment regimens for *C. trachomatis* infection

The recommended regimens for genital and rectal infections with *C. trachomatis* serovars D-K are either doxycycline (100mg, twice daily, for 7 days) or azithromycin (1g, orally, in a single dose). Other antibiotic regimens are also possible, such as ofloxacin (200mg, twice daily, or 400mg, once daily, for 7 days), and erythromycin (500mg, twice daily, for 10-14 days) (Horner & Boag 2006), but the efficacy of azithromycin and doxycycline have been most rigorously investigated. Azithromycin, erythromycin and doxycycline inhibit bacterial protein synthesis, either by binding to the large ribosomal subunit of the bacteria (azithromycin and erythromycin) (Chopra *et al.*, 2001), or by preventing the aminoacyl-tRNA from adhering to the ribosome (doxycycline). In contrast, ofloxacin is a second-generation fluoroquinolone that inhibits DNA gyrase and Topoisomerase IV required for DNA replication and transcription (Blondeau, 2004).

The *in vitro* efficacy of an antibiotic is assessed by calculating the Minimum Inhibitory Concentration (MIC) and the Minimum Chlamydicidal Concentration (MCC). The MIC is the concentration of drug required to inhibit the development of detectable inclusions in cell-

cultures (Andrews *et al.*, 2001), whereas the MCC is the lowest concentration of an antimicrobial required to ensure that *Chlamydia* cannot be recovered from a culture (Misyurina *et al.*, 2004). In practice, detecting inclusions in the presence of antibiotics tends to be variably interpreted (Suchland *et al.*, 2003; Misyurina *et al.*, 2004).

Clinically, over 95% of infected individuals treated with azithromycin or doxycycline test negative by NAAT or *C. trachomatis* culture methods 2-5 weeks post-treatment and comparative studies have demonstrated that azithromycin and doxycycline have a similar efficacy (Lau *et al.*, 2002). Ofloxacin has a similar efficacy to doxycycline, but only one randomised control trial has been conducted (Boslego *et al.*, 1988) and it is not currently recommended as first-line treatment (Horner & Boag 2006; Horner, 2008). Erythromycin is less efficacious than either azithromycin or doxycycline clinically (efficacy between 73-95%) (Tobin *et al.*, 2004). However, as *Chlamydia* has the ability to enter into a persistent state under stressful conditions, it has been suggested that a negative test soon after treatment may not indicate eradication (Horner, 2006; Horner, 2008). Consistent with this hypothesis, Dean *et al* observed from retrospective case-examination of 11,212 patients that 1% of women treated for *C. trachomatis* had had three or more infections with the same serovar which they suggest is the result of reactivation following treatment failure (Dean *et al.*, 2000).

Two types of antimicrobial resistance have been identified in *Chlamydia*: heterotypic and homotypic. Heterotypic resistance was defined by Wang *et al* as “the replication of a heterogeneous population of resistant and susceptible bacteria derived from a subculture of a single resistant organism propagated on an antimicrobial-containing medium” (Wang *et al.*, 2005). Heterotypic resistance occurs when there is a large infecting organism load and typically involves the survival of around 1% of microbes in high antimicrobial concentrations

(Suchland *et al.*, 2003). The mechanism behind this is unknown as it is not genetically inherited. In contrast, “homotypic resistance” refers to replication of a homogeneous, clonal population of resistant organisms from a subculture of a single resistant organism and is genetically inherited (Wang *et al.*, 2005).

Suchland *et al.*, 2003, found that at high loads, *C. trachomatis*, *C. pneumoniae* and *C. psittaci* demonstrated resistance to doxycycline, azithromycin, erythromycin and ofloxacin, but at low loads, recovered isolates showed no increase in MIC (i.e. they were still sensitive). Horner has argued there is evidence of heterotypic resistance *in vivo* if the infectious load is high and noted that mass treatment trials of trachoma found high chlamydial loads to be associated with treatment failure (Horner, 2006).

Case Management in the UK

As no diagnostic test is 100% sensitive and specific, positive NAAT tests should ideally be confirmed in the laboratory using a different NAAT platform (Skidmore *et al.*, 2006), however, antibiotics should be offered to all patients with unconfirmed positive NAAT results immediately following diagnosis (Johnson *et al.*, 2002). Patients should be advised to avoid sexual intercourse (including oral sex) until they and their partners have received treatment and patients diagnosed with *C. trachomatis* should be encouraged to be screened for other sexually transmitted infections (Horner & Boag 2006). Partner notification is also an essential component of case management (ECDC, 2009) as it reduces rates of re-infection and provide one mechanism for targeting people who are at high risk of infection (Trelle *et al.*, 2007).

Control of *C. trachomatis* Infection

Four levels of *Chlamydia* control have been identified by the European Centre for Disease Prevention and Control (ECDC) (ECDC, 2009). The first level, Level A, is entitled ‘primary prevention’, which includes health promotion and education, school programmes and condom distribution. Level B is described as ‘case management’ where, in addition to level A, routine case surveillance is conducted and cases are managed according to evidence-based practices. Level C is classed as ‘opportunistic testing’ which builds on level B but, in addition to case management and surveillance, aims to identify asymptotically infected individuals by offering testing to individuals attending other clinical services. Level D is a ‘screening programme’ which, in addition to opportunistic screening, aims to provide regular *Chlamydia* testing to a substantial proportion of a defined population with the aim of reducing the prevalence in the population.

The organisation of *Chlamydia* control throughout Europe varies widely. Thirteen countries (45%) do not have national guidelines for diagnosis or management, 5 (17%) have some guidelines on diagnosis and treatment, 3 (10%) have some guidelines on case management and 6 (21%) have opportunistic screening programmes that aim to identify asymptotically infected people. Only 2 (7%) have introduced national screening programmes: England and the Netherlands (ECDC, 2009).

As *C. trachomatis* is common, treatable and there are means of detecting it, yet left untreated it can lead to devastating complications, it is an infection that constitutes an ideal candidate for screening programmes (Low, 2007). Not only do mathematical models predict that screening should reduce transmission (Turner *et al.*, 2006), systematic screening for *Chlamydia* has been shown to halve the incidence of PID one year later (Low *et al.*, 2004;

Ostergaard *et al.*, 2000; Scholes *et al.*, 1996). Due to high and increasing rates of *C. trachomatis* in the UK, a National *Chlamydia* Screening Programme (NCSP) was established in 2003 with the objective of “controlling *Chlamydia* through the early detection and treatment of asymptomatic infection, thus preventing the development of sequelae and reducing onward disease transmission.” (NCSP website).

To achieve these aims, a screening programme must cover enough of the target population and be regular enough to detect and treat re-infections to prevent transmission. There is, however, no firm empirical data on the impact of screening on transmission and it is unclear how many people would need to be tested in order to reduce transmission effectively (ECDC, 2009). It is also difficult to monitor the prevalence of *Chlamydia* as it requires repeated population surveys, which are expensive and technically challenging (Low *et al.*, 2007). It is, therefore, difficult to evaluate the success of the NCSP. Moreover, mathematical models that guide screening decisions rely on assumptions of pathogenesis, disease burden, transmission etc. As discussed in the pages above, our knowledge of *in vivo* persistence, pathogenesis, and the human immune response to *C. trachomatis*-specific antigens is incomplete and our current non-invasive means of detecting infection fail to distinguish between a current and previous infection of the fallopian tubes meaning the true contribution of *Chlamydia* to PID and TFI remains unknown.

Lymphogranuloma Venereum

Burden of Disease

Lymphogranuloma venereum is endemic in East, West and Southern Africa (Mabey *et al.*, 1987; Ndinya-Achola *et al.*, 1996; Dangor *et al.*, 1990; O'Farrell *et al.*, 1994), Madagascar (Behets *et al.*, 1999), India (Ray *et al.*, 1993) and South-East Asia (Viravan *et al.*, 1996).

Most cases in industrialised settings were traditionally imported via travellers, sailors or soldiers (as in the case of the USA during the wars in Korea or Vietnam (reviewed by Mayaud, 2006)) however, this situation changed in 2003, with an outbreak of LGV in The Netherlands (Nieuwenhuis *et al.*, 2004). Since 2003, there have been a series of outbreaks in several European cities, including the UK (Ward *et al.*, 2007), mostly among human immunodeficiency virus (HIV)-1 sero-positive MSM (Gotz *et al.*, 2004). An international surveillance alert was launched in October 2004 as a result and more than 290 cases had been confirmed in the UK by December 2005 (French *et al.*, 2005). The majority of these cases were diagnosed in GUM clinics in London (72%), and Brighton (14%) and all the cases identified occurred among white MSM belonging to large sexual networks, and were not obviously linked with known endemic countries. The majority of cases (>75%) were already known to be HIV positive, and many patients were co-infected with other sexual transmitted infections, for example gonorrhoea or hepatitis C virus (HCV) (Mayaud, 2006).

When the strain responsible was further analysed, it was found to be a variant of the classical *C. trachomatis* L2 serovar and termed serovar L2b (Spaargaren *et al.*, 2005a). Moreover, this strain appeared to have been circulating in Amsterdam in 2000 and had previously been identified in 1980s in San Fransisco (Spaargaren *et al.*, 2005b) indicating that rather than a

sudden outbreak in 2003, this disease represented a slowly evolving epidemic that had largely gone unnoticed in the population.

Clinical features of LGV

Classical LGV has an extremely variable incubation period of 3 to 30 days from the time of contact with an infected individual. The primary lesion is a painless papule, pustule or ulcer that may go unnoticed by the patient, however, the infection may disseminate to the draining lymph nodes, leading to inflammation and swelling. The most common clinical manifestation of classical LGV is inguinal or femoral lymphadenopathy that is typically unilateral. When both the inguinal and femoral lymph nodes are enlarged, the inguinal ligament that separates them may give rise to a “groove sign”. Systemic spread of *C. trachomatis* L2 may be associated with fever, arthritis, and pneumonitis and, in a few patients, the persistence of *C. trachomatis* infection leads to a chronic inflammatory response giving rise to proctitis, proctocolitis, fistulae, and strictures (Mayaud, 2006).

In the recent outbreaks occurring among MSM in Western Europe and the USA, almost all cases have presented with proctitis. Symptoms include severe rectal pain, mucoid and/or haemorrhagic rectal discharge, tenesmus, constipation and other signs of lower gastrointestinal inflammation. Genital ulcers and inguinal symptoms are, however, rare, for reasons that remain unknown (Mayaud, 2006).

Detection of LGV

Typically, patients are initially identified as infected with *C. trachomatis* by a positive NAAT result using a rectal swab. Subsequently, a nested PCR and restriction fragment length polymorphism (RFLP) analysis is performed, or the *ompA* gene is sequenced in order

to ascertain the genotype of the infecting strain. Alternatively, a qPCR using LGV-specific primers and probes may be conducted. The first qPCR assay to be developed was limited to detecting LGV in samples that were previously found to be *C. trachomatis*- positive by NAAT (Morré *et al.*, 2005), however, subsequent assays were multiplex qPCRs that could determine both LGV and non-LGV *C. trachomatis* serovars (Halse *et al.*, 2006; Chen *et al.*, 2007; Jalal *et al.*, 2007; Chen *et al.*, 2008). The latter of these assays is also capable of detecting mixed infections.

Treatment Regimens for LGV

The current recommended treatment regimen for LGV is a 3 week course of doxycycline (100mg, twice daily) (Mayaud, 2006) which is in contrast to rectal infection with serovars D-K, where a one week course is given. The shorter course fails to clear LGV infection (de Vries *et al.*, 2008; Van der Bji *et al.*, 2006), emphasising the importance of correct diagnosis of the disease. The activity of azithromycin against *C. trachomatis* also suggests that it may be effective in multiple doses over 2-3 weeks but clinical data on its use are lacking and more randomised control trials are needed (Mayaud, 2006).

Patient Management

Individuals who have had sexual contact with an LGV-infected patient within the 30 days prior to the onset of symptoms should be examined, tested for chlamydial infection and treated. Patients should be followed clinically until signs and symptoms have resolved and individuals should be advised to avoid unprotected sexual intercourse until they and their partners have completed treatment and follow-up. Patients with fibrotic lesions or fistulae may require surgical repair. Many cases of the recent LGV outbreaks in Europe were

associated with HIV, hepatitis C infection and gonorrhoea and screening for these infections is encouraged (Mayaud, 2006).

1.10 *Chlamydia* and co-infection

In vitro, studies have been conducted of *C. trachomatis* co-infection with herpes simplex virus-2 (HSV-2) and human immunodeficiency virus-1 (HIV-1).

The HSV-2 virus belongs to the *Herpesviridae* family of enveloped DNA viruses. The replication cycle takes from 12 to 24 h and is initiated by viral attachment to one of several host cell receptors. The virus uncoats into the cytoplasm and the viral DNA enters the nucleus, whereupon viral genes are expressed in a specific temporal order. New virions are then assembled, enveloped and released from the cell (Roizman and Knipe, 2001).

Genital HSV-2 infections usually occur on the skin or mucous membranes, leading to inflammation, but the virus may subsequently establish latency in the sacral ganglia.

Typically, individuals infected with HSV-2 experience reactivation on average five times per year, during which lesions and virions are present (Corey *et al.*, 1983). In addition, HSV-2 may also cause keratitis, meningitis and disseminated herpes infection.

A number of studies have established that co-infections with HSV-2 and *C. trachomatis* occur in the human population. Both organisms have been simultaneously isolated from the genital tract of women suffering from endometritis and salpingitis (Paavonen *et al.*, 1985) as well as cystitis (Tait *et al.*, 1985) and several serological studies indicate that HSV-2-positive

individuals are likely to be *C. trachomatis*-positive as well (Paroli *et al.*, 1990; Vetter *et al.*, 1990; Silins *et al.*, 2002).

Co-infection of *C. trachomatis*-infected HeLa cells with HSV-2 induces chlamydial persistence (Deka *et al.*, 2006) and leads to significantly larger *C. trachomatis* inclusions compared to cultures infected with *C. trachomatis* alone. These inclusions contain enlarged, swollen reticulate bodies that show a loss of infectivity, but no alteration in genomic replication or in the accumulation of unprocessed 16S rRNA transcripts.

As *Chlamydia* persistence is known to be caused by amino acid and iron deficiencies, certain antibiotics and cytokines such as IFN- γ , the authors initially surmised that the HSV-2 virus could be competing with the *Chlamydia* for amino acids, or could be causing the production of cytokines that could influence the development of *C. trachomatis* (Deka *et al.*, 2006). Subsequent work, however, revealed that persistence was induced even if cultures were inoculated with UV-inactivated, replication incompetent HSV-2, or cells were infected in the presence of cycloheximide (that inhibits eukaryotic ribosomes and, hence, viral replication). Therefore, a productive viral infection was not necessary for the induction of persistence. The authors concluded that an early event in the HSV-2 life cycle was responsible for the induction of persistence (Deka *et al.*, 2007) and demonstrated that viral binding to host-cell receptors could initiate intracellular signalling events that lead to *C. trachomatis* persistence (Vanover *et al.*, 2010). Candidates for such persistence-inducing pathways include those initiated by HSV-2 binding of Nectins 1 and 2, the herpes viral entry mediator (HVEM), or toll-like receptors 2 and 9 (Deka *et al.*, 2007).

The HIV-1 virus belongs to the *Lentivirus* genus within the *Retroviridae* family. The virus binds to the host-cell receptor, CD4, and co-receptors, CXCR4 or CCR5, via the envelope protein, gp120. The virus manipulates the host-cell cytoskeleton in order to gain entry to the cytoplasm, where the virion uncoats and the single-stranded RNA genome is reverse-transcribed to pro-viral DNA. Viral proteins and nucleic acid are transported to the nucleus where proviral DNA integrates into the host-cell genome and viral proteins are subsequently transcribed and translated by host cell machinery. Progeny virions assemble in the cytoplasm and bud from the plasma membrane, to mature extracellularly (Freed *et al.*, 2006).

The virus, which is present in the semen of infected men (Krieger *et al.*, 1991; Zagury *et al.*, 1984) is deposited into the rectal or vaginal lumen during unprotected intercourse. It enters the submucosa, either by traversing the epithelial barrier (Fantini *et al.*, 1993), or directly due to epithelial cell-loss resulting from intercourse-related trauma or ulcerative lesions from other sexually transmitted diseases (Plummer *et al.*, 1991). Once in the sub-mucosa, HIV-1 enters cells of the reticuloendothelial lineage and is carried to the draining lymph node where it infects CD4⁺ T-cells.

Recent studies of MSM have found that 38% of individuals with rectal *C. trachomatis* are also co-infected with HIV-1 (Annan *et al.* , 2009). This association is more pronounced for individuals infected with the L2 serovar, with 74-76% of infected individuals co-infected with HIV-1 (Jebbari *et al.*, 2007; Ward *et al.*, 2007). The reason for the association is unknown. While it is possible that high-risk sexual behaviour in distinct sexual networks comprised of HIV-positive individuals contribute to the epidemiology (Pimenta 2003), it is possible that *C. trachomatis* has a different clinical course in individuals who are co-infected with HIV-1.

It is plausible that HIV-1 and *C. trachomatis* co-infect cells *in vivo*, as HIV-1 virions may traverse epithelial cells that are infected with *C. trachomatis*, or infect reticuloendothelial cells in the submucosa that are infected with *C. trachomatis* L2. The two organisms may, therefore, impact on the replication of one another, and *in vitro* models of HIV-1 and *C. trachomatis* co-infection have been established to investigate this: In 1995, *C. trachomatis* serovar L2 was shown to act synergistically with polymorphonuclear cells (PMNs) to increase the replication of HIV-1 in chronically infected monocytic cells (Ho *et al.*, 1995). The addition of *C. trachomatis* to cell-cultures enhanced viral replication an additional nine-fold at 24 hours post co-infection compared to the addition of PMNs alone. The presence of the PMNs in the culture was required for the increase in viral replication and the authors concluded that, *in vivo*, a *C. trachomatis* infection of the rectum or lower genital tract could serve to recruit PMNs to the area and facilitate an increase in the replication of HIV-1 in infected semen deposited in the lower genital tract or rectal lumen. The mechanism of this synergy was not determined, but the authors speculated that the phagocytosis of *Chlamydia* organisms by PMNs caused the secretion of increased reactive oxygen intermediates that, in turn, enhanced viral replication in infected monocytes.

The addition of *C. trachomatis* to HIV-1 chronically infected monocytes did not significantly alter viral replication when PMNs were omitted from the cultures, suggesting that *C. trachomatis* does not directly impact upon viral replication (Ho *et al.*, 1995). However, Bianchi *et al.*, showed that *C. trachomatis* infection of HIV-1 chronically infected monocytes lead to a decrease in viral replication, despite the absence of PMNs (Bianchi *et al.*, 1998). One possible explanation for the conflicting results is that Bianchi *et al.* used an unusually large *C. trachomatis* multiplicity of infection (MOI) (25-100) and centrifuged cultures at

3,500 g for 30mins to achieve infection, whereas Ho *et al* simply added the bacteria to the cultures at 5 ID₅₀s (determined by titration on L-cells). Bianchi *et al* document a decrease in the number of viable cells per ml in cultures infected with *C. trachomatis* compared to cells that were mock-infected with sucrose phosphate buffer. This decrease in cell-viability may have lead to the reduction in viral replication, as opposed to a direct impact of *C. trachomatis* infection on HIV-1 replication.

The effect of HIV-1 on the natural history of *Chlamydia* disease, surprisingly, remains unknown. Ho *et al* did not investigate this, however Bianchi *et al* attempted to. They claimed that HIV-1 infection made U-937 cells more susceptible to *C. trachomatis* lytic infection and suggested that the presence of the HIV-1 accelerated the *C. trachomatis* infection cycle (Bianchi *et al.*, 1998). These conclusions were based on the fact that the rate of cell lysis increased in co-infected cell-cultures compared to single infections. There was, however, no quantification of *Chlamydia* inclusions by microscopy, or quantification of genomic replication by molecular techniques and, as such, it is not possible to draw conclusions regarding the susceptibility of cells to *C. trachomatis* infection, or the kinetics of *Chlamydia* replication as they propose.

The impact of HIV-1 co-infection on *C. trachomatis* replication *in vitro*, therefore, remains unknown.

1.11 Quantification of *C. trachomatis* load

Attempts to quantify chlamydial infection in patient samples were made initially using quantitative cell-culture (Barnes *et al.*, 1990; Eckert *et al.*, 2000; Geisler *et al.*, 2001) and

later qPCR (Gomes *et al.*, 2005; Michel *et al.*, 2007; Wiggins *et al.*, 2009). The genital tract chlamydial load shed by infected patients is associated with the presence of clinical symptoms (Geisler *et al.*, 2001; Michel *et al.*, 2007), transmissibility and persistence of infection (Geisler *et al.*, 2008; Rogers *et al.*, 2008) and the risk of developing chronic sequelae (Geisler *et al.*, 2001), however, to our knowledge there have been no studies conducted that evaluate *C. trachomatis* load in rectal infections.

Rectal *C. trachomatis* infection is not uncommon in MSM (approximately 1% for LGV (Anan *et al.*, 2009; Ward *et al.*, 2009) and 6.5 to 8.2% for serovars D-K (Anan *et al.*, 2009; Benn *et al.*, 2007; Manavi *et al.*, 2004) . Moreover, in contrast to LGV, the majority of patients with serovar D-K infections are asymptomatic (Anan *et al.*, 2009; Kent *et al.*, 2005; Ward *et al.*, 2009). Asymptomatic individuals are likely to be unaware of their infection, remaining undiagnosed and untreated, thus representing a reservoir of infection in the community. It is not known, however, the extent to which they transmit infection and it would be of interest to know how the chlamydial load varies between individuals with rectal symptoms and asymptomatic infections, and between those with LGV and non-LGV infections.

1.12 Novel ways of studying *C. trachomatis* immunobiology and detecting infection

To date, there have been no studies investigating *ex vivo* human cellular immune responses to *C. trachomatis*-specific antigens, and gaps in our knowledge of chlamydial immunobiology remain. An IFN- γ ELISpot assay is one means of quantifying *ex vivo* antigen-specific cellular immune responses by enumerating T-cells in the peripheral blood of patients that recognise, and respond to, specific antigens by secreting IFN- γ (Lalvani *et al.*, 1997). The approach has

been of use in the investigation of immune responses to several pathogens, but *Mycobacterium tuberculosis*-specific IFN- γ is also capable of discerning a current infection from a past infection as, once an infection has been cleared by effective treatment, the number of T-cells circulating in the peripheral blood that recognise *M. tuberculosis*-specific antigens decline (Pathan *et al.*, 2001; Millington *et al.*, 2007).

Both *M. tuberculosis* and *C. trachomatis* are intracellular pathogens in which IFN- γ is believed to play an important role in the immune response (reviewed by Cooper, 2009 and Brunham Rey-Ladino, 2005) and, therefore, quantification of peripheral blood T-cells that recognise *C. trachomatis*-specific antigens and secrete IFN- γ could potentially be useful as a biomarker for current *C. trachomatis* infection. Such a biomarker is desirable as, although sexually transmitted infection with *C. trachomatis* at the lower genital tract, rectum or oropharynx can be detected by NAAT, *C. trachomatis* is capable of migrating from the portal of entry to other anatomical sites such as the endometrium, salpinges and peritoneal cavity of women, epididymi and testicles of men, and the joints of those afflicted with SARA. The extent to which detection of *C. trachomatis* by NAATs reflects *Chlamydia* infection at other anatomical sites is unknown (Taylor-Robinson *et al.*, 2009). An *ex vivo* IFN- γ ELISpot assay may, therefore, be of benefit in detecting such infection.

1.13 Aims of the Thesis

This thesis is comprised of 3 main aims of investigation surrounding chlamydial biology:

- 1.) determine whether *Chlamydia trachomatis* and HIV-1 impact upon the replication of one another in co-infected cell cultures.
- 2.) quantify the extent to which rectally infected individuals shed *Chlamydia trachomatis* and investigate differences between patient groups.
- 3.) investigate the human *ex vivo* cellular immune response to a *Chlamydia trachomatis* – specific antigen and investigate the possibility of using *C. trachomatis*-specific IFN- γ as a biomarker for infection as part of a non-invasive assay to detect current chlamydial infection.

Chapter 2

Materials and Methods

Molecular Biology Materials and Methods

2.1 DNA extraction

DNA was extracted with a DNA mini-kit (Qiagen, UK). Briefly, 200µl of 10% Proteinase K in tissue lysis buffer (ATL) were added to a cell suspension or pellet from which DNA was to be extracted. The mixture was vortexed and incubated overnight at 56°C, after which 200µl lysis buffer AL were added and the mixture incubated for 10 minutes at 70°C. Following sample lysis, 200µl of 96-100% molecular grade ethanol (Sigma-Aldrich, UK) were added and the mixture applied to a QiAmp spin column (Qiagen, UK) for centrifugation at 6000g for 1 minute. The DNA bound to the column was washed in buffers AW1 and AW2 and eluted into 50µl buffer AE following a 5 minute incubation at room temperature.

2.2 Plasmid DNA extraction

Bacterial plasmid DNA was extracted using a QIAprep Spin Miniprep Kit (Qiagen, UK). Briefly, a suspension of transformed *E. coli* bacteria were pelleted at 17,900g for 3 minutes and re-suspended in 250µl buffer P1 to which 250µl of the alkaline lysis buffer P2 were added. This was neutralised by the addition of 350µl of buffer N3, which also precipitated denatured proteins, cellular debris and chromosomal DNA that were subsequently removed by centrifugation (at 17,900g for 10 minutes). The supernatant fluid (containing the plasmid) was applied to a QIAprep spin column that was washed sequentially with buffers PB and PE before plasmid DNA was eluted into 50µl of Buffer EB.

2.3 RNA extraction

RNA was extracted from cell monolayers by using TRIZOL Reagent (Invitrogen, UK). Briefly, cells grown in 24 well-plates were either lysed *in situ* by the addition of 500µl of TRIZOL Reagent or were removed from the bottom of the wells by incubation with trypsin (Section 2.5), pelleted by centrifugation at 500g, 5 mins and re-suspended in 200µl TRIZOL Reagent. Following 5 minutes incubation at room temperature, chloroform was added (200µl per ml of TRIZOL Reagent) and the mixture centrifuged at 10,000g for 4 minutes in order to separate it into an upper aqueous phase (containing the RNA) and a lower organic phase. The aqueous phase was transferred to another microcentrifuge tube (VWR, USA) and the RNA was precipitated by the addition of isopropanol (500µl per 1 ml TRIZOL Reagent). The suspension was incubated for 10 minutes at room temperature and RNA pelleted at 10,000g for 10 minutes. The RNA pellet was washed twice in 500µl 75% ethanol, air-dried and re-suspended into 100µl nuclease-free water (Qiagen, UK).

RNA was purified with an RNeasy Mini kit (Qiagen, UK). Briefly, buffer RLT (350µl) and 96-100% ethanol (250µl) were added to the RNA solution and the mixture applied to an RNeasy spin column (Qiagen, UK) that was centrifuged at 8,000g for 15 seconds to permit RNA binding to the column. The column was washed with buffer RW1(350µl) before genomic DNA contamination was eliminated by addition of 80µl DNase I (Qiagen, UK) and incubation at room temperature for 15min. The column was washed once with buffer RW1 (350µl), twice with Buffer RPE (500µl) and the RNA eluted into 30µl of nuclease free water (Qiagen, UK).

2.4 Reverse Transcription of RNA to cDNA

A 12µl aliquot of RNA solution was mixed with 2µl of genomic DNA wipeout buffer (Qiagen, UK), incubated at 42°C and placed on ice (as an additional genomic DNA elimination step). Quantiscript Reverse Transcriptase (1µl), Quantiscript RT buffer (4µl) and RT Primer Mix (1µl) (Qiagen, UK) were added and the mixture incubated at 42°C for 15 minutes to permit reverse transcription of RNA to cDNA. The Quantiscript Reverse Transcriptase was inactivated by incubating at 95°C for 3 minutes and the cDNA was stored at -20°C.

Table 2.1 Oligonucleotide primers used in the this thesis

Amplified Region	Primer	Sequence (5'-3')	Reference
<i>C. trachomatis</i> <i>pmpH</i>	Forward	TTYGGAACAGTTTTTCGAGGTAA	In house
	Reverse	CCTTCATTGTCTTTGAAAAGCAC	
<i>C. trachomatis</i> plasmid <i>orf2</i>	Forward	CAGCTTGATGTCCTGCTTGAGAGA	Pickett <i>et al</i> 2005
	Reverse	CAAGAGTACATCGGTCAACGAAGA	
<i>C. trachomatis</i> plasmid <i>orf5</i>	Forward	GGATTGACTCCGACAACGTATTC	Chen <i>et al</i> 2008
	Reverse	ATCATTGCCATTAGAAAAGGGCATT	
<i>C. trachomatis</i> 16S rRNA	Forward	GTCGAGAATCTTTGCAATGG	Ouellette <i>et al</i> 2006
	Reverse	ACAACCCTAGAGCCTTCATCACA	
<i>C. trachomatis</i> <i>ompA</i>	Forward	GGTTTCGGCGGAGATCCT	Ouellette <i>et al</i> 2006
	Reverse	AGTAACCAACACGCATGCTGAT	
<i>C. trachomatis</i> <i>omcB</i>	Forward	GAATATGTGATCTCCGTTTCCAATC	Ouellette <i>et al</i> 2006
	Reverse	CCGGGAGAAAGAGTGTCTTCAA	
<i>C. trachomatis</i> <i>euo</i>	Forward	GGCTTTTATTCCGTGGGACA	Ouellette <i>et al</i> 2006
	Reverse	AATGCGGTAGCATGATAGTAAATCTTCTG	
λ -phage	Forward	GGAAGTGAAGAATGCCAGAGACTC	In house
	Reverse	AGCCCGTCGAGAATACTGGCAAT	
HIV-1	Forward	GGTCTCTTTTAGAGATTTACAGTG	Pizzato <i>et al</i> 2008
	Reverse	CGTGTTGACACGCAGACCTCTTAC	

Table 2.1 The oligonucleotide primers used in the study are listed. Citations are given for primers that have been previously described in the literature whereas “in-house” primers have been identified. All primers were synthesised by Invitrogen, UK.

Cell Culture

2.5 Cell lines used in the investigation

The cell lines used in this study are listed in Table 2.2 Adherent cell lines were cultured in Dulbecco's Modified Eagle Medium (D-MEM) (Gibco, UK) supplemented with 0.1% gentamicin (Invitrogen, UK) and 10% fetal bovine serum (FBS) (Gibco, UK) that had been heated for at least 30 minutes at 56°C to inactivate components of the complement cascade according to the manufacturer's instructions (heat-inactivated (hi) FBS). Cells were cultured at 37°C in an atmosphere of 5% CO₂ in tissue culture flasks (Nunc, UK) and were passaged at 80-90% confluence. Briefly, the medium was removed and cells were washed in trypsin-ethylenediaminetetra acetic acid (trypsin EDTA) (Gibco, UK) prior to incubation at 37°C in an atmosphere of 5% CO₂ in sufficient trypsin-EDTA to cover the cells until they were detached from the tissue culture flask. The D-MEM supplemented with gentamicin and hiFBS (complete D-MEM) was added to the cell suspension to give a final volume of 10ml and a 1 ml aliquot was placed into a new tissue culture flask and complete D-MEM added to a volume of 15ml.

Non-adherent cell lines were cultured in Roswell Park Memorial Institute (RPMI) medium (Gibco, UK) supplemented with 10% hiFBS and 0.1% gentamicin (complete RPMI). Cells were cultured at 37°C in an atmosphere of 5% CO₂ in tissue culture flasks and were passaged at 80-90% confluence. Briefly, 1 ml of cell suspension was dispensed into a new tissue culture flask and diluted 1:10 in complete RPMI.

Table 2.2 Cell lines used in the investigation

Cell line	Type	Description	Use in this study	Reference
BGMK	Adherent	Buffalo Green Monkey Kidney epithelial cells.	Growth and titration of <i>Chlamydia trachomatis</i> stock.	Hobson <i>et al</i> 1982
C8166	Non-adherent	Human T cell- line. Forms syncytia when infected with HIV.	HIV-1 stock titration	Sodroski <i>et al</i> 1986
H9	Non-adherent	Human T cell -line.	Growth of HIV-1 (strain MN) stock.	Popovic <i>et al</i> 1984
Jurkat E6.1	Non-adherent	Human leukaemic T cell lymphoblast	Investigating <i>C. trachomatis</i> L2 tropism	Alkhatib <i>et al</i> 1996
MAGI P4R5	Adherent	HeLa cell- line (immortalized cervical cancer cell line) stably expressing the HIV-1 receptor (CD4) and co-receptors (CXCR4 and CCR5) and transformed with β -galactosidase under the control of the HIV-1 long terminal repeat (LTR)	HIV-1 and <i>C. trachomatis</i> co-infection	Kimpton & Emerman 1992
THP-1	Non-adherent	Human monocytic cell line	Investigating <i>C. trachomatis</i> L2 tropism.	Tsuchiya <i>et al</i> 1980
U937	Non-adherent	Cell line of histiocytic origin with monocyte-like characteristics	Investigating <i>C. trachomatis</i> L2 tropism	Sundström & Nilsson 1976

Table 2.2 The cell lines used in this study. Their provenance and original citation in the literature are shown.

2.6 Freezing and Thawing of cell lines

Adherent cell lines were grown in 75cm² tissue culture flasks until 80- 90% confluent. Cells were removed from the bottom of the flask by incubation with trypsin-EDTA, suspended in complete D-MEM, and pelleted by centrifugation at 400g for 5 minutes. The cell pellet was re-suspended in 8ml of an ice-cold freezing mixture comprised of 50% hiFBS, 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and 40% D-MEM, and 1ml aliquots of the cell suspension were added to cryotubes (Merck) on ice. Cryotubes were placed in a cryofreezing container (Jencons) lined with isopropanol (Sigma-Aldrich) that was placed at -80°C overnight to freeze cells slowly at a rate of approximately 1°C per minute. Cryotubes were subsequently placed in liquid nitrogen for long-term storage.

Non-adherent cell lines were grown in 75cm² tissue culture flasks until 80-90% confluent, whereupon cells were pelleted by centrifugation at 400g for 5 minutes, re-suspended in 8ml of freezing mixture and aliquotted into cryotubes that were treated in the same manner as described above for adherent cell lines. All cells were frozen in aliquots containing 10⁶-10⁷ cells/ml.

Frozen cells were rapidly thawed at 37°C in a water bath and decanted into a 50ml Falcon centrifuge tube (Invitrogen) containing 45ml complete medium. Cells were pelleted by centrifugation at 400g for 5 minutes and re-suspended in 5ml complete D-MEM (adherent cell lines) or complete RPMI (suspension cell lines) before transfer to a 25cm² flask for culture at 37°C in an atmosphere of 5% CO₂.

2.7 *Mycoplasma* Testing of cell lines

Cells were tested for *Mycoplasma* contamination at regular intervals with the MycoAlert® *Mycoplasma* detection kit (Lonza). When cells were 80-90% confluent, a 1.5 ml sample of culture medium was centrifuged at 400g for 5 minutes and 100µl of the supernatant fluid was transferred to a white 96 well-plate (Nunc, UK). One hundred microlitres of MycoAlert Reagent were added and the plate incubated at room temperature for 5 minutes, then placed in a microplate luminometer (Turner Designs) for a single 1 second reading. One hundred microlitres of MycoAlert Substrate were added and the plate incubated for 10 minutes at room temperature. A second luminometer reading was taken and the cell line was considered negative for *Mycoplasma* contamination if the ratio of the second luminometer reading over the first was less than 1. Only *Mycoplasma*-negative cell cultures were used in subsequent experiments.

2.8 Determining cell viability and density

A 10µl aliquot of non-adherent cell-suspension (or adherent cells that had previously been removed from tissue culture flasks by incubation with trypsin-EDTA and suspended in complete medium) was mixed with an equal volume of Trypan Blue (Sigma-Aldrich, UK). The mixture was applied to a haemocytometer and viewed under the x20 objective of a light microscope in order to determine the concentration of viable cells (that excluded Trypan Blue and appeared white). The average number of viable cells per haemocytometer square was determined and multiplied by 2 to account for the Trypan Blue dilution factor. This figure represented the number of viable cells x 10⁴ per ml.

2.9 Immunofluorescence Microscopy (IFM)

For IFM, cell cultures were washed once in PBS (Sigma-Aldrich, UK) and fixed at room temperature for 30 minutes in 2% paraformaldehyde (Sigma-Aldrich, UK) in PBS. Following a PBS wash, cells were permeabilised at room temperature for 10 minutes in 0.1% Saponin (Sigma-Aldrich, UK) in fluorescence activated cell sorting (FACS) buffer (1% bovine serum albumin (BSA) (Sigma-Aldrich, UK), 0.1% sodium azide (Sigma-Aldrich, UK) and 5mM EDTA (Sigma-Aldrich, UK)) in PBS). Cells were stained with anti *C. trachomatis* lipopolysaccharide (LPS) antibody conjugated to fluoro-iso-thiocyanate (FITC) (anti-LPS-FITC) (Virostat, ME) and a Phycoerythrin (PE)-conjugated antibody raised against the HIV-1 core antigens (55, 39, 33 and 24KDa proteins) (anti-P24-PE) (Coulter clone KC57-RD1, Beckman Coulter, CA). Both antibodies were diluted in FACS buffer + 0.1% Saponin (anti-LPS-FITC 1:30, anti-P24-PE 1:40) and cells were stained at room temperature for 1 hour in the dark. Following staining, cells were washed 3 times in FACS buffer + 0.1% Saponin, followed by one wash in FACS buffer alone. Cells were viewed under the x20 objective of a Nikon Eclipse TE2000-S fluorescence microscope at excitation/emission wavelengths of 535/565 nm (PE, red) and 470/505 (FITC, green) and photographed with a Nikon digital camera (DXM1200F) using Lucia Software (Version 4.81, Nikon, DE). The anti-P24-PE red fluorescence localised to the cytoplasm in cells infected with HIV-1, whereas the anti-LPS-FITC green fluorescence localised to the inclusion in cells infected with *Chlamydia trachomatis*. Images were merged using the Photoshop software (Adobe Systems Inc., USA).

2.10 *Chlamydia trachomatis* culture

Chlamydia trachomatis, serovar L2 (strain 434/Bu, source ATCC VR-902B) (Schachter & Meyer, 1969) was supplied by Professor Ian Clarke (Molecular Microbiology Group, University of Southampton Medical School). A crude stock was generated by infecting one 175cm² tissue culture flask (Nunc, UK) containing BGMK cells at approximately 80% confluence with *C. trachomatis* L2 at an multiplicity of infection that would lead to >90% cell infection. Infected cells were incubated at 37 °C in 5% CO₂ for 40-48 hours, scraped into complete medium, decanted into a 50ml Falcon tube, and homogenised by vortexing in the presence of glass beads to release intracellular *Chlamydia* organisms. Cellular debris was removed from the cell-lysate by centrifugation at 500g for 5 minutes, and aliquots of the supernatant-containing *C. trachomatis* organisms were stored at -80 °C in 10% D- sorbitol (Sigma-Aldrich, UK).

2.11 *Chlamydia trachomatis* Elementary Body Purification

A stock of purified *Chlamydia trachomatis* L2 Elementary bodies (EBs) was obtained by infecting fourteen T175 flasks containing BGMK cells at approximately 80% confluence with crude *C. trachomatis* L2 and incubating at 37 °C in 5% CO₂ for 40 hours. Infected cells were detached from the surface of the tissue culture flasks by incubation in trypsin-EDTA at 37 °C in an atmosphere of 5% CO₂, whereupon they were pooled, pelleted (at 400g, 5 minutes), and re-suspended in a hypotonic solution (10% Dulbecco's sterile (s)PBS) (Sigma-Aldrich, UK) in Milli-Q_{PLUS} Ultra pure water (Millipore, UK)). Infected cells were homogenised by vortexing in the presence of glass beads to release the intracellular bacteria that were subsequently separated from the cellular debris by centrifugation at 500g for 5 minutes. The supernatant, containing *C. trachomatis* organisms, was layered onto 30ml of 20% Urografin 370 (Shering, UK) in sPBS and subjected to ultracentrifugation at 60,000g for

2 hours at 4°C. The supernatant fluid was decanted, the pellet re-suspended in 2ml sPBS, layered onto a triple gradient (consisting of 35%, 45% and 55% urografin in sPBS) and ultracentrifuged at 60,000g for 2 hours at 4°C. Elementary bodies formed a band at the interface between 45% and 55% urografin and this was removed with a Pasteur Pipette (alpha Laboratories, UK), washed in water (molecular biology reagent, Sigma-Aldrich, UK) and ultracentrifuged at 40,000g for 30 minutes, without break. The resultant pellet was re-suspended in a freezing mixture (comprised of 0.2M sucrose, 6mM NaH₂PO₄, 15mM Na₂HPO₄ and 5mM L-glutamine) and stored at -80°C.

2.12 Determination of *Chlamydia trachomatis* titre

Ninety-six well plates (Nunc, UK) were seeded with BGMK cells at 1.5×10^4 cells per well and incubated at 37°C in 5% CO₂ overnight. Monolayers were inoculated with 100µl of 10-fold serial dilutions of purified *Chlamydia trachomatis* L2 EBs that were removed 4 hours post -infection, after which cells were washed with complete D-MEM and incubated at 37°C in 5% CO₂ for a further 26-30 hours. Cells were subsequently prepared for immunofluorescence microscopy (Section 2.9) and the number of infectious forming units (IFU) determined for a known dilution of *C. trachomatis* by counting the number of chlamydial inclusions in 3 infected wells and calculating the mean value. The titre was expressed IFU per millilitre of undiluted stock (IFU/ml). From this value, the multiplicity of infection (MOI) could be determined, which represented the ratio of IFU per cell.

2.13 HIV-1 culture

An aliquot of human immunodeficiency virus-1 (HIV-1) (laboratory adapted strain MN (Gallo *et al.*, 1984)) was cultured in the T-lymphocyte cell line, H9 for 14 days. Briefly, 5×10^5 H9 cells were infected with HIV-1 in a total volume of 1.5 ml complete RPMI in one

well of a 24 well-plate. Infected cells were cultured at 37°C in an atmosphere of 5%CO₂ for 2-3 days, after which uninfected H9 cells were added to the infected culture in a ratio of 4:1 (uninfected cells: infected cells) in a total volume of 5 ml complete RPMI and the cells cultured in a 25cm² flask. Thereafter, infected cells were supplemented with uninfected H9 cells as described every 4-6 days and the medium subsequently changed 2-3 days later. Cell densities were maintained between 1-2 million cells per ml and the volume expanded accordingly. After 14 days in culture, cells were pelleted at 500g for 5 minutes and the supernatant, containing the HIV-1, was aliquotted and stored at -80°C.

2.14 HIV-1 titration

The tendency for HIV-1 strain MN to form clearly identifiable and quantifiable syncytia in the T-lymphocyte cell line C8166 was exploited in order to determine the viral stock titre in a modification of the bioassay described by Japour *et al.*,(1993). Briefly, each well of a 96 well-plate (Nunc, UK) was seeded with 150µl of C8166 cells (at a density of 4 x 10⁵ cells per ml) to which 50µl of either 10-fold serially diluted HIV-1 strain MN or complete RMPI were added. Cells were infected with each MN dilution in replicates of six and incubated at 37°C in an atmosphere of 5% CO₂ for 3 days after which the medium was changed and cultures incubated for a further 3 days. The number of syncytia in each well was determined and wells scored either positive or negative. A culture was scored as positive if 3 or more syncytia were observed. The tissue culture infectious dose 50 per ml (TCID₅₀/ ml) was subsequently determined using the Spearman-Karber formula:

$$M = xk + d [0.5 - (1/n) (r)]$$

Where

xk = lowest viral concentration

d = spacing between dilutions (for a 10-fold serial dilution, d = 1)

n = number of replicate wells per dilution

r = sum of the number of negative wells

The TCID₅₀ per well (10^M) was multiplied by 4 to determine TCID₅₀/ml by accounting for the viral dilution.

2.15 Quantification of HIV-1 by SYBR Green I-based product -enhanced reverse transcriptase (SG-PERT) assay

To quantify HIV-1 activity, an in-house SYBR Green I-based product-enhanced reverse transcriptase (SG-PERT) assay was developed (Pizzato *et al* 2008). Virus supernatant (5µl) was lysed with an equal volume of lysis buffer (100mM Tris-HCl (pH7.4), 0.25% Triton X-100, 50mM KCl, 40% glycerol, 0.4U/µl RNase inhibitor) and incubated at room temperature for 10 to 15 minutes. The lysate was diluted 10-fold with dilution buffer (20mM Tris-HCl (pH8.3), 5mM (NH₄)₂SO₄, 20mM KCl) and 10µl applied to duplicate lightcycler capillaries (Roche) containing 10µl of a master mix (40mM Tris-HCl (pH8.3), 10mM (NH₄)₂SO₄, 40mM KCl, 10mM MgCl₂, 0.2mg/ml BSA, 1/10000 SYBR Green-I, 400µM dNTPs, 1µM sense primer (SGF1, Table 2.1) , 1µM antisense primer (SGR1, Table 2.1), 1.2µg/ml BMV RNA and 0.2U hotstart Taq). Samples were run on a LightCycler 2.0 (Roche Diagnostics) machine with the following cycling conditions: 1 cycle of RT reaction at 37°C for 30 minutes, 1 cycle of polymerase activation at 95°C for 5 minutes and 45 cycles of amplification (denaturation at 95°C for 5 seconds, annealing at 55°C for 5 seconds, extension at 72°C for 15 seconds and acquisition at 83°C for 7 seconds). Recombinant HIV-1 RT was serially diluted 10-fold with dilution buffer and subjected to the SG-PERT assay to generate a standard curve. Amplification curves and melting temperatures were generated by recording the fluorescence intensity at 530nm and analyzed using the LightCycler software 4. The units of RT in the sample were calculated from the standard curve.

2.16 Quantification of *C. trachomatis* genome copy number *in vitro*

The *C. trachomatis* genome copy number was quantified *in vitro* by a qPCR assay, carried out as follows: Firstly, a fragment of the *C. trachomatis* *pmpH* gene was cloned into a recombinant plasmid. Briefly, DNA was extracted from a 10µl aliquot of purified *C. trachomatis* elementary bodies by using a DNA mini-Kit (Qiagen, UK). A 189 base-pair fragment of the *pmpH* gene, Pubmed Accession AY184168, was amplified from *Chlamydia trachomatis* L2 genomic DNA by the polymerase chain reaction (PCR) using the forward primer: TTYGGAACAGTTTTTCGAGGTAA, and reverse primer: CCTTCATTGTCTTTGAAAAGCAC (Invitrogen, UK). The PCR product was applied to a 2% agarose gel and the DNA separated by electrophoresis. A single band was detected at a position close to the 200bp marker. This band was excised from the gel and the DNA extracted and purified as per the protocol in the QIAquick Gel Extraction Kit (Qiagen, UK). Briefly, the excised band was incubated at 50°C in the presence of 3 gel-volumes of buffer QG until dissolved. One gel-volume of isopropanol (Sigma-Aldrich, UK) was added, whereupon the sample was applied to a QIAquick column and centrifuged at 17,900g for 1 minute. The column was sequentially washed in buffers QC and PE, and DNA was eluted in 50µl of elution buffer and stored at -20°C. A 10µl aliquot of the product from gel extraction was applied to an additional 2% agarose gel and DNA separated by electrophoresis in order to confirm the efficiency of the gel extraction.

The gel extraction product was diluted 1 in 3 with water (Sigma-Aldrich, UK), and 2µl added to 0.5µl of the vector pCR[®]4-TOPO[®] (Invitrogen, UK) and 0.5µl of a salt solution (1.2M NaCl, 0.06M MgCl₂) for ligation. One vial of One Shot[®] TOP10 Competent cells (Invitrogen, UK) were transfected with 2µl of the recombinant plasmid produced from the ligation

reaction (TOPO-*pmpH*) by incubating on ice for 5 minutes and subsequently heat-shocking at 42°C for 30 seconds. Super Optimal Broth with Catabolyte suppression (SOC) medium (Invitrogen, UK) was added, and bacteria were shaken at 37°C for 1 hour before inoculation into a Luria Bertani (LB) – Agar (Melford, UK) plate (supplemented with 0.1% ampicillin (Sigma –Aldrich, UK)) and incubation at 37°C for approximately 16 hours.

One sample from each of 5 resultant colonies was placed in 3ml LB broth (Melford, UK) supplemented with 0.1% ampicillin and shaken at 37°C for approximately 16 hours, after which recombinant plasmids were purified by means of a QIAprep Spin Miniprep Kit (Qiagen, UK) (Section 2.2). An analytical digest of the construct (with EcoR1 (New England Biolabs (NEB), USA)) at 37°C for 1 hour, followed by gel electrophoresis on a 2% agarose gel revealed two bands consistent with a pCR[®]4-TOPO[®] vector (3956bp in length) containing a *Chlamydia trachomatis* L2 *pmpH* fragment (189 bp in length). Five of the miniprep samples were prepared for sequencing (by the addition of T3 and T7 primers to approximately 300ng of the construct) to confirm this.

The concentration of the TOPO-*pmpH* recombinant plasmid was quantified using an Ultraspec 2000 UV/visible Spectrophotometer (Pharmacia Biotech) and the number of plasmid copies per microlitre determined by dividing the plasmid concentration (µg/µl) by the weight of 1 plasmid (µg). The weight of one plasmid was determined by dividing the plasmid molecular weight calculated in Daltons (g/mole) (gained from a knowledge of the DNA sequence and the molecular weight of each nucleotide) by Avagadro's constant (6.022×10^{23} molecules/mole).

The recombinant plasmid TOPO-*pmpH* was serially diluted 10-fold in water in order to create a standard curve. Five microlitre aliquots of each plasmid dilution were applied to Lightcycler capillaries (Roche, UK) containing the forward primer TTYGGAACAGTTTTTCGAGGTAA (1µl), and reverse primer CCTTCATTGTCTTTGAAAAGCAC (1µl) (Invitrogen, UK), as well as a ready- to-use hot start PCR reaction mix (4µl) (Roche, UK) consisting of Taq DNA Polymerase, a deoxyribose nucleotide (dNTP) mix, a reaction buffer containing MgCl₂, and SYBR Green I. Samples were run on a LightCycler 1.5 instrument (Roche) and the crossing point (the number of amplification cycles taken to reach a pre-determined threshold) for each plasmid concentration was determined, in order to create a standard curve. The following cycling conditions were used during sample amplification: 1 hot-start cycle of 95°C for 10 minutes and 50 cycles of amplification (denaturation at 95°C for 10 seconds, annealing at 55°C for 5 seconds and extension at 72°C for 8 seconds, after which the fluorescence signal was acquired.

In order to determine the analytical sensitivity of the assay (the lowest concentration of plasmid that was amplified in every instant), the TOPO-*pmpH* recombinant plasmid was diluted to 10,000, 1,000, 100, 70, 50, 30, and 10 copies per reaction and each dilution amplified in quadruplicate. As there is one copy of *pmpH* per *C. trachomatis* organism, the sensitivity could be expressed in terms of *C. trachomatis* organisms per reaction.

In order to determine intra-assay variability, three dilutions of DNA extracted from purified *C. trachomatis* EBs were amplified. Two dilutions were amplified 9 times each and one was repeated 5 times. The mean crossing point and standard deviation was determined for each

dilution and the coefficient of variation (the standard deviation as a percentage of the mean) calculated.

In order to determine inter-assay variability, three dilutions of DNA extracted from purified *C. trachomatis* EBs were amplified. Each dilution was amplified in duplicate on four separate occasions. The mean crossing point and standard deviation was determined for each dilution and the coefficient of variation calculated.

2.17 Change in *C. trachomatis* genome copy number over time

BGMK cells were seeded into 24-well plates at 1×10^5 cells per well, incubated at 37°C, 5% CO₂ overnight to permit cells to adhere to the bottom of the wells, and infected with 1ml purified *Chlamydia trachomatis* L2 EBs suspended in complete D-MEM at an MOI of 0.1 in duplicate. Cells were incubated at 37°C in an atmosphere of 5% CO₂ for 4 hours after which the inoculum was replaced with complete medium. Cells were further incubated at 37°C in 5% CO₂ and at 0, 6, 16, 24, 30, 40, 48, 64 and 72 hours post-infection, cells were detached from the wells by incubation with trypsin, transferred to microcentrifuge tubes, pelleted at 3,300g for 3 minutes, and DNA extracted with a DNA mini-Kit (Qiagen, UK) (Section 2.1). A total of 5µl DNA from each sample was applied to a lightcycler capillary and amplified as described (Section 2.16). The number of pmpH copies was plotted against time to generate a growth curve for *C. trachomatis*. DNA was also extracted from duplicate cells that were mock-infected (with complete medium) as negative controls.

2.18 Development of an assay to quantify infectious *C. trachomatis* progeny

Twenty-four well plates were seeded with BGMK cells at 1×10^5 cells per well and incubated at 37°C in 5% CO₂ overnight to permit cells to adhere to the bottom of the wells. Cells were

infected with 1ml purified *Chlamydia trachomatis* L2 EBs in complete D-MEM at an MOI of 0.1 in duplicate. The inoculum was removed 4 hours post-infection, replaced with complete D-MEM, and cells were further incubated at 37°C in 5% CO₂. At 0, 6, 16, 24, 30, 40 and 48 hours post-infection, cells were detached from the wells by incubation with trypsin, transferred to microcentrifuge tubes, pelleted at 3,300g for 3 minutes and re-suspended in complete D-MEM. Cell suspensions were subjected to two rounds of water-bath sonications, each consisting of 3 x 15 seconds in duration, in order to release intracellular organisms. Cellular debris was pelleted by centrifugation at 500g for 3 minutes, and the supernatant, containing *Chlamydia* serially diluted in complete D-MEM and inoculated onto a second monolayer of BGMK cells that were subsequently incubated at 37°C in 5% CO₂ for 30 hours. Cells were fixed, permeabilised and stained with anti-LPS-FITC, as described (Section 2.9). The number of IFUs for a particular dilution of cell lysate was determined by IFM, from which the number of IFU per ml of undiluted cell lysate could be extrapolated and plotted on a graph against time.

Three methods of lysing cells were compared and the yield of infectious *C. trachomatis* organisms determined by passage onto BGMK cells. Briefly, 5 x 10⁴ cells were seeded per well of a 24 well-plate and cells were incubated overnight at 37°C in 5% CO₂ to allow them to adhere to the bottom of the wells. Cells were infected, in duplicate for each lysis condition, with 1ml *C. trachomatis* serovar L2 in complete D-MEM at an MOI of 1 and incubated for 4 hours at 37°C in 5% CO₂, after which, the inoculum was replaced with complete D-MEM and cells incubated for an additional 36 hours. At 40 hours post-infection, the cells were removed from the bottom of the wells by incubation with trypsin, as described, and subject to either one freeze-thaw cycle (between room temperature and -80°C), two rounds of sonication, or one freeze-thaw cycle followed by two rounds of sonication. Cellular debris was pelleted at

500g for 5 minutes and an aliquot of the supernatant (containing the *C. trachomatis*) was serially diluted and passaged onto BGMK cell monolayers. Cells were incubated for 30 hours at 37°C in 5% CO₂ after which they were fixed, permeabilised, stained with α -LPS-FITC and subject to immunofluorescence microscopy. The number of IFUs per ml of undiluted cell-lysate was determined and plotted graphically.

If the cells had been co-infected with HIV-1, the cell-lysate would also contain virus, so lysates were further processed in a way that would remove the viral contamination. Briefly, following the removal of cellular debris from the lysate by centrifugation at 500g for 5mins, the supernatant was transferred to another microcentrifuge tube and the *C. trachomatis* pelleted at 10,000g for 30 mins whereupon the supernatant was removed and the pellet re-suspended in complete D-MEM and subject to centrifugation at 10,000g for an additional 30 mins. The washed pellet was re-suspended in complete D-MEM once more, serially diluted and passaged onto BGMK cells as described above. The yield of infectious *C. trachomatis* was then compared with the previous protocol to determine whether any loss resulted.

2.19 Validating the assays using a known inducer of persistence, Penicillin

The ability of the assays to detect chlamydial persistence was tested to ensure they were adequate for future experiments. Briefly, 1×10^5 MAGI P4R5 cells were seeded per well of a 24 well-plate and cells were incubated overnight at 37°C in 5% CO₂ to allow them to adhere to the bottom of the wells. Cells were infected with 1ml *C. trachomatis* serovar L2 in complete D-MEM at an MOI of 2 and incubated for 1 hour at 37°C in 5% CO₂, after which, the inoculum was replaced with either complete D-MEM, or a final concentration of 5 μ g/ml Penicillin (Invitrogen, UK) in complete D-MEM and cells were incubated for an additional 24 hours at 37°C, 5% CO₂.

Cells were removed from the bottom of the wells by incubation with trypsin and the cell-suspensions either lysed by sonication or divided into two equal aliquots and pelleted. Cell-lysates were passaged onto monolayers of BGMK cells as described (Section 2.18) and the number of infectious progeny determined.

The DNA was extracted from one cell pellet (Section 2.1) and the number of genomic copies determined (Section 2.16). The number of gene copies of *euo*, *ompA*, *omcB* and *16S rRNA* were also determined (Section 2.22). The RNA was extracted from the other cell pellet as described (Section 2.3), reverse transcribed to cDNA (Section 2.4) and the number of chlamydial transcripts of *euo*, *ompA*, *omcB* and *16S rRNA* determined (Section 2.22). Gene transcription was quantified by dividing the number of transcripts by the number of genes (cDNA copies/DNA copies). The number of infectious chlamydial progeny, the genome copy number and the transcription of chlamydial genes were quantified in the presence and absence of penicillin, which is known to induce a persistence phenotype (Matsumoto & Manire, 1970; Lambden *et al.*, 2006) and plotted graphically.

2.20 Investigating *C. trachomatis* tropism in CD4-positive cell-lines

In order to select a suitable cell-line to investigate co-infection, CD4⁺ Cell lines were compared in how permissive they were to *C. trachomatis* serovar L2 infection. Twenty four well-plates were seeded with the epithelial cell lines BGMK and MAGI P4R5, the T-cell lines C8166 and Jurkat, and the monocytic cell lines U937 and THP-1 (Table 2.2) at a density of 5×10^4 cells per well (1ml per well) in duplicate. Cells were incubated overnight to allow cells to settle to the bottom of the wells and adherent cells to stick. Five hundred microlitres of the medium were removed and replaced with 500µl of *C. trachomatis* in

complete medium at a final concentration of 10 IFU per cell. Cells were incubated at 37°C, in an atmosphere of 5% CO₂ for 4 hours, after which 800µl of the medium + *C. trachomatis* was replaced with complete medium and the cells incubated for 30 hours, whereupon they were fixed, permeabilised and stained with α-LPS-FITC antibody solution as described (Section 2.9). Suspension cells were transferred to microcentrifuge tubes and pelleted at 500g for 5 minutes between wash, permeabilisation and staining steps and, following post-staining washes, cells were re-suspended into 100µl PBS and 10µl aliquotted into a haemocytometer slide in order for cells to be viewed by immunofluorescence microscopy.

At least 5 fields of view were photographed and the number of cells (visible under white light) and the number of *C. trachomatis* inclusions (visible at excitation/emission wavelengths of 470/505) were counted. The percentage of infected cells was determined for each field of view and the mean value plotted on a graph (+/- standard error of the mean (SEM)).

2.21 HIV-1 infection of MAGI P4R5 cells

Cells of the epithelial cell line, MAGI P4R5 were infected with HIV-1 at increasing MOIs and the percentage of infected cells determined for a given MOI. Briefly, 5 x 10⁴ MAGI P4R5 cells were seeded per well of a 24 well-plate and incubated overnight at 37°C in an atmosphere of 5% CO₂ to allow cells to adhere to the bottom of the wells. Cells were infected with 1 ml HIV-1_{MN} in complete D-MEM at MOIs of 0, 0.5, 1, 5 and 10 in duplicate for 2 hours at 37°C, 5%CO₂, whereupon the inoculum was removed and replaced with 1ml complete D-MEM and the cells incubated for a further 20 hours at 37°C, 5% CO₂. Cells were fixed, permeabilised and stained with anti-P24-PE as described in Section 2.2.6. The total number of cells (visible under white light) per field of view in at least 5 fields for any given

MOI were counted and the number of cells that were infected with HIV-1 (cells possessing a red cytoplasm when viewed at excitation/emission wavelengths of 535/565 nm) were determined. The percentage of infected cells was determined for each field and the mean value plotted graphically (+/- SEM) for each MOI. In addition, MAGI P4R5 cells infected with HIV-1 in the same manner as above were fixed in 0.5% glutaraldehyde at 37°C for 15 minutes, washed in PBS and stained in X-gal + Buffer for 2 hours at 37°C. Cells were viewed by light microscopy and imaged. Infected cells stained with X-gal appeared blue, whereas uninfected cells remained colourless.

2.22 Quantification of *C. trachomatis* gene expression

Cell cultures were removed from a 24 well-plate by incubation with trypsin, divided into two aliquots and each aliquot pelleted by centrifugation at 500g for 5 mins. One pellet was subjected to DNA extraction (Section 2.1) and one pellet was lysed in TRIZOL Reagent and RNA extracted (Section 2.3) and reverse-transcribed to cDNA (Section 2.4).

The DNA extracted from each cell-pellet was used as a template for the amplification of the following *C. trachomatis* genes by qPCR: *16S ribosomal RNA*, *omcB*, *ompA* and *euo*. The Primer sequences with which to amplify these genes have been published (Ouellette *et al* 2006) and are in Table 2.1. For each gene to be amplified, 1.5µl DNA from one sample was applied to Lightcycler capillaries containing 13.5µl of master mix comprising of 7.5µl SYBR Green (Qiagen, UK), 0.75µl of the relevant forward and reverse primers (Invitrogen, UK) and 5.25µl of nuclease-free water (Qiagen, UK). Samples were amplified with a LightCycler 2.0 instrument (Roche) and the crossing-points determined for each sample. The following cycling conditions were used: 1 hot-start cycle at 95°C for 15 minutes and 50 cycles of

amplification (denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 15 seconds with a single acquisition at 72°C).

The number of organisms in an aliquot of purified *C. trachomatis* elementary bodies was determined by extracting the DNA and quantifying the number of *pmpH* genes present by using the *pmpH* qPCR (Section 2.16). The DNA was 10-fold serially diluted to create a standard curve that was amplified by each primer pair to determine the number of *16S rRNA*, *omcB*, *ompA* and *euo* genes in each DNA sample.

The cDNA was used as a template for amplification of the following transcripts:

Unprocessed *16S rRNA*, *omcB*, *ompA* and *euo*. Primers used to amplify these transcripts were the same as those used to amplify the DNA (Table 2.1.). For each transcript to be amplified, 1.5µl cDNA from one sample was applied to the Lightcycler capillaries containing 13.5µl of a master mix comprising 7.5µl SYBR Green (Qiagen, UK), 0.75µl of the relevant forward and reverse primers (Invitrogen, UK) and 5.25µl of nuclease-free water (Qiagen, UK). Samples were amplified by a LightCycler 1.0 instrument (Roche) with the same cycling conditions described above and with the same standard curve. Thus, the number of *16SrRNA*, *omcB*, *ompA* and *euo* transcripts in the cDNA samples was determined. The number of transcripts were normalised to the number of genes and gene expression expressed in terms of cDNA copies per DNA copy.

2.23 Transmission Electron Microscopy

Duplicate samples were processed for transmission electron microscopy (TEM) at 5, 10 and 15 hours post co-infection. Unless otherwise stated, reagents were purchased from TAAB Laboratories Equipment Ltd, UK. Cultures were grown on sterile cover-slips and fixed in

2.5% glutaraldehyde and 2% paraformaldehyde (in 0.1M sodium cacodilate buffer, pH 7.4) for 15 minutes at 37°C, 45 minutes at room temperature and 4°C overnight, after which cells were washed in 0.1M sodium cacodilate buffer and incubated for 1 hour at 4°C in 1% osmium tetroxide and 1.5% potassium ferrocyanide as a secondary fixation step. Following additional washes, cells were incubated in 1% tannic acid in 0.05M sodium cacodilate buffer at room temperature for 45 minutes in the dark as a further fixation/staining step. Cells were washed in 1% sodium sulphate in 0.05M cacodilate buffer and the tannic acid quenched by incubation at room temperature. The sodium sulphate solution was removed and cells were dehydrated by incubation in a series of increasing ethanol concentrations (10%, 25%, 50%, 70% and 90% ethanol in Milli-Q_{PLUS} Ultra pure water and 100% ethanol. Following ethanol dehydration, cells were infiltrated with a mixture of Epon 812 resin and propylene oxide (PO) at increasing ratios of resin (1:3, 1:1, 3:1 (resin:PO)). The mixture was then replaced with 100% resin. Cover slips were subsequently mounted on the top of solidified resin blocks and baked at 60°C for 48 hours to attach the cover slip to the block. Each coverslip was removed from the block by snap-freezing in liquid nitrogen. Cells were left embedded *en face* of the solidified resin block, which was mounted onto a stand and trimmed to size before being cut into sections approximately 80nm thick with an ultramicrotome. The sections were collected onto copper grids and stained for 10 minutes at room temperature in 2% aqueous (w/v) uranyl acetate, washed Milli-Q_{PLUS} Ultra pure water, and stained with Reynold's lead citrate solution for 20 minutes at room temperature and washed. Grids were air-dried and viewed using a Tecnai G2 Spirit Transmission Electron Microscope (FEI, USA), operating at a voltage of 120kV. Cells were imaged at 21,000, 27,000 and 44,000 X magnification with an FEI Eagle CCD camera (2k x 2k) and analysed using TEM Imaging and Analysis (TIA) software (Tecnai, FEI).

2.24 Co-infection of HIV-1 and *C. trachomatis* serovar L2

Wells of a 24 well-plate were seeded with 5×10^4 MAGI P4R5 cells per well and incubated at 37°C in an atmosphere of 5% CO₂ overnight to allow the cells to adhere to the substratum. Cells were either mock-infected (with complete D-MEM alone) or infected with HIV-1 (MN) at an MOI of 5 and incubated at 37°C, 5% CO₂ for 20 hours after which cells were either mock-infected with D-MEM alone or were infected with *C. trachomatis* at an MOI of 0.1. Four hours post-co-infection, the inoculum was replaced with complete D-MEM (Time point 0) and the cells incubated at 37°C in 5% CO₂. At various times (6, 12, 24, 30, 36 and 48 hours) post-infection, cells were either fixed, permeabilised, stained and viewed by immunofluorescence microscopy (IFM) (Section 2.9) or they were removed from the wells by incubation with trypsin. Cell suspensions were either lysed and the liberated *C. trachomatis* passaged in BGMK cells (Section 2.18), to establish the quantity of infectious *C. trachomatis* present, or the cells were pelleted at 500g, 5 mins and DNA extracted to quantify the chlamydial genome copy number in each sample. In addition, the quantity of HIV-1 virus present in the cell supernatants at each time-point was quantified by titration onto C8166 cells (Section 2.14) and the SG-PERT assay (Section 2.15).

In an alternative protocol, cells were seeded as above and either mock-infected or infected with *C. trachomatis* at an MOI of 10 for 24 hours, after which cells were either mock-infected with D-MEM, or were infected with HIV-1 at an MOI of 10. Two hours post-co-infection, the inoculum was replaced with complete D-MEM (Time point 0) and the cells incubated at 37°C in 5% CO₂. At 5, 10, 15 and 20 hours post-inoculum removal, cells were either fixed, permeabilised, stained and viewed by IFM, or they were removed from the bottom of the wells by incubation with trypsin. Cell suspensions were either lysed and the liberated *C. trachomatis* passaged in BGMK cells to establish the quantity of infectious *C.*

trachomatis present, or the cells were pelleted and DNA extracted to quantify the chlamydial genome copy number in each sample. The quantity of HIV-1 present in the supernatants was quantified by the SG-PERT assay and by titration onto C8166 cells. Cells from time points 5, 10 and 15 hours post-co-infection were also processed and viewed by transmission electron microscopy (Section 2.23) and RNA was extracted 15 hours post-co-infection, reverse-transcribed to cDNA and the relative expression of unprocessed *16SrRNA*, *omcB*, *ompA* and *euo* determined (Section 2.22).

Patient Recruitment

2.25 Patient Recruitment for the ELISpot study

Eighty five patients were recruited from St Mary's Hospital, London. Sixty nine adults were recruited from the Jefferiss Wing, Department of Genito-urinary medicine (GUM) and sixteen children, under the age of 12, were recruited as negative controls from the Paediatric Outpatients "GP bloods" clinic, Imperial College Healthcare NHS Trust. Clinical information was recorded on a proforma for the adults and ethical approval was obtained from both the Hounslow & Hillingdon Local Research Committee (file number: 07/H0709/55) and the St. Mary's Research and Ethics Committees (REC ref no: 09/H0712/58) for adults and children, respectively (Appendices 2 and 3).

Adults recruited to the study (n = 69) were all male. The mean age was 34 years old (range 18 to 77) and the patients were from ethnically diverse backgrounds: 21 (30.4%) were British, 4 (5.8 %) were African, 4 (5.8 %) were from the Indian Subcontinent, 8 (11.6 %) were from the Caribbean, 27 (39.1 %) were whites of other nationalities, while there were no data on the ethnicity for 5 (7.2 %) patients. Men were recruited if they had symptoms and/or signs of urethritis or proctitis (symptomatic), or if they had no symptoms and no detectable inflammation (asymptomatic). The signs or symptoms of urethritis included urethral discharge and or dysuria, whereas signs or symptoms of proctitis included rectal discomfort, discharge, or bleeding. Ten (45%) of the 22 NAAT-positive individuals were symptomatic (5 with proctitis and 5 with urethritis), while twelve (55%) were asymptomatic. Fifteen (32%) of the 47 NAAT-negative individuals were symptomatic (7 with proctitis and 8 with urethritis), while 32 (68%) were asymptomatic.

Children recruited to the study (n = 16) were all male to match the adult population. The mean age was 6 years old (range 2 to 10) and subjects were from ethnically diverse backgrounds: 2 (12.5 %) were British, 4 (25 %) were African, 1 (6.3 %) was from the Indian Subcontinent, 5 (31.3 %) were whites of other nationalities and 4 (25 %) were non-whites of other nationalities. Children were excluded from the study if they had signs or symptoms of acute or chronic inflammatory conditions.

2.26 Patient Recruitment for the qPCR study

Ninety-one patients were recruited to the study, of which qPCR could be conducted on 86 (94.6%), 54 from the Bristol Sexual Health Centre (BSHC) and 32 from the Jefferiss Wing, Department of Genito-urinary medicine, St Mary's Hospital, London. Ethics approval was obtained from the Frenchay Research Ethics Committee (Ref 09/H0107/6) for BHSC (Appendix 1) and from the Hounslow & Hillingdon Local Research Committee (file number:

07/H0709/55) for St. Mary's Hospital (Appendix 2). Seventy-nine (91.9%) of the 86 subjects were male. The mean age was 33 years old (range 16 to 63), and 73 (84.9%) were Caucasian, 10 (11.6%) were non-Caucasian while the ethnicity of 3 (3.5%) was undocumented.

A rectal swab was obtained from each patient for quantification of chlamydial load. In addition, a rectal Gram-stained smear was undertaken. Proctitis was defined as greater than or equal to 5 polymorphonuclear leucocytes per high power (x1000) field of view when a Gram-stained rectal smear was viewed by light microscopy.

2.27 Sample Processing and Storage

When recruiting paediatric patients, parental informed consent was obtained and a 5 ml venous blood sample was collected from each child into tubes lined with EDTA for the extraction of PBMCs. When recruiting adult patients from the GUM clinic, informed consent and a clinical questionnaire were obtained and a venous blood sample (30ml) was collected from each patient into tubes coated with EDTA for the extraction of PBMCs. An additional venous blood sample (3ml) was collected into a plain glass tube for the separation of serum. A rectal swab was obtained from every patient with proctitis or who were asymptomatic and had partaken in passive anal intercourse. A first-void urine sample was obtained from adults who did not have proctitis and had not partaken in passive anal intercourse. The samples collected for each patient group are tabulated in Table 2.3

Table 2.3 Patient recruitment for the ELISpot study

Group	Blood collected into EDTA tubes for PBMC extraction	Blood collected into a plain tube for serum separation	First-void Urine sample	Rectal Swab
Symptomatic proctitis	Yes (30ml)	Yes (3ml)	No*	Yes
Symptomatic urethritis	Yes (30ml)	Yes (3ml)	Yes	No
Asymptomatic with a history of receptive anal intercourse	Yes (30ml)	Yes (3ml)	No*	Yes
Asymptomatic with no history of receptive anal intercourse	Yes (30ml)	Yes (3ml)	Yes	No
Paediatric negative controls	Yes (5ml)	No [#]	No	No

[#]Where no serum could be obtained, plasma was separated from the blood collected into EDTA tubes

* Samples were obtained for routine screening but were not available to our laboratory for research use

Table 2.3 The clinical samples obtained from each patient group are listed.

Patient PBMCs were extracted from whole blood by centrifugation over Histopaque and the cells were stored in liquid nitrogen. Serum was separated from whole blood by centrifugation and the serum stored at -80°C. Where no serum sample was obtained, plasma was separated from whole blood and stored at -80°C. Rectal swab and urine samples were stored at -80°C.

First-void urine samples were stored at -20°C. Urine was thawed at room temperature, mixed thoroughly and 1.5ml aliquots centrifuged at 15,000g for 10 minutes to pellet any *Chlamydia* present in the sample. DNA was extracted from the pellet using a DNA mini-kit (Qiagen, UK) (Section 2.1).

Rectal swabs collected at St Mary's Hospital, London were collected using a BD ProbeTec ET *C. trachomatis* and *N. gonorrhoeae* (CT/NC) Amplified DNA Assay Collection Kit (Beckton Dickinson, USA) with a polyurethane-tipped collection swab that was placed into 2 ml of fluid (supplied with the swab). The swab in the fluid was frozen at -20°C until further processing. Swabs were thawed at room temperature, mixed by vortexing, then discarded. A 700µl aliquot of swab fluid was centrifuged at 15,000g for 10 minutes to pellet any *Chlamydia* in the sample and DNA was extracted from the pellet using a DNA mini-kit (Qiagen, UK).

Rectal swabs collected from the Bristol Sexual Health Centre were placed into Bijou tubes and frozen dry at -20°C until further processing. Each Swab was thawed at room temperature and placed in a microcentrifuge tube to which 400µl PBS were added. The DNA was extracted using a DNA mini-kit Buccal Swab Spin Protocol (Qiagen, UK). Briefly, Proteinase K (20µl) and buffer AL (400µl) were added to the sample, vortex-mixed and

incubated at 56°C for 10 minutes. Molecular-grade ethanol (400µl) was added, and the mixture applied to a QiAmp spin column (Qiagen, UK) for centrifugation at 6000g for 1 minute. The column was washed in buffers AW1 and AW2 and DNA eluted into 50µl buffer AE following 5-minutes incubation at room temperature.

Blood (3ml) was collected from each patient into a silicone-coated tube that permitted clotting. Each sample was centrifuged at 800g for 10 minutes to pellet cellular components of the blood, leaving a serum supernatant. One microlitre aliquots of the serum were stored frozen in cryovials (Nunc, DK) at -80°C.

Approximately 30 ml of blood from each adult patient were collected into EDTA-coated tubes (Becton Dickinson, UK). Blood was transferred into two 50ml Falcon tubes (Becton Dickinson) and diluted 1 in 2 with sterile Dulbecco's PBS (sPBS) (Sigma-Aldrich, UK). Diluted blood was layered onto an equal volume of Histopaque (Sigma-Aldrich, UK) and centrifuged at 800g for 30 minutes without break. The PBMCs (located at the interface of Histopaque and diluted plasma) were removed with a Pasteur pipette (alpha Laboratories, UK) and transferred to a 50ml Falcon tube to which sterile phosphate-buffered saline (sPBS) was added. Cells were pelleted (by centrifugation at 500g for 5 minutes) and repeatedly washed in sPBS until the supernatant was clear. An aliquot of cell suspension was mixed with an equal volume of Trypan Blue (Sigma-Aldrich, UK) to assess cell viability and the number of viable cells (excluding Trypan Blue) were counted in a haemocytometer (by light microscopy under x 20 objective).

Approximately 5 ml of blood from each paediatric patient were collected into EDTA-coated tubes (Becton Dickinson, UK) and the sample centrifuged at 800g for 10 minutes to pellet

cellular components of the blood. The plasma supernatant was stored at -80°C in cryovials. The remaining pellet was re-suspended to 7 ml in sPBS and diluted blood was layered onto an equal volume of Histopaque (Sigma-Aldrich, UK) and centrifuged at 800g for 30 minutes without break. The PBMCs were then processed as described above.

2.28 Freezing and thawing PBMCs

Suspensions of washed PBMCs were pelleted at 500g for 5 minutes, re-suspended in ice-cold freezing mix (90% hi FCS and 10% dimethyl sulphoxide (DMSO) (Sigma- Aldrich, UK)) and decanted into cryotubes at 1×10^7 cells per tube. Cryotubes were placed in a cryofreezing container (Jencons) lined with isopropanol (Sigma-Aldrich) and kept at -80°C overnight in order for cells to freeze slowly at a rate of approximately 1°C per minute. Cryotubes were subsequently placed in liquid nitrogen for long-term storage.

Frozen PBMCs were rapidly thawed at 37°C in a water-bath and decanted into a 15ml Falcon centrifuge tube (Invitrogen) containing 10ml ice-cold sPBS (Sigma, UK) + 0.5% hiFBS (Gibco, UK). Cells were pelleted by centrifugation at 500g for 5 minutes and re-suspended in 5ml sPBS+0.5% hiFBS before viability was assessed by Trypan blue exclusion and the number of viable cells counted.

2.29 Chlamydial load quantification in patient samples by qPCR assay

2.29.1 Selecting a suitable *C. trachomatis* gene target

Chlamydia trachomatis was cultured and purified and DNA extracted from an aliquot of bacterial suspension. The DNA was serially diluted 10-fold, and 3 dilutions (1 in 100, 1 in 1000 and 1 in 10,000) were amplified by qPCR with primers targeting the *C. trachomatis*

plasmid open reading frame 5 (*orf5*) (Chen *et al.*, 2008) and *orf 2* (Pickett *et al.*, 2005) (Table 2.1). Briefly, each dilution of DNA extracted from *C. trachomatis* organisms (2µl) was applied to triplicate lightcycler capillaries containing SYBR green (10µl) (Qiagen, UK), nuclease-free water (6µl) (Qiagen, UK), one forward primer (1µl) and the relevant reverse primer (1µl). Samples were amplified with a LightCycler 2.0 instrument (Roche) and the crossing-points determined in order to discern the gene target that gave the earliest crossing-point for a given dilution of *C. trachomatis* DNA. The following cycling conditions were used for amplification: 1 hot-start cycle of 95°C for 15 minutes and 50 cycles of amplification (denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 15 seconds followed by a single acquisition).

2.29.2 Generation of *C. trachomatis* plasmid standard curve

The plasmid DNA of *C. trachomatis* serovar L1 (Hatt *et al.*, 1988) was provided by Professor Ian Clarke, University of Southampton. The concentration of the plasmid (µg/µl) was quantified using an Ultraspec 2000 UV/visible Spectrophotometer (Pharmacia Biotech) and the number of plasmid copies per microlitre determined by dividing the plasmid concentration (µg/µl) by the weight of 1 plasmid (µg). The weight of one plasmid was determined by dividing the plasmid molecular weight calculated in Daltons (g/mole) (gained from a knowledge of the DNA sequence and the molecular weight of each nucleotide) by Avogadro's constant (molecules/mole). The DNA (of a known plasmid copy number) was 10-fold serially diluted in water and 2µl of each dilution was applied to lightcycler capillaries (Roche) containing SYBR green (10µl) (Qiagen, UK), nuclease-free water (6µl) (Qiagen, UK), *orf5* forward primer (1µl of 10µM) and *orf5* reverse primer (1µl of 10µM). Samples were amplified with a LightCycler 2.0 instrument (Roche), using the same cycling conditions in Section 2.29.1, and the crossing-points determined to generate a standard curve.

2.29.3 End point serial dilutions

The *C. trachomatis* plasmid DNA was diluted to 10,000, 1,000, 200, 100, 50 and 10 copies per reaction and each dilution amplified in quadruplicate as described (Section 2.29.2). The sensitivity of the assay was defined as the lowest concentration of plasmid that was amplified in every instant. As there are a mean number of 7.72 plasmids per *C. trachomatis* organism +/- 0.68 (Michel *et al* 2007), the sensitivity could be expressed in terms of *C. trachomatis* organisms per reaction.

2.29.4 Intra-assay variation

Three dilutions of DNA extracted from purified *C. trachomatis* EBs were amplified as described (Section 2.29.2), 6 times per dilution. The mean crossing point and standard deviation was determined for each dilution and the coefficient of variation (the standard deviation as a percentage of the mean) calculated.

2.29.5 Inter-assay variation

Three dilutions of DNA extracted from purified *C. trachomatis* EBs were amplified as described (Section 2.29.2). Each dilution was amplified in duplicate on four separate occasions. The mean crossing point and standard deviation was determined for each dilution and the coefficient of variation calculated.

2.29.6 Orf 5 qPCR with patient samples

DNA was extracted from patient samples as described (Section 2.1) and 2µl of DNA from each sample were applied to duplicate lightcycler capillaries (Roche) containing SYBR green (10µl) (Qiagen, UK) , nuclease-free water (6µl) (Qiagen, UK), *orf5* forward primer (1µl of

10 μ M) and *orf5* reverse primer (1 μ l of 10 μ M) (Table 2.1). Samples were amplified using a LightCycler 2.0 instrument (Roche) (Section 2.29.2) and the crossing points determined. A standard curve (Section 2.29.2) was simultaneously produced to quantify the number of *orf5* copies per sample which, in turn, could be used to deduce the number of *C. trachomatis* organisms per ml of urine or per swab.

2.29.7 Inhibition Assay

The existence of PCR inhibitors in patient samples was investigated by use of a lambda (λ)-phage inhibition assay. The assay involved amplifying DNA from the Lambda phage in a qPCR reaction and spiking the reaction with patient samples. The crossing-point of the Lambda DNA PCR product in the presence of a clinical sample was compared to the crossing point in the presence of AE buffer (Qiagen, UK). Briefly, 2 μ l of a patient sample was applied to duplicate lightcycler capillaries (Roche) containing a master mix of SYBR green (Qiagen, UK) (10 μ l), Lambda phage DNA (1 μ l), Lambda phage forward primer (1 μ l), Lambda phage reverse primer (1 μ l) (Table 2.1) and nuclease –free water (5 μ l). Additionally, 2 μ l of AE buffer (Qiagen, UK) were added to 4 lightcycler capillaries containing the same master mix. Samples were amplified with a LightCycler 2.0 instrument (Roche) and the crossing points determined. The following cycling conditions achieved amplification: 1 hot-start cycle of 95 $^{\circ}$ C for 15 minutes and 50 cycles of amplification (denaturation at 95 $^{\circ}$ C for 15 seconds, annealing at 55 $^{\circ}$ C for 30 seconds and extension at 72 $^{\circ}$ C for 15 seconds followed by a single acquisition).

2.30 Pgp3 antigen

Proteins encoded by the *C. trachomatis* genome that did not have a significant BLAST hit (e value <0.001) with the *C. pneumoniae* genome were identified by using the STDgen database *Chlamydia* proteome comparisons section (<http://stdgen.northwestern.edu/>). The *C. trachomatis*-specific protein Pgp3 (Pubmed Accession P10557) was selected.

Fifty one overlapping peptides, spanning the entire length of the Pgp3 protein (Table 5.1) were purchased (GenScript, USA), to ensure the observed IFN- γ responses were not restricted to, or biased by, certain HLA types. The identify and purity of each peptide was confirmed by mass-spectrometry and high performance liquid chromatography (HPLC). Each peptide was 15 residues in length (termed a “15mer”), with the exception of the last peptide (that was 14 residues in length), and the sequence of each peptide overlapped the previous by 10 residues, with the exception of the first peptide. Peptides were suspended in DMSO and pooled in 4 groups of 10 peptides and one group of 11 peptides at a working stock concentration of 20 μ g/ml in RPMI and frozen at -80°C.

2.31 Detection of anti-Pgp3 antibodies in patient serum by Enzyme-Linked

Immunosorbent Assay (ELISA)

An in-house ELISA was developed and optimised by Dr. Gillian Wills (Wills *et al.*, 2009).

Briefly, wells of an Immunosorb 96-well plate (Nunc, DK) were coated with 100µl of the Pgp3 protein (20ng/ml) in 100mM NaHCO₃ (Sigma-Aldrich, UK), pH 8.4 for 1 hour at 37°C in an atmosphere of 5% CO₂. Free antigen was discarded and the plate washed 3 times in a solution of 0.05% Tween 20 (Sigma-Aldrich, UK) in PBS (PBST) and blocked at 37°C, 5% CO₂ for 2 hours in a solution of 1% Hammerstein casein in PBST (blocking buffer).

Following 3 additional washes as described, 50µl patient sera (diluted 1:100 in blocking buffer) were added to each well in duplicate and the plate incubated for 1 hour at 37°C, 5% CO₂. In addition to the test sera, serum samples from 7 individuals (2 testing highly-positive, 2 medium positive and 3 low positive by the ELISA) were added in duplicate to each plate as inter-assay variability controls and duplicate wells contained no human serum (i.e. blocking buffer alone) as negative controls.

Antigen and bound antibody were washed 3 times before 100µl of a goat anti-human antibody (Fc fragment) conjugated to horseradish peroxidase (HRP) (Sigma-Aldrich, UK) were added at a 1:8000 dilution in blocking buffer before incubating at 37°C, 5% CO₂ for 1 hour. Following 6 additional washes, 100µl of complete Tetramethylbenzidine (TMB) solution were added to each well and the plate incubated for 10 minutes at room temperature while the HRP metabolised the TMB to a blue product. The reaction was terminated by the addition of 50µl 2.5M H₂SO₄, and the reaction was read in a Biotrack II plate reader (Amersham Biosciences) at a wavelength of 450nm.

2.32 Detection of Effector T-cell responses to Pgp3 by an *ex vivo* IFN- γ Enzyme-Linked Immunoabsorbant Spot-forming (ELISpot) Assay.

A Pgp3 –peptide- specific *ex vivo* IFN- γ ELISpot assay was developed to assay T-cell responses to *C. trachomatis* Pgp3. Two hundred microlitres of RPMI + 10% hiFBS + 1% penicillin/streptomycin were added to wells of a 96-well polyvinylidene difluoride-backed ELISpot plate that was supplied pre-coated with anti-IFN- γ monoclonal antibody 1-D1K (Mabtech, SE). The plate was blocked for 90-120 minutes at 37°C in an atmosphere of 5% CO₂ after which 100 μ l of each peptide pool (at a concentration of 20 μ g/ml in RPMI) were added to duplicate wells in addition to negative (unstimulated) controls comprised of complete RPMI only and positive controls consisting of 5 μ g/ml phytohaemagglutinin (Pha) (ICN Biomedicals) in RPMI.

For each patient sample, an aliquot of frozen PBMCs was thawed rapidly, washed and the number of viable cells counted. Cells were pelleted and re-suspended in RPMI + 20% hiFBS + 2% penicillin/streptomycin at a density of 2.5x10⁶ PMBCs/ml. One hundred microlitres of cell suspension (2.5 x 10⁵ PBMCs) were added to each well of the ELISpot plate that contained peptide pools or controls and the plate was incubated overnight (16-18 hours) at 37°C in an atmosphere of 5% CO₂.

Following the overnight incubation, cells were washed 6 x in PBS and 100 μ l of the biotinylated anti- IFN- γ monoclonal antibody 7-B6-1 biotin (Mabtech, SE) were added at a concentration of 1 μ g/ml in PBS + 0.5% hiFBS. The plate was incubated for 2 hours at 37°C in an atmosphere of 5% CO₂ and washed 6 x in PBS prior to the addition of streptavidin-alkaline phosphatase conjugate (Mabtech, SE) at a dilution of 1:1000 in PBS + 0.5% hiFBS.

The plate was incubated in the dark at room temperature for 45 minutes and washed 6 times with PBS, after which 100µl of the alkaline phosphatase substrate 5-Br-4-chloro-3-indolyl-phosphate-nitro blue tetrazolium chloride (BCIP/NBT (PLUS) (Europa, UK) were added to each well and the plate incubated at room temperature for 5-10 minutes after which the reaction was terminated by rinsing in tap water and air-drying.

Where a cell had produced IFN-γ during the overnight incubation, a spot was visible on the membrane lining the relevant well. Spots were visualised using an ELISpot plate reader (AID, USA), in conjunction with AID software (Version 3.0) and the number in each well counted. The average number of spots per peptide pool was determined for each patient and peptide pools scored as either positive or negative. A pool was considered to be positive if the average number of spots was five more than the average number of spots in the negative, unstimulated, control wells (the background) and, additionally, this number was double the average number of spots in negative control wells.

The number of spots above the background was summated for each pool deemed to be positive for a given patient. This figure represented the number of spot-forming cells (SFCs) per 2.5×10^5 PBMCs that responded to the pgp3 antigen by producing IFN-γ. The number of SFCs per million PBMCs that responded to pgp3 was determined for each patient.

2.33 Comparison of spot enumeration using pre-coated and non-pre-coated ELISpot plates

The *Ex Vivo* IFN-γ ELISpot assay was conducted in a 96-well polyvinylidene difluoride-backed ELISpot plate. Two types of plate were used during the course of the study; one that was supplied pre-coated with the anti- IFN-γ monoclonal antibody 1-D1K (Mabtech, SE) and

one that was not (Millipore, UK). In the case of the latter, plates were coated overnight at 4 °C with 10µg/ml anti- IFN-γ monoclonal antibody 1-D1K (Mabtech, SE) in sterile PBS (100µl per well) and washed 5 x in sterile PBS. Peripheral blood mononuclear cells were thawed from 3 patients known to be Pgp3-ELISpot positive and the number of SFCs per million PBMCs determined (Section 2.36) using either plates that were supplied pre-coated or that were manually coated with anti- IFN-γ monoclonal antibody 1-D1K (Mabtech, SE).

2.34 Identification of *Chlamydia pneumoniae* antibodies in patient serum

The presence of anti- *C. pneumoniae* IgG antibodies in patient sera was tested using a *Chlamydia pneumoniae* IgG/IgM Micro-IF Test kit (Ani Labsystems). Briefly, the kit contained glass slides, each containing a series of wells. Each well was dotted with three chlamydial antigens: *C. pneumoniae*, *C. trachomatis* and *C. psittaci*. As anti-LPS antibodies cross-react between serovars, *C. pneumoniae* and *C. trachomatis* antigens were depleted of LPS. Undepleted *C. psittaci* served as a control to test for the presence of any anti-LPS antibodies. Serum from each patient was diluted 1:32 with sample diluent and 10µl applied to each well on the slide. Positive and negative controls were supplied with the kit. Wells were incubated at 37 °C, 5% CO₂ in a moist chamber for 30 minutes after which wells were washed 4 times in sterile PBS (Sigma-Aldrich), 2 times in Milli-Q_{PLUS} Ultra pure water (Millipore) and air-dried. A FITC-conjugated secondary anti-human antibody (supplied) was applied to each well and the slide incubated at 37 °C, 5% CO₂ in a moist chamber for 30 minutes before washing and air-drying. Cover-slips were mounted onto the slides and antigen dots viewed under the x100 objective of a Nikon Eclipse TE2000-S fluorescence microscope (under oil emersion) at excitation/emission wavelengths of 470/505 (FITC, green). Sera were considered positive for *C. pneumoniae* if the *C. pneumoniae* antigen dot appeared bright green, and negative if the dot appeared a dull, faint green.

Chapter 3

Co-infection of *C. trachomatis* and HIV-1 *in vitro*

3.1 Introduction

Background

Recent studies of MSM have found that 74-76% of individuals with LGV are co-infected with HIV-1 (Mayaud, 2006; Jebbari *et al.*, 2007; Ward *et al.*, 2007). This high degree of co-infection is puzzling and may be the result of biological synergy between *C. trachomatis* and HIV-1, high-risk behaviour within sexual networks of HIV-positive MSM, a clinical selection bias, or a combination of factors (Ward *et al.*, 2009a; Ward *et al.*, 2009b; Annan *et al.*, 2009).

As both pathogens are capable of traversing the epithelial barrier (Dessus-Babus *et al.*, 2008; Richardson & Goldmeier 2007; Fantini *et al.*, 1993; Plummer *et al.*, 1991) and infecting reticuloendothelial cells (Matyszak *et al.*, 2002; Numazaki *et al.*, Bianchi *et al.*, 1997; Bianchi *et al.*, 1998), there is the potential for HIV-1 and *C. trachomatis* serovar L2 to impact upon the replication of one another. Yet, there have only been a limited number of *in vitro* models of HIV-1 and *C. trachomatis* co-infection conducted (Ho *et al.*, 1995; Bianchi *et al.*, 1998). These have been designed to investigate the effect of *C. trachomatis* co-infection on HIV-1 replication, however, Bianchi *et al.*, 1998 suggest that HIV-1 accelerates the replication of *C. trachomatis in vitro*. This study was based solely on the rate of cell-lysis and, consequently, *C. trachomatis* replication in HIV-1 infected cells remains uncharacterised.

Viral co-infection with HSV-2 can lead to *C. trachomatis* persistence, characterised by the presence of viable, but non-cultivable, organisms that are morphologically abnormal (Deka *et*

al., 2006), however, it remains unknown whether HIV-1 also causes a *C. trachomatis* persistent phenotype in co-infected cells.

3.1.2 Hypothesis

HIV-1 co-infection induces *C. trachomatis* persistence *in vitro*.

3.1.3 Aims of the Project

This project aimed to establish assays that characterised *C. trachomatis* growth *in vitro* by means of several experimental parameters, including chlamydial inclusion size, inclusion number, EB and RB morphology, EB infectivity, genome copy number and the transcription of unprocessed *16S rRNA*, *ompA*, *omcB*, and *euo*. A cell-culture model of *C. trachomatis* and HIV-1 co-infection was developed and used to assess whether HIV-1 induced *C. trachomatis* persistence *in vitro*.

3.2 Results

3.2.1 Quantification of *C. trachomatis* by immunofluorescence microscopy (IFM)

The compound cycloheximide inhibits eukaryotic DNA and protein synthesis and enhances the growth of *C. trachomatis* serovars A- I, L1, L2 and L3 in cell-culture (Ripa & Mårdh 1977). However, as HIV-1 replication is dependent on the action of eukaryotic transcriptional and translational machinery (reviewed by Freed *et al.*, 2006), cycloheximide use is precluded in studies of HIV-1 and *C. trachomatis* co-infection.

In order to demonstrate that *C. trachomatis* serovar L2 could grow in the absence of the compound, monolayers of BGMK cells were infected with 10-fold serial dilutions of *C. trachomatis* in triplicate, in the presence or absence of cycloheximide (1 µg/ml) (Figure 3.1). Cells were incubated for 30 hours before they were subject to immunofluorescence microscopy (IFM). The number of inclusions was counted for each dilution in the presence and absence of cycloheximide and no significant difference found, demonstrating that *C. trachomatis* could grow in the absence of the compound.

Figure 3.1 *C. trachomatis* serovar L2 can grow in the absence of cycloheximide

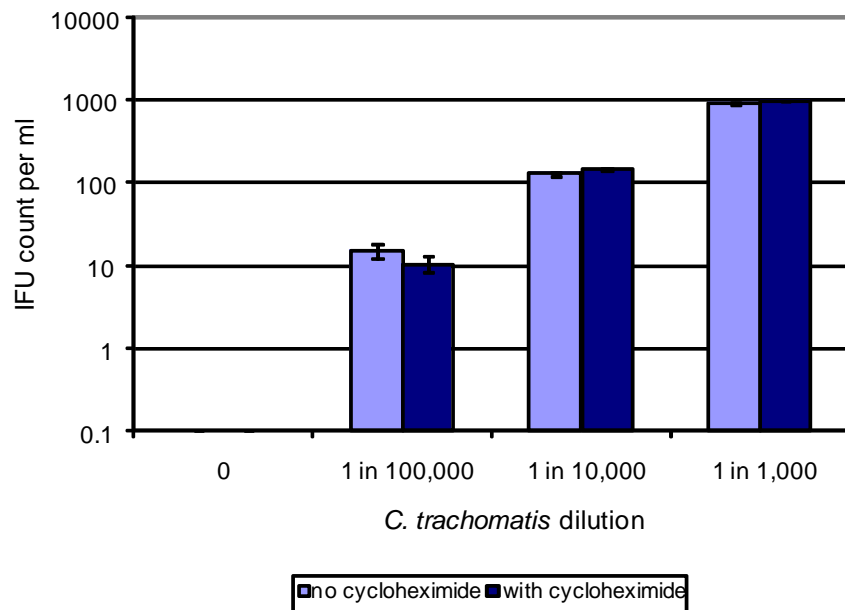


Figure 3.1 *C. trachomatis* was serially diluted 10-fold and monolayers of BGMK cells infected with each dilution in triplicate. Cells were infected in the presence and absence of cycloheximide and subject to IFM 30 hours post-infection. The number of inclusions was determined for each dilution of *C. trachomatis* and the mean plotted graphically (+/- standard error of the mean (SEM)).

3.2.2 Quantification of *C. trachomatis* replication by qPCR

The replication of *C. trachomatis* was quantified by determining the chlamydial genome copy number in infected cell-lysates at various time-points post-infection. The genome copy number was enumerated by using a quantitative polymerase chain reaction (qPCR) that targeted a 189 base-pair (bp) section of the *pmpH* gene (*pmpH*-qPCR). This gene encodes the polymorphic membrane protein H (PmpH) that is present in the inclusion membrane (Mygind *et al.*, 2000) of all serovars of *C. trachomatis*, but is absent from *C. pneumoniae*, making the gene a useful target for *C. trachomatis* diagnosis (Morré *et al.*, 2005). As the gene is encoded by the chromosome, rather than the plasmid, it is present in only one copy per *C. trachomatis* organism. By determining the number of *pmpH* copies in a sample it was, therefore, possible to determine the number of organisms in that sample.

In order to quantify the number of *pmpH* gene copies in a sample, a standard curve was generated, using a recombinant plasmid containing the target region of *pmpH*, termed pCR[®]4-TOPO[®]*pmpH* (constructed as described in Chapter 2, Section 2.16)..

The concentration of the pCR[®]4-TOPO[®]*pmpH* construct (copy number/ml) was determined and the plasmid was serially diluted 10-fold. Aliquots were added to a reaction mixture containing SYBR Green I dye, the *pmpH* forward primer and reverse primer and water, and standards were processed in a LightCycler 1.5 instrument. The SYBR Green I dye fluoresced when bound to double- stranded DNA. As the number of PCR amplification cycles increased, the number of amplification products increased and, hence, the fluorescence signal increased. When reagents became limiting, amplification decreased and the fluorescent signal reached a plateau, giving rise to a sigmoidal amplification plot (Figure 3.3 A). As the plasmid was serially diluted, it took an increasing number of amplification cycles for a given fluorescence

to be reached, so the sigmoidal curve shifted progressively to the right. The point at which fluorescence crossed a pre-determined threshold (the crossing point) was determined and plotted against plasmid number to produce a standard curve showing a linear relationship between crossing point and cycle number, with a gradient of -3.33 (Figure 3.3 B).

To confirm the specificity of the amplified products, a melting curve (Figure 3.3 C) and melting peak (Figure 3.3 D) were constructed. Briefly, the temperature of the post-amplification reaction-mix was gradually increased. At a temperature of approximately 83 °C, the double strands of the DNA product separated. As SYBR Green I only binds double-stranded DNA, the fluorescence dropped sharply (Figure 3.3 C), confirming the products to be of the same constituency. The negative control (water) did not give rise to a sigmoidal amplification plot, a melting curve or a melting peak.

The analytical sensitivity of the assay was determined by diluting the pCR[®]4-TOPO[®] *pmpH* plasmid to concentrations of 10,000, 1,000, 100, 70, 50, 30 and 10 copies per 5µl (the volume added to each lightcycler capillary) and amplifying the target DNA in quadruplicate. The lowest concentration of plasmid that amplified in all 4 replicates was 50 copies per reaction (Table 3.1) and, as there is one *pmpH* gene copy per *C. trachomatis* organism, the sensitivity of the assay was 50 organisms per reaction. This is consistent with the sensitivities of previously published qPCR assays that target the *pmpH* gene (Chen *et al.*, 2008) and was adequate for subsequent *in vitro* experiments.

Assay reproducibility was determined by amplifying 3 dilutions of DNA extracted from an aliquot of purified *C trachomatis* EBs between 5 and 9 times in one experiment to determine intra-assay variation, and in 4 separate experiments to determine inter-assay variation. The

mean crossing point, standard deviation and coefficient of variation per dilution were determined. Intra-assay variability and inter-assay variability were low, with co-efficient of variations ranging from 1.93-3.75% for intra-assay variation (Table 3.2) and 1.21-2.24% for inter-assay variation (Table 3.3).

The replication of *C. trachomatis* in BGMK cells was determined by qPCR. Briefly, BGMK monolayers were infected with *C. trachomatis* at an MOI of 0.1 in duplicate. At various times (0, 6, 16, 24, 30, 40, 48 and 64 hours) post-infection, DNA was extracted from the cells and amplified by qPCR. The number of *pmpH* copies in each sample was determined from the standard curve, and the mean number of organisms present at each time-point plotted graphically (+/- standard error of the mean (SEM)) in order to characterise the growth of *C. trachomatis* over time (Figure 3.4). Organisms divided in a logarithmic fashion between 0 and 40 hours post-infection, reflecting one replication cycle, after which cell-lysis and a second round of replication likely occurred.

Figure 3.3 Standard Curve for the quantification of *C. trachomatis* by qPCR

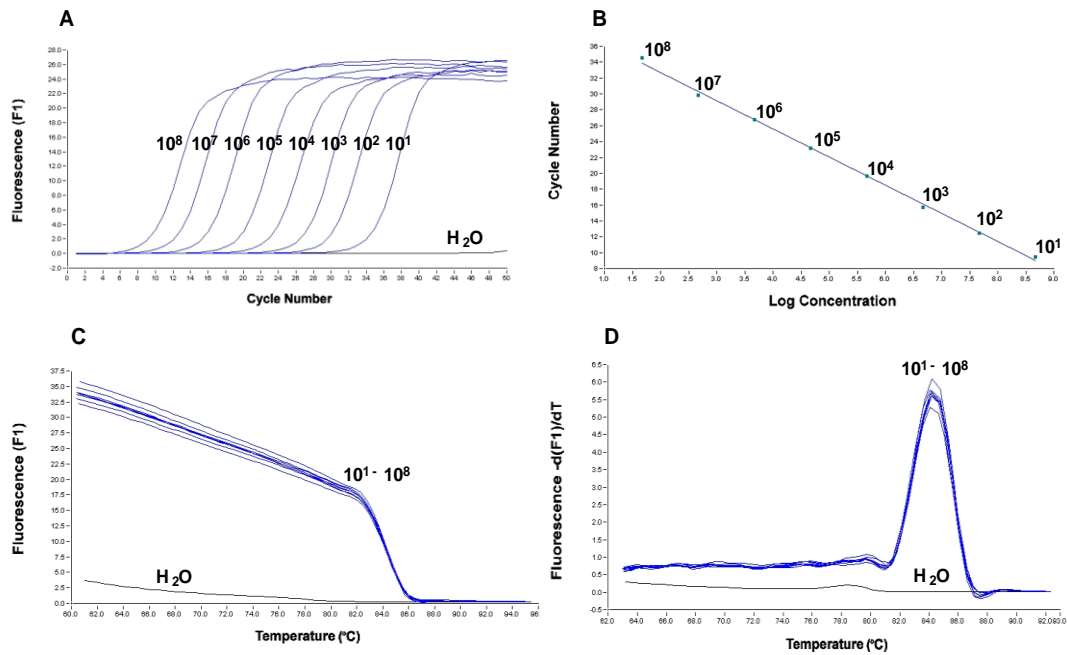


Figure 3.3 The pCR[®]4-TOPO[®] *pmpH* recombinant plasmid was quantified and serially diluted 10-fold in nuclease-free water. For each dilution (10^1 - 10^8 copies per reaction), the amplification plot of fluorescence over cycle number (A) showed a typical sigmoidal curve, from which the crossing point was determined. A standard curve with a gradient of -3.33 was generated of crossing point against DNA copy number (B). The melting curve (C) and melting peak (D) showed a single peak for plasmid dilutions 10^1 - 10^8 copies per reaction, whereas the negative control (H_2O) did not give rise to a sigmoidal amplification plot, a melting curve or melting peak.

Table 3.1 End-point serial dilutions of pCR[®]4-TOPO[®]*pmpH*

Number of <i>pmpH</i> copies per reaction	Repeats			
	1	2	3	4
10,000	+	+	+	+
1,000	+	+	+	+
100	+	+	+	+
70	+	+	+	+
50	+	+	+	+
30	+	+	-	-
10	+	-	-	-

Table 3.1 The pCR[®]4-TOPO[®]*pmpH* recombinant plasmid was diluted to 10,000, 1,000, 100, 70, 50, 30 and 10 copies per 5µl reaction and each dilution amplified in quadruplicate. The lowest concentration of plasmid that was amplified in each replicate reflected the sensitivity of the assay (50 copies per reaction). As there is 1 *pmpH* gene per *C trachomatis* organism, the sensitivity of the assay is 50 organisms per reaction.

Table 3.2 *pmpH*-qPCR intra-assay variation

Sample	Intra-assay variation		
	Mean crossing point	SD	Coefficient of variation (%)
1	12.24	0.46	3.75
2	16.39	0.50	3.06
3	23.32	0.45	1.93

Table 3.2 Three dilutions of DNA extracted from purified *C. trachomatis* EBs (samples 1, 2 and 3) were amplified with the *pmpH* qPCR. Samples 1 and 2 were amplified 9 times each, and sample 3 was amplified 5 times (due to an insufficient volume of DNA for 9 repeats). The mean crossing point, standard deviation and coefficient of variation for each sample were determined. The intra-assay variability was low, with the highest co-efficient of variation being 3.75%.

Table 3.3 *pmpH*-qPCR inter-assay variation

Sample	Inter-assay variation		
	Mean crossing point	SD	Coefficient of variation (%)
1	20.32	0.45	2.24
2	24.39	0.41	1.69
3	28.32	0.34	1.21

Table 3.3 Three dilutions of DNA extracted from purified *C. trachomatis* EBs (samples 1, 2 and 3) were amplified in duplicate with the *pmpH* qPCR in 4 independent experiments. The mean crossing point, standard deviation and coefficient of variation for each sample were determined. The inter-assay variability was low, with the highest coefficient of variation being 2.24%.

Figure 3.4 Replication kinetics of *C. trachomatis* in BGMK cells

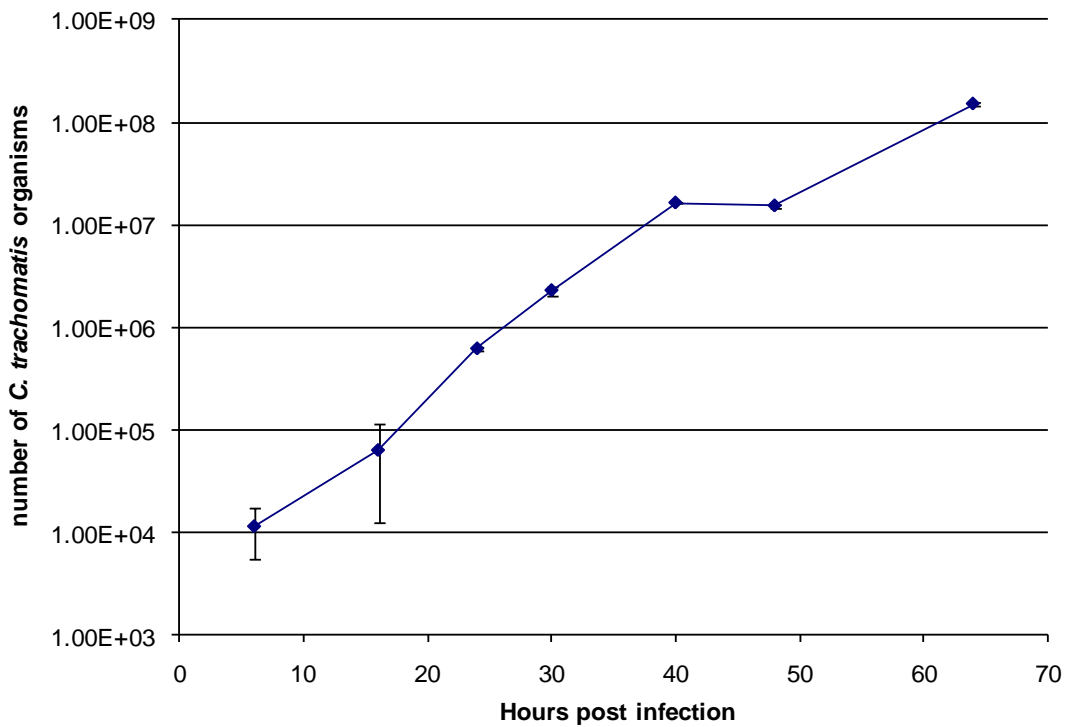


Figure 3.4 At 0, 6, 16, 24, 30, 40, 48 and 64 hours post-*C. trachomatis* infection, DNA was extracted from infected BGMK cells and the number of organisms in each sample determined by qPCR in duplicate. A graph of the mean number of *C. trachomatis* organisms over time (+/- SEM) was plotted to determine the replication dynamics of *C. trachomatis* in BGMK cells.

3.2.3 Quantification of the infectivity of *C. trachomatis* progeny by sub-culture

Optimisation of sub-culture

The infectivity of *Chlamydia* progeny is routinely quantified by lysing infected cell-cultures, and inoculating cell-lysates onto reporter cell-lines to quantify the number of IFUs that subsequently develop. Methods for lysing cells to release intracellular *C. trachomatis* include multiple freeze-thaw cycles (Suchland *et al.*, 2003), sonication (Rothermel *et al.*, 1983) or a combination of the two (Deka *et al.*, 2006; Deka *et al.* , 2007; Vanover *et al.*, 2008).

The yields of *C. trachomatis* (IFU per ml undiluted cell-lysate) resulting from the different methods of cell-lysis were compared. Briefly, BGMK cells were infected with *C. trachomatis* at an MOI of 1, in duplicate. Forty hours post-infection, cells were subject to either one freeze-thaw cycle, two rounds of sonication, or one freeze-thaw cycle followed by two rounds of sonication. Additional BGMK monolayers were inoculated with the lysates. These were fixed, stained and viewed by IFM 30 hours post-infection in order to determine the number of IFUs per ml cell-lysate for each lysis condition. Sonication yielded a titre that was 100 times higher than other lysis techniques and was the method of choice in subsequent experiments (Figure 3.5, blue bars).

In order to remove viral contamination from HSV-2/*C. trachomatis* co-infected cell-lysates, Deka *et al.*, 2006 pelleted the *C. trachomatis* organisms, discarded the supernatant, and washed and re-suspended the *C. trachomatis* in complete medium prior to passage (purified cell-lysates). Following this protocol, the titre of *C. trachomatis* in purified cell-lysates was found to be the same log-factor as un-purified cell-lysates (Figure 3.5, pink bars).

Figure 3.5 Comparison of *C. trachomatis* yield from different cell-lysis techniques

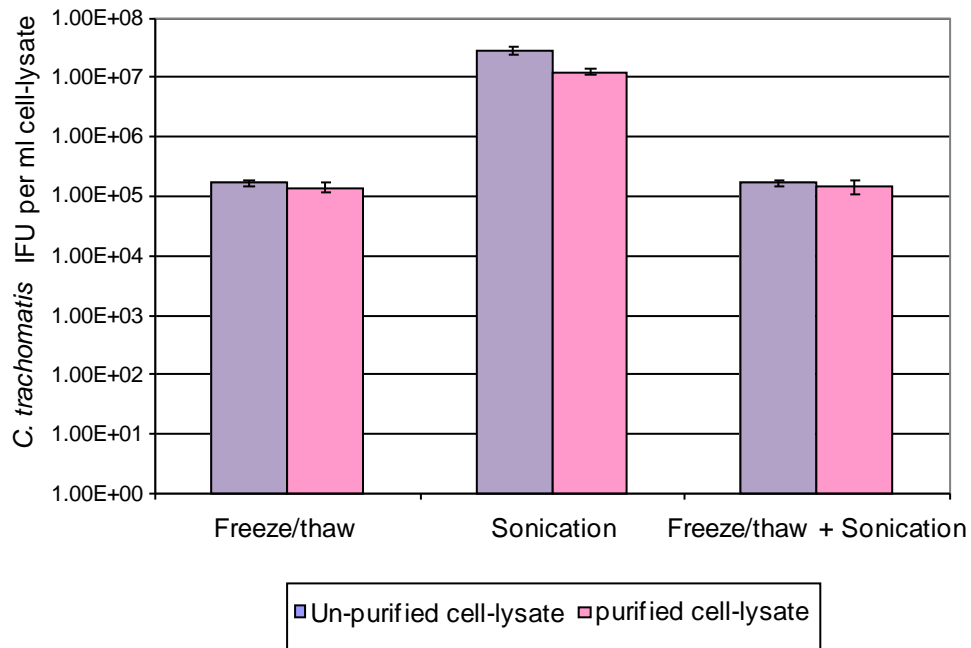


Figure 3.5 BGMK cells were infected in duplicate with *C. trachomatis* at an MOI of 1. Forty hours post-infection, cells were subjected to either freeze/thawing, sonication, or freeze-thawing and sonication. Additional BGMK cell-monolayers were inoculated with the lysates and the number of IFUs/ml cell-lysate for each lysis condition was compared to determine which gave the highest yield. Lysates were either un-purified (blue bars) or purified (pink bars) by pelleting the *C. trachomatis* organisms in the lysate, discarding the supernatant, washing the bacteria and re-suspending them in complete medium prior to BGMK-cell inoculation as per Deka *et al.*, 2006.

The infectivity of *C. trachomatis* progeny over 48 hours

BGMK cells were infected with *C. trachomatis* at an MOI of 0.1 in duplicate. At various times (6, 16, 24, 30, 40 and 48 hours) post-infection, cells were lysed by sonication and additional BGMK monolayers inoculated with 10-fold serially diluted cell-lysates in triplicate. Cells were fixed, stained and viewed by IFM 30 hours post-infection (Figure 3.6 A). The average number of IFUs/ml of undiluted lysate was determined and plotted graphically over time (+/- SEM) (Figure 3.6 B). Between 6 and 24 hours post-infection, no infectious organisms were present in the cell lysates, despite a logarithmic growth curve throughout this period (Figure 3.4). This is consistent with the intracellular presence of non-infectious, replicative RBs. Between 24 hours and 40 hours post-infection, the number of infectious organisms increased, consistent with the differentiation of RBs to EBs. At later time-points, the number of infectious organisms reached a plateau, despite a continued increase in replication of *Chlamydia* genomes, possibly as infected cultures became asynchronous, with some cells lysing to begin a second round of replication while other RBs continuing to differentiate into non-replicative, infectious EBs.

Figure 3.6 The infectivity of *C. trachomatis* progeny over time

Figure 3.6 A

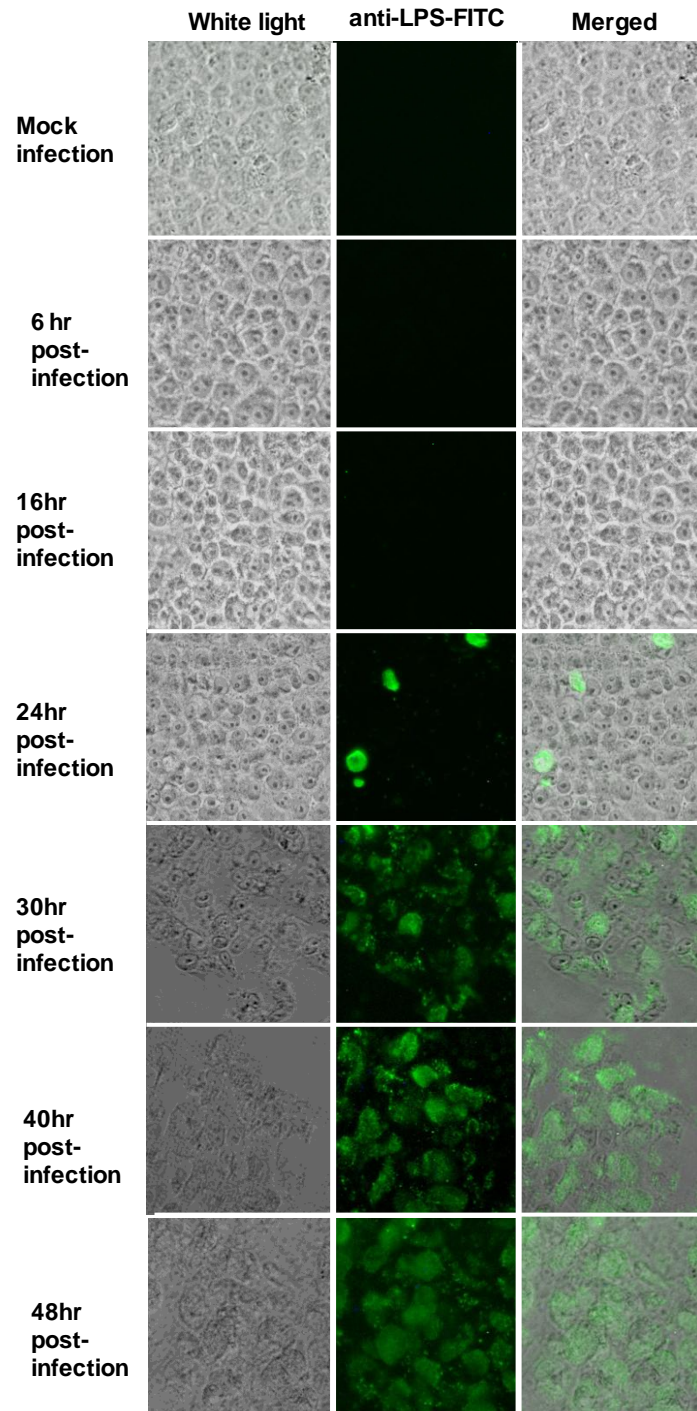


Figure 3.6 B

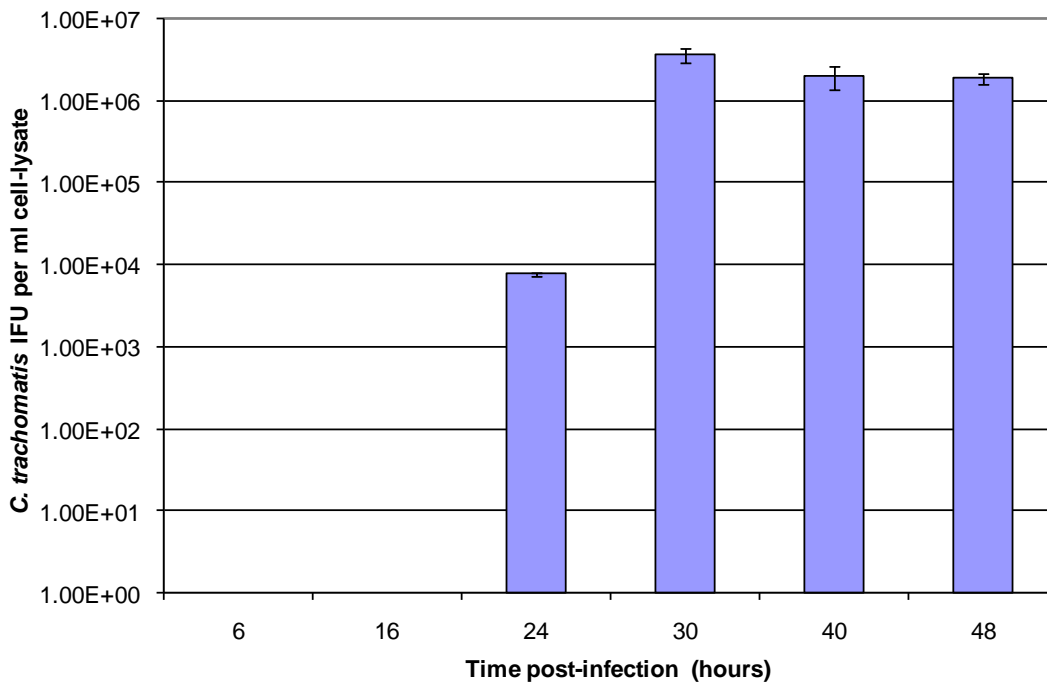


Figure 3.6. BGMK cells were infected with a *C. trachomatis* at an MOI of 0.1. At 6, 16, 24, 30, 40, and 48 hours post-infection, cells were lysed by sonication and cell a second monolayer of BGMK cells inoculated with serial dilutions of cell-lysate in triplicate. Thirty hours post-inoculation, cells were fixed, stained and observed by IFM (Figure 3.6 A). The average number of IFU in the BGMK monolayer was determined for a given dilution and extrapolated to the number of IFU per ml of undiluted cell-lysate. This was plotted graphically for each time-point (Figure 3.6B).

3.2.4 Development of a *C. trachomatis* model of persistence with Penicillin

The assays described above characterised the growth of *C. trachomatis* during a lytic cycle of infection. The growth of *C. trachomatis* during a persistent infection was subsequently assessed. Penicillin is known to induce *C. trachomatis* persistence (Matsumoto & Manire, 1970; Lambden *et al.*, 2006; Ouellette *et al.*, 2006). During penicillin-induced chlamydial persistence, the genes *ompA* and *omcB* (encoding the EB outer membrane proteins, MOMP and OMP2, respectively) are down-regulated and the expression of the gene '*euo*' (whose product silences late-gene expression) is upregulated (Ouellette *et al.*, 2006). In addition, there is no significant reduction in chlamydial viability, as determined by the continued presence of unprocessed *16S rRNA* transcripts (Ouelette *et al.*, 2006), but there is a significant reduction in the number of infectious progeny.

Monolayers of BGMK cells were infected with *C. trachomatis* at an MOI of 2 in the presence or absence of 5µg/ml penicillin (Sigma-Aldrich, UK). Twenty four hours post-infection, cells were lysed and DNA and RNA extracted. Additional BGMK monolayers were also inoculated with the cell-lysates to determine the number of infectious organisms present in the samples.

The RNA was reverse-transcribed to cDNA that was amplified by qPCR using primers against *Chlamydia* unprocessed *16s rRNA*, *ompA*, *omcB* and *euo*, in order to determine the number of cDNA transcripts of each gene in the sample. The DNA extracted from the cells was also amplified by qPCR using the same primers in order to determine the number of DNA copies of each gene present. The number of cDNA transcripts for each gene was divided by the number of DNA copies, in order to quantify gene expression in the sample

(cDNA copies/DNA copies) in the presence and absence of penicillin. This was plotted graphically (Figure 3.7).

In the presence of penicillin, the infectivity of *C. trachomatis* was reduced (Figure 3.7 A), and there was no change in the genome copy number (Figure 3.7 B). In addition, although the expression of unprocessed *16S ribosomal RNA (rRNA)* was slightly reduced in the presence of penicillin, viable organisms continued to remain in the culture (Figure 3.7 C).

The expression of *euo* was upregulated (Figure 3.7 D), whereas *ompA* and *omcB* were down-regulated (Figure 3.7 E and F). These observations were consistent with penicillin inducing a persistent phenotype in the *C. trachomatis* organisms and demonstrated that the assays developed for characterising *Chlamydia* growth *in vitro* could detect *C. trachomatis* persistence.

Figure 3.7 The effect of Penicillin on chlamydial infectivity, genome copy number, and the transcription of *16srRNA*, *euo*, *ompA* and *omcB*

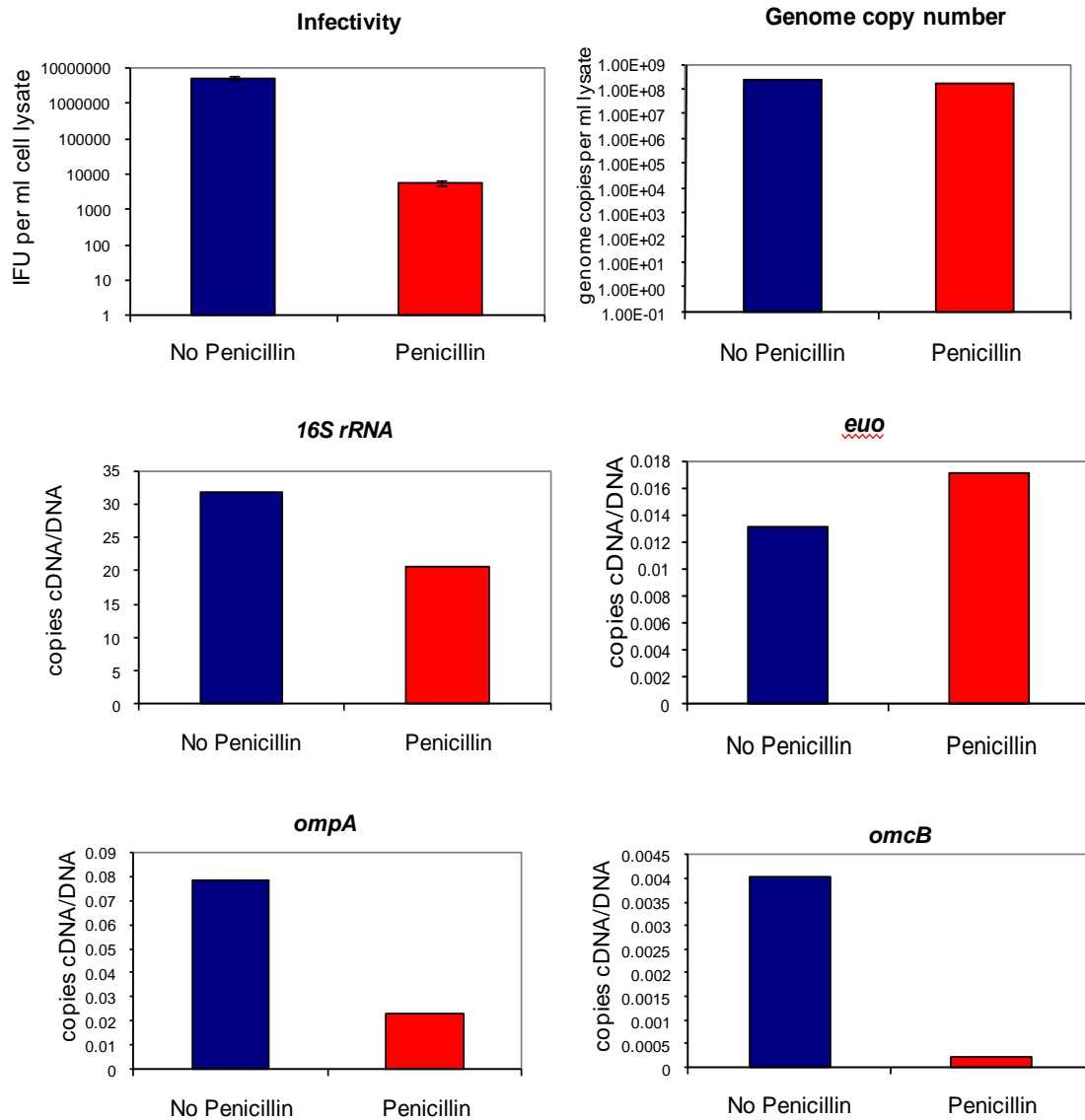


Figure 3.7 Monolayers of BGMK cells were infected with *C. trachomatis* at an MOI of 2 in the presence or absence of penicillin. Cells were either lysed and titrated onto additional BGMK cells to quantify the number of infectious organisms present in the samples (A) or the DNA and RNA were extracted in order to determine the *C. trachomatis* genomic copy number (B), and the expression of unprocessed *16SrRNA* (C), *euo* (D), *ompA* (E), and *omcB* (F) in the presence (red bars) and absence (blue bars) of penicillin.

3.2.5 Identification of a cell-line to investigate *C. trachomatis* and HIV-1 co-infection

Cell-lines expressing CD4 that are known to be permissive to HIV-1 infection were investigated for their ability to support the replication of *C. trachomatis*. The BGMK epithelial cell-line (Hobson *et al.*, 1982) was chosen as the standard against which the other cell-lines were compared, as it is known to be permissive for *C. trachomatis* infection. The following CD4-positive cell-lines were investigated: MAGI P4R5 (epithelial cell-line) (Kimpton & Emerman 1992), C8166 (T-cell-line) (Sodroski *et al.*, 1986), Jurkat E6.1 (T-cell-line) (Alkhatib *et al.*, 1996), U937 (monocytic cell-line) (Sundström & Nilsson 1976) and THP-1 (monocytic cell-line) (Tsuchiya *et al.*, 1980) (Table 2.2).

Cells were infected in duplicate with *C. trachomatis* at an MOI of 10 for 30 hours. Cells were fixed, permeabilised, stained with anti-LPS-FITC antibody solution and viewed by IFM with a x 20 objective lens. At least 5 fields of view were imaged under white light (phase contrast) and at excitation/emission wavelengths of 470/505nm (Figure 3.8 A). The number of cells (visible under white light) and the number of inclusions (that fluoresced green at excitation/emission wavelengths of 470/505nm) were counted and the average percentage of infected cells per field of view determined for each cell-line and plotted graphically +/- SEM (Figure 3.8 B).

The MAGI P4R5 cell-line was as permissive to *C. trachomatis* infection as BGMK cells, and was used in subsequent co-infection experiments.

Figure 3.8 Identification of a cell-line to investigate *C. trachomatis* and HIV-1 co-infection

Figure 3.8 A – IFM Images of *C. trachomatis*-infected CD4+ cells

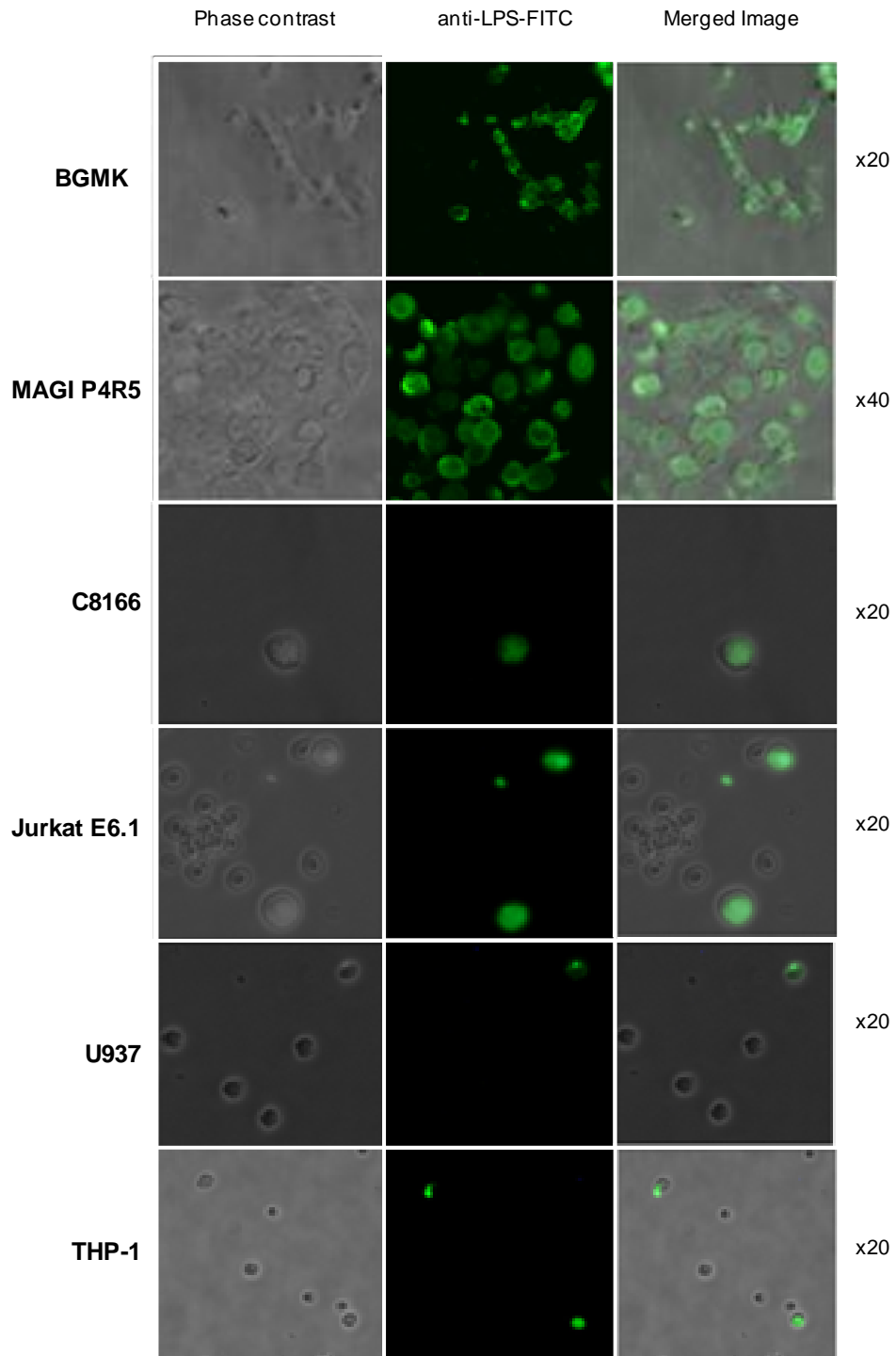


Figure 3.8 B – Quantification of *C. trachomatis* infection of CD4+ cell-lines

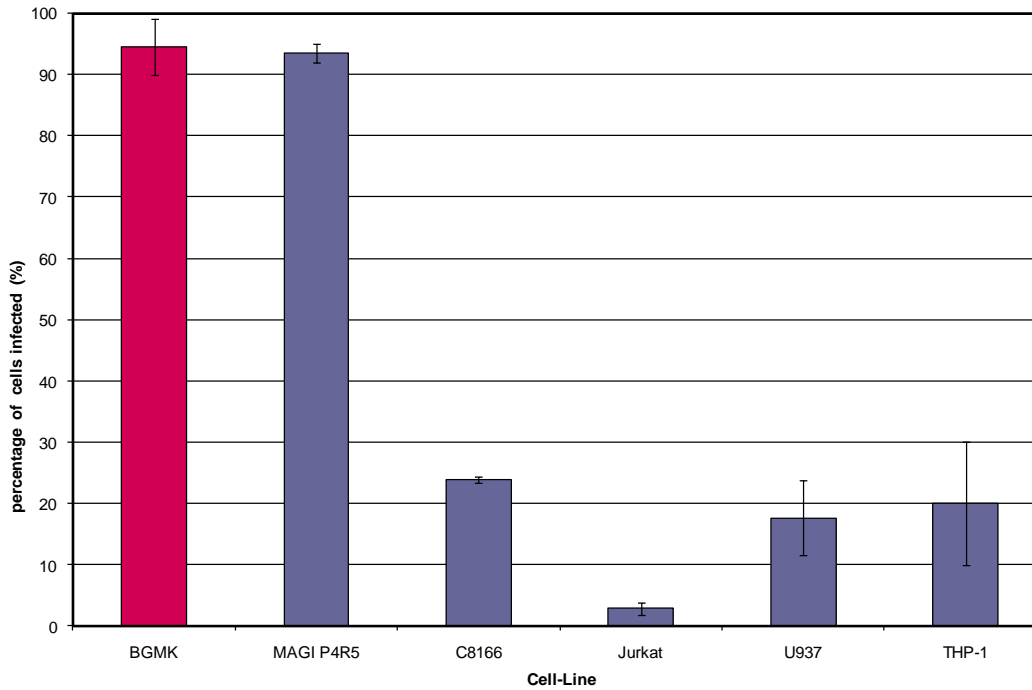


Figure 3.8 BGMK cells and the CD4-positive cell-lines MAGI P4R5, C8166, Jurkat E6.1, U937 and THP-1 were infected with *C. trachomatis* at an MOI of 10. Thirty hours post-infection, cells were fixed, stained with anti-LPS-FITC and subject to IFM (Figure 3.6A). Cells were viewed by white light (phase contrast), blue light (anti-LPS-FITC) and the images merged (Figure 3.8A). The percentage of CD4-positive cells infected with *C. trachomatis* (blue bars) was compared with BGMK cells (red bar) (Figure 3.8 B).

3.2.6 Quantification of HIV-1 infection in MAGI P4R5 cells

The MAGI P4R5 cells are transformed with a β -galactosidase gene under the control of the HIV-1 long terminal repeat (LTR). When they are incubated in the presence of a solution containing X-gal, infected cells catabolise the substrate to a coloured product and the cells' cytoplasm appear blue (Kimpton & Emerman 1992). This feature was exploited in quantifying viral infection (Figure 3.9 A). However, in co-infected cells, *C. trachomatis* inclusions were not clearly visible against the blue cytoplasmic background. An antibody raised against the HIV-1 core antigens (55, 39, 33 and 24KDa proteins) conjugated to the red fluorophore phycoerythrin (anti-P24-PE) was therefore used to visualise the HIV-1 infection (Figure 3.9 B).

In order to validate the antibody staining, monolayers of MAGI P4R5 cells were infected with HIV-1 at increasing MOIs and the number of cells with a blue cytoplasm after X-gal staining was compared with the number of cells fluorescing red after staining with anti-P24-PE at 20 hours post-infection. The percentage of infected cells was calculated for each field of view and the mean value plotted graphically (+/- SEM) for each MOI (Figure 3.9 C).

There was no significant difference in the percentage of infected cells as determined by X-gal staining (yellow bars) or anti-P24-PE antibody staining (red bars).

Figure 3.9 HIV-1_{MN} infection of MAGI P4R5 cells

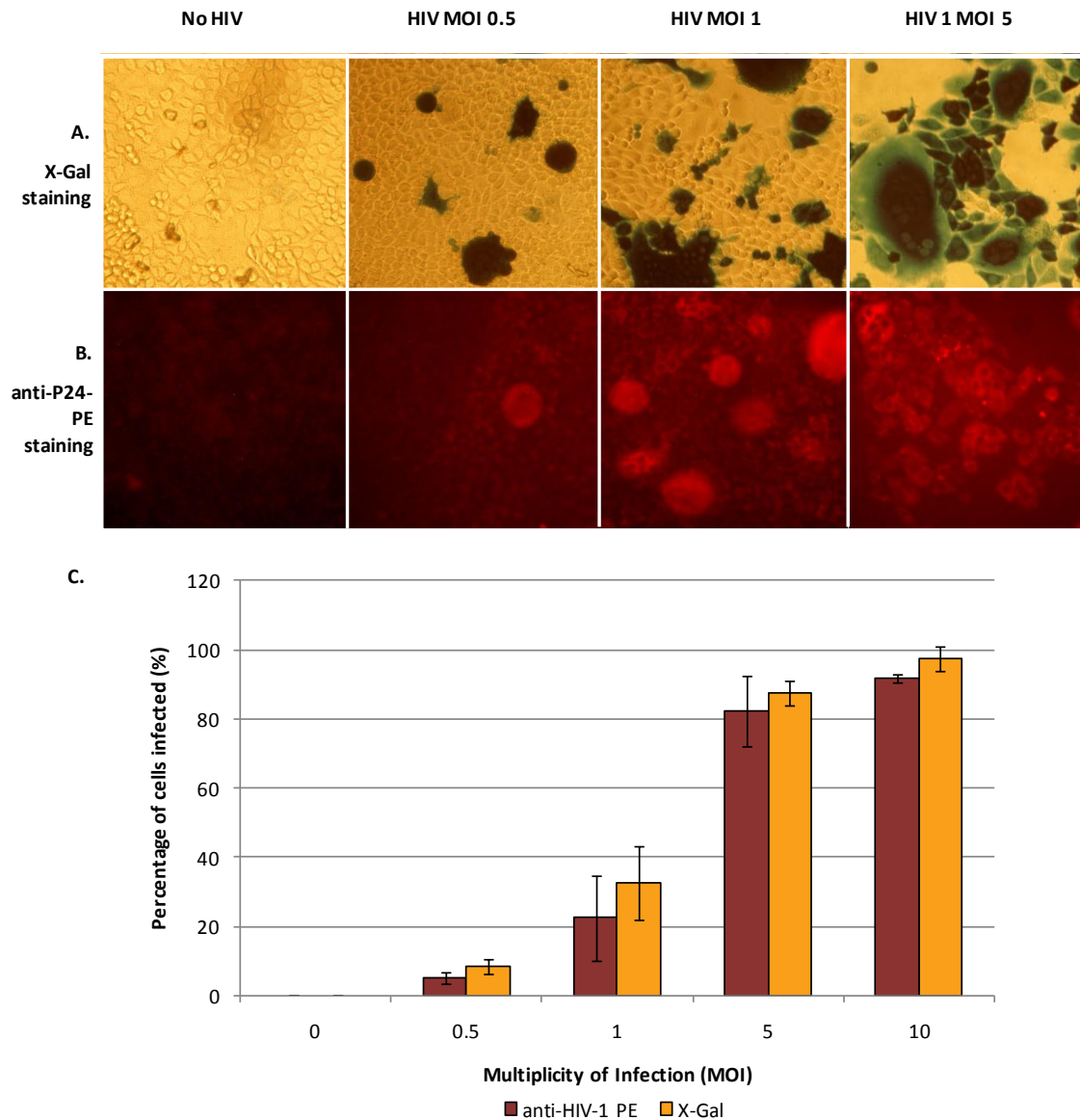


Figure 3.9 MAGI P4R5 cells were infected with HIV in duplicate at MOIs of 0, 0.5, 1, 5 and 10. Twenty hours post-infection, cells were either fixed and stained with X-gal in X-gal buffer, or subject to IFM with anti-P24-PE antibody. Infected cells possessed a blue cytoplasm when cultures were stained with X-Gal (Figure 3.9 A), and a red cytoplasm when cultures were stained with anti-P24-PE antibody and viewed at excitation/emission wavelengths of 535/565 nm by a fluorescence microscope (Figure 3.9 B). The percentage of infected cells was determined for each field of view and the mean value plotted graphically (+/- SEM) for each MOI (Figure 3.9 C).

3.2.7 Co-infection of HIV-1-infected cells with *C. trachomatis*

The growth of *C. trachomatis* in MAGI P4R5 cells infected with HIV-1 was compared to when the virus was absent. Briefly, duplicate monolayers of MAGI P4R5 cells were either mock-infected with complete medium (Figure 3.10 A, grey circle) or infected with HIV-1 at an MOI of 5 for 2 hours (Figure 3.10 A, red diamond). Cultures were incubated for 20 hours, after which cells were either mock-infected once more, or were infected with *C. trachomatis* at an MOI of 0.1 (Figure 3.10 A, green square). Four hours post-co-infection, the inoculum was replaced with complete medium (Time point 0). At various times (6, 12, 24, 30, 36 and 48 hours) post-co-infection, cells were either subject to IFM, or removed from the substratum by incubation with trypsin. Cell suspensions were either lysed by sonication and BGMK cells inoculated with the cell-lysates to establish the quantity of infectious *C. trachomatis* present, or DNA was extracted to quantify the chlamydial genome copy number in each sample. In addition, the quantity of HIV-1 virus present in the cell supernatants at each time-point was quantified by the SG-PERT assay and by titration onto C8166 cells.

At 6, 12, 24, 36 and 48 hours post-co-infection, cells were subject to IFM with anti-LPS-FITC and anti-P24-PE. The cells were viewed with a fluorescence microscope (x20 objective lens) (Figure 3.10 B). The *C. trachomatis* inclusions fluoresced green at excitation/emission wavelengths of 470/505nm and the cytoplasm of cells infected with HIV-1 fluoresced red at excitation/emission wavelengths of 535/565 nm. The number of *C. trachomatis* inclusions per well was counted for each time point and the mean plotted graphically (+/- SEM) in the presence and absence of HIV-1 (Figure 3.10 C).

There were significantly fewer *C. trachomatis* inclusions at 36 and 48 hours post-co-infection in cells that were infected with HIV-1, compared to when HIV-1 was absent (Student's t-test $P < 0.01$).

C. trachomatis inclusions in at least 5 fields of view were imaged for each well and the surface area of each inclusion was determined (pixels²) by the IFM computer software (Lucia). The median inclusion surface area for each time point, where inclusions were visible, in the presence and absence of HIV-1 was plotted graphically (Figure 3.10 D). In HIV-1 infected cells, *C. trachomatis* inclusions were significantly larger at 36 and 48 hours post-co-infection, compared to when HIV-1 was absent (Mann-Whitney U $P < 0.01$).

At 6, 12, 24, 36 and 48 hours post-co-infection, DNA was extracted from cell cultures and amplified with primers against the *C. trachomatis* *pmpH* gene (Table 2.1) by qPCR. The genome copy number in each sample was determined and the mean for each time point plotted graphically (\pm SEM) for each time point in the presence (red line) and absence (blue line) of HIV-1 (Figure 3.10 E). There was no significant difference in the replication of *Chlamydia* genomes in the presence or absence of HIV-1.

Infected cell-cultures were lysed by sonication at 24, 36 and 48 hours post-co-infection. Lysates were purified as described (Section 3.2.3) and monolayers of BGMK cells were infected with 10-fold serial dilutions of the lysates. Cells were incubated for 30 hours prior to IFM, whereupon the average number of IFUs per ml of undiluted cell-lysate was calculated and plotted graphically for each time point in the presence (red bar) and absence (blue bar) of HIV-1 (Figure 3.10 F). There was no significant difference in the infectivity of *Chlamydia* progeny in the presence or absence of HIV-1. As persistence is characterised by a loss of

infectivity Johnson & Hobson, 1977; Beatty *et al.*, 1993), this result is not consistent with a persistent phenotype.

At 6, 12, 24, 36 and 48 hours post-co-infection, an aliquot of supernatant was taken from each well and the activity of HIV-1 reverse transcriptase quantified by means of an in-house SYBR Green I-based product-enhanced reverse transcriptase (SG-PERT) assay. Additionally, the tissue culture infectious dose 50 (TCID₅₀) of HIV-1 in the supernatants was also calculated as described (section 2.4.2) (Figure 3.10 G). The addition of *C. trachomatis* to MAGI P4R5 cells did not alter the replication of HIV-1 as determined by either the SG-PERT or TCID₅₀ assays.

Figure 3.10 A – Co-infection of HIV-1 infected cells with *C. trachomatis*

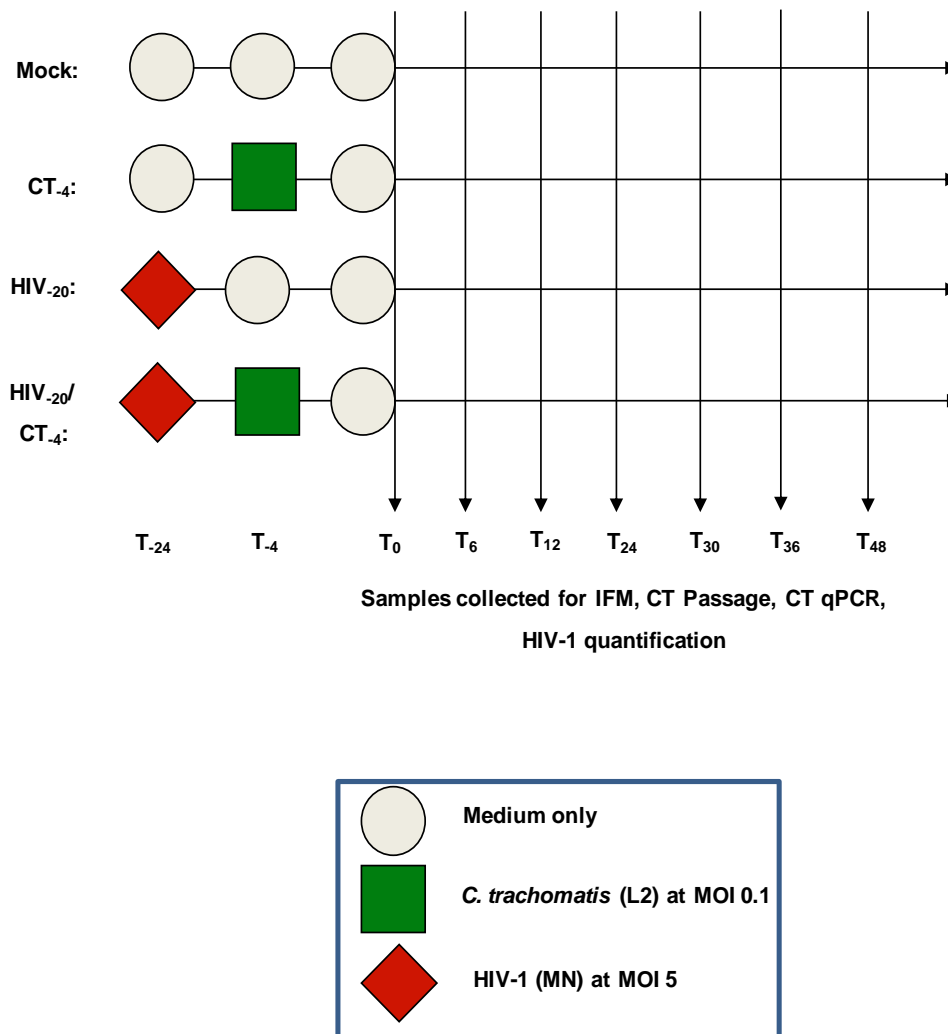


Figure 3.10 A MAGI P4R5 cells were either mock infected with complete medium (grey circle) or infected with HIV-1 (red diamond) and incubated for 20 hours, after which cells were either mock infected with complete medium (grey circle), or infected with *C. trachomatis* (green square). Four hours post- *C. trachomatis* infection, the inoculum was replaced with complete medium (grey circle) (Time point 0) and the cells incubated at 37°C in 5% CO₂. At 6, 12, 24, 30, 36 and 48 hours post co-infection, cells were processed for the following assays: IFM, passage onto BGMK cells to establish the quantity of infectious *C. trachomatis* in the samples (CT Passage), *Chlamydia* genome copy number quantification by qPCR (CT qPCR), and HIV-1 quantification by the SG-PERT assay and titration onto C8166 cells (HIV-1 quantification).

Figure 3.10 B - IFM of *C. trachomatis* inclusions in cells that were either mock infected, or co-infected with HIV-1.

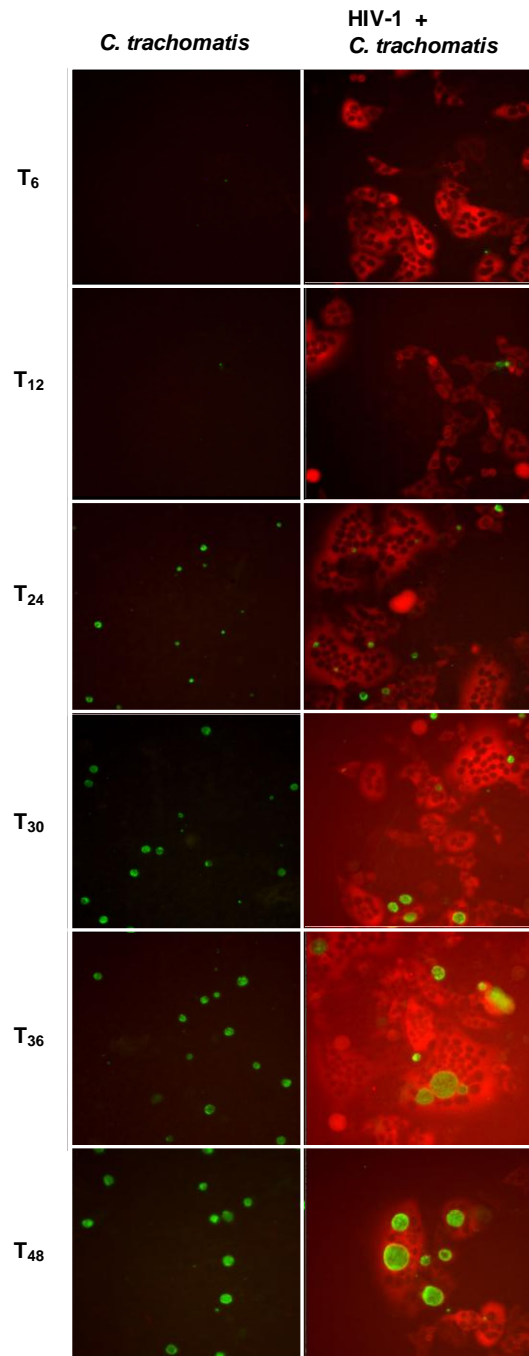


Figure 3.10 B MAGI-P4R5 cells were either mock infected with complete medium, or infected with HIV-1 at an MOI 5 for 20 hours. Cells were co-infected with *C. trachomatis* at an MOI of 0.1 and at various times (T 6, 12, 24, 30, 36 and 48 hours) post co-infection, cell cultures were subject to IFM with anti-LPS-FITC and anti-P24-PE. Cells were viewed with a fluorescence microscope (x20 objective). *C. trachomatis* inclusions fluoresced green at excitation/emission wavelengths of 470/505nm and the cytoplasm of cells infected with HIV-1 fluoresced red at excitation/emission wavelengths of 535/565 nm.

Figure 3.10 C - Significantly fewer *C. trachomatis* inclusions develop in cells infected with HIV-1, compared to when HIV-1 is absent

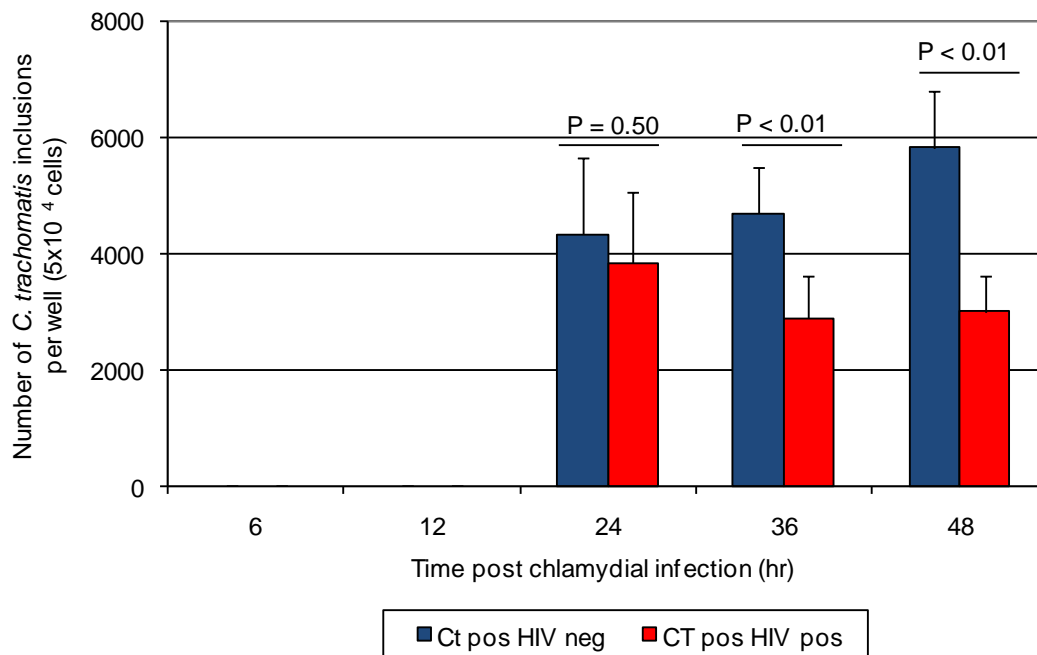


Figure 3.10 C MAGI P4R5 cells that had previously been mock-infected with complete medium, or infected with HIV-1, were infected with *C. trachomatis*. At 6, 12, 24, 36 and 48 hours post-co-infection, cells were subject to IFM and the number of *C. trachomatis* inclusions per well counted. The mean value was plotted graphically (+/- SEM) for each time point in the presence (red bars) and absence (blue bars) of HIV-1. Ct pos, *C. trachomatis* infected cells; HIV pos, HIV-1 infected cells; HIV neg, cells not infected with HIV-1.

Figure 3.10 D- Significantly larger *C. trachomatis* inclusions develop in cells infected with HIV-1, compared to when HIV-1 is absent

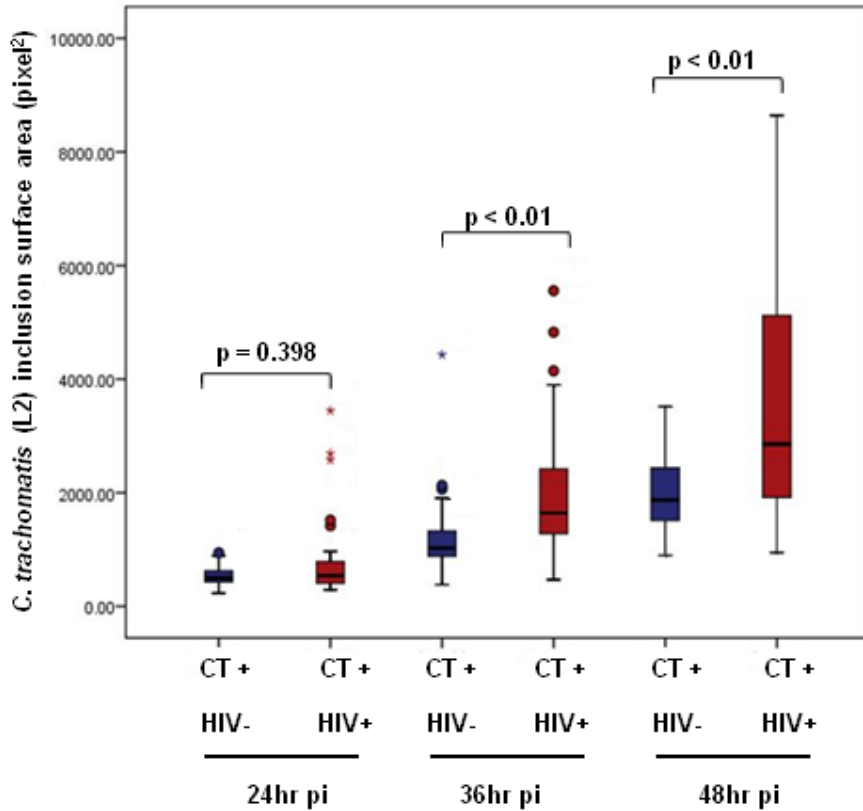


Figure 3.10 D MAGI P4R5 cells that had either been mock-infected with complete medium, or infected with HIV-1, were co-infected with *C. trachomatis*. At 6, 12, 24, 36 and 48 hours post-co-infection, cells were subject to IFM and imaged in at least 5 fields of view per well. The surface area of each inclusion (pixel²) was determined by the IFM computer software (Lucia) and the median inclusion surface area for each time point where inclusions were visible (24, 36 and 48 hours post co-infection) were plotted graphically in the presence (red plots) and absence (blue plots) of HIV-1. CT +, *C. trachomatis* infected cells; HIV +, HIV-1 infected cells; HIV-, cells not infected with HIV-1; pi, post-infection. Dots and stars are out-lier points.

Figure 3.10 E -The replication of *C. trachomatis* genomic copies remains unchanged in cells infected with HIV-1 compared to when HIV-1 is absent.

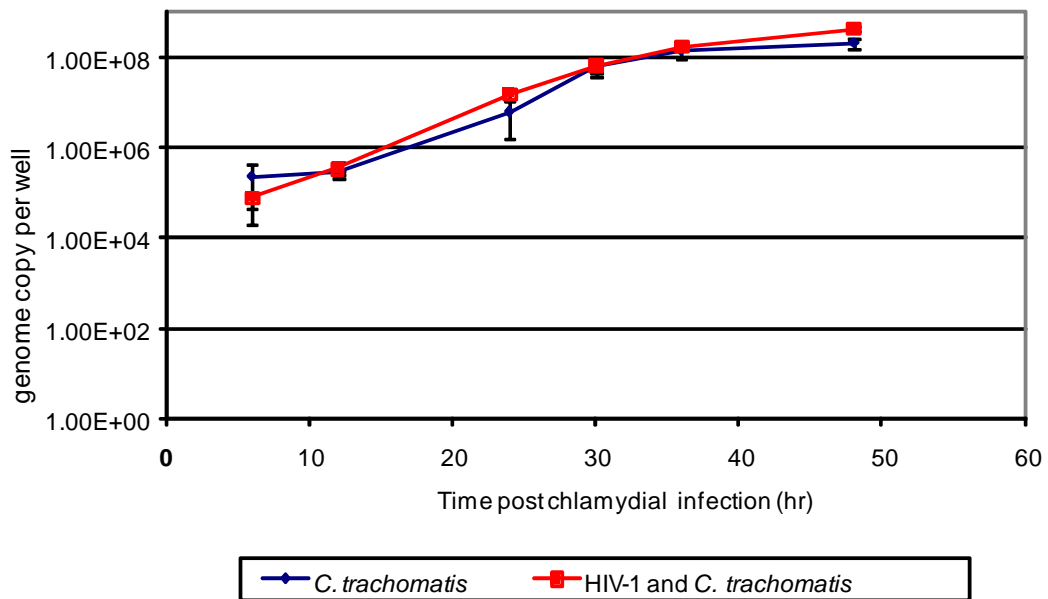


Figure 3.10 E MAGI P4R5 cells that had previously been mock-infected with complete medium, or infected with HIV-1, were co-infected with *C. trachomatis* in duplicate. At 6, 12, 24, 36 and 48 hours post-co-infection, DNA was extracted from the cells and amplified with primers against the *C. trachomatis* *pmpH* gene by qPCR in order to determine the genome copy number in each sample. The mean *Chlamydia* genome copy number was plotted graphically (+/- SEM) for each time point in the presence (red line) and absence (blue line) of HIV-1.

Figure 3.10 F- The infectivity of *C. trachomatis* progeny remains unchanged in cells that infected with HIV-1 compared to when HIV-1 is absent.

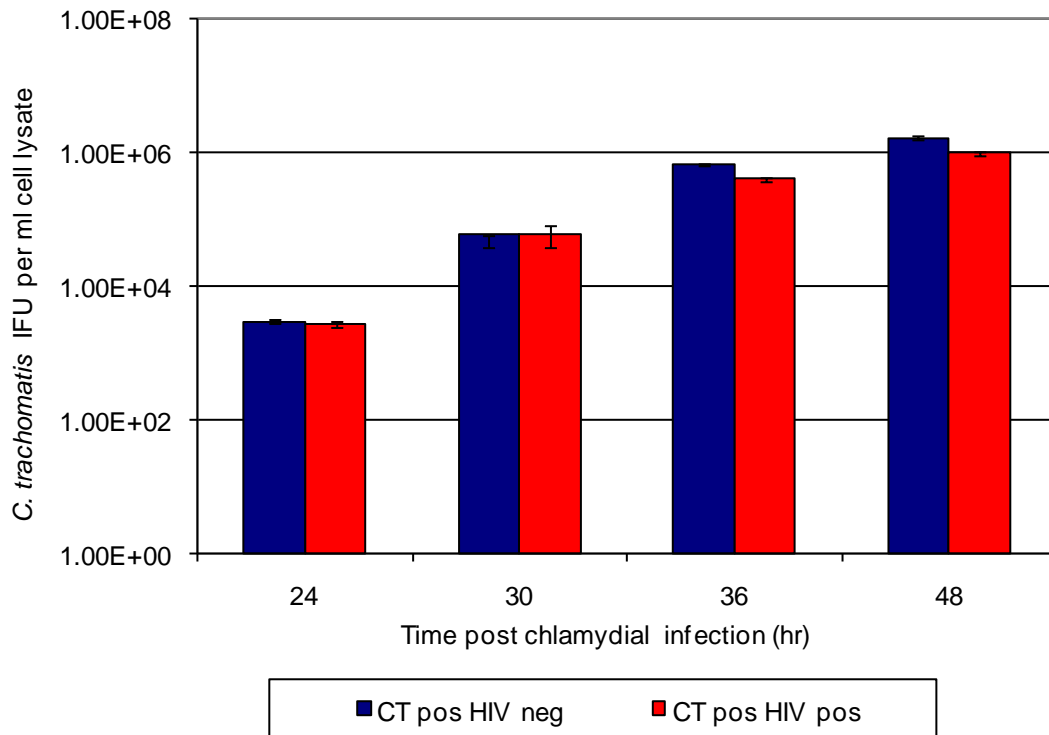


Figure 3.10 F MAGI P4R5 cells that had been mock-infected with complete medium, or infected with HIV-1, were co-infected with *C. trachomatis* in duplicate. At 24, 36 and 48 hours post-co-infection, cell-suspensions were lysed by sonication. Monolayers of BGMK cells were inoculated with 10-fold serial dilutions of the cell-lysates, and the number of IFUs counted. The number of *C. trachomatis* IFUs per ml of undiluted cell-lysate was calculated and plotted graphically for each time point in the presence (red bar) and absence (blue bar) of HIV-1.

Figure 3.10 G - The replication of HIV-1 remains unchanged by the addition of *C. trachomatis*

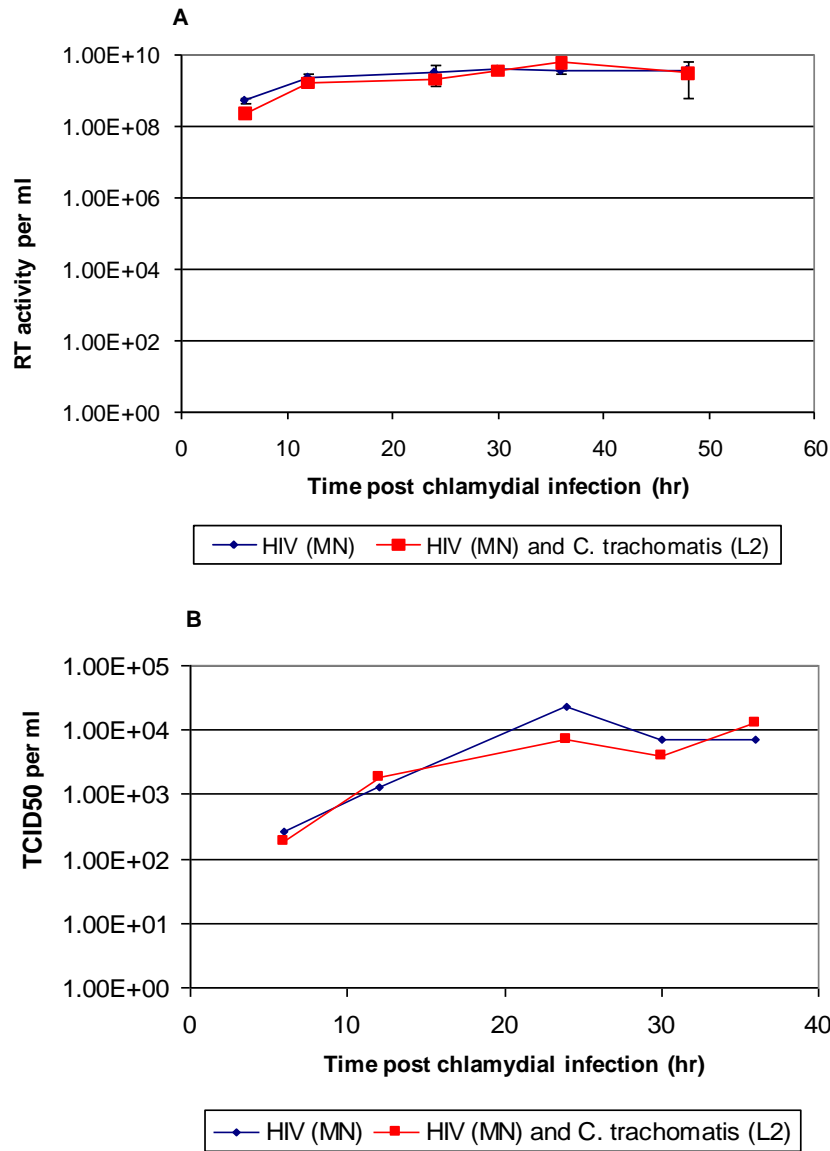


Figure 3.10 G MAGI P4R5 cells were either mock-infected with complete medium, or infected with HIV-1. Cells were co-infected with *C. trachomatis* and, at 6, 12, 24, 36 and 48 hours post-co-infection, aliquots of cell supernatants were obtained from each well. The activity of HIV-1 RT in the supernatant was quantified by means of an in-house SG-PERT assay (A), and the TCID₅₀ of HIV-1 was calculated (B). The RT activity and TCID₅₀ of HIV-1 was compared in the presence (red lines) and absence (blue lines) of *C. trachomatis*.

3.2.7 Co-infection of *C. trachomatis*-infected cells with HIV-1

The effect of HIV-1 co-infection on *C. trachomatis* growth in MAGI P4R5 was investigated. Briefly, cells were either mock infected with complete medium (Figure 3.11 A, grey circle) or infected with *C. trachomatis* at an MOI of 10 (Figure 3.11 A, green square) and incubated for 24 hours, after which cells were either mock infected with complete medium (Figure 3.11 A, grey circle), or co-infected with HIV-1 at an MOI of 10 (Figure 3.11 A, red triangle). Two hours post co-infection, the inoculum was replaced with complete medium (Time point 0). At various times (0, 5, 10, 15 and 20 hours) post co-infection, cells were either subject to IFM, or they were removed from the substratum by incubation with trypsin. Cell suspensions were either lysed by sonication and BGMK cells inoculated with the cell lysates to establish the quantity of infectious organisms in the samples, or DNA was extracted to quantify the chlamydial genome copy number present. In addition, RNA was extracted from cells 15 hours post-co-infection and reverse transcribed to cDNA in order to determine the relative expression of *Chlamydia* genes in the presence and absence of HIV-1. Cells were also subject to transmission electron microscopy (TEM) 5, 10 and 15 hours post-co-infection to characterise the morphology of intracellular organisms in the presence and absence of HIV-1. Finally, the quantity of HIV-1 virus present in the cell supernatants at each time point was quantified by the SG-PERT assay and by titration onto C8166 cells in the presence and absence of *C. trachomatis*.

Infected cell cultures were subject to IFM with anti-LPS-FITC and anti-P24-PE and viewed with a fluorescence microscope (x20 objective lens) (Figure 3.11 B). The *C. trachomatis* inclusions fluoresced green at excitation/emission wavelengths of 470/505nm and the cytoplasm of cells infected with HIV-1 fluoresced red at excitation/emission wavelengths of

535/565 nm. The number of *C. trachomatis* inclusions in at least 5 fields of view per well was counted at 0, 5, 10, and 15 hours post-infection, and the mean plotted graphically (+/- SEM) (Figure 3.11 C). There were significantly fewer *C. trachomatis* inclusions at 10 and 15 hours post-HIV-1 co-infection compared to when HIV-1 was absent (Student's t-test $P = 0.03$).

C. trachomatis inclusions in at least 5 fields of view were imaged for each well at excitation/emission wavelengths of 470/505nm with the fluorescence microscope x 20 objective lens. For each inclusion imaged, the surface area was determined (pixels²) by the IFM computer software (Lucia). The median inclusion surface area was determined for each time point and plotted graphically (Figure 3.11 D). In the presence of HIV-1, *C. trachomatis* inclusions were significantly larger at 5, 10 and 15 hours post-co-infection, compared to when HIV-1 was absent (Mann-Whitney U $P < 0.01$).

At 0, 5, 10, and 15 hours post-co-infection, duplicate cell-cultures were removed from the substratum by incubation with trypsin in 3 independent experiments. In the first, DNA was extracted from the cells and amplified with primers against the *C. trachomatis* *pmpH* gene by qPCR in order to determine the number of genome copies in each sample. The mean for each time point was plotted graphically in the presence and absence of HIV-1 (+/- SEM) (Figure 3.11 E). The presence of HIV-1 made no significant difference to the replication of *Chlamydia* genomes, indicating that the virus did not accelerate the *C. trachomatis* life cycle as has been suggested (Bianchi *et al.*, 1998). In the second, cell-suspensions were lysed by sonication and purified as described (Section 3.2.3). Monolayers of BGMK cells were inoculated with 10-fold serial dilutions of the lysates and incubated for 30 hours prior to IFM with anti-LPS-FITC. The number of *C. trachomatis* IFUs per ml of undiluted cell-lysate was

determined and plotted graphically for each time point (Figure 3.11 F). The presence of HIV-1 did not significantly alter the infectivity of *C. trachomatis* organisms in the lysates at 0, 5 and 10 hours post-infection, however, there was a slight decrease in chlamydial infectivity at 15 hours post-co-infection. When BGMK cells were inoculated with the supernatant from cell cultures 15 hours post-co-infection, the number of *C. trachomatis* IFUs recovered per ml was not significantly altered by the presence of HIV-1 (Figure 3.11 G). As persistence is characterised by a loss of infectivity (Johnson & Hobson, 1977; Beatty *et al.*, 1993), this result is not consistent with a persistent phenotype

In the third, the cell-suspension from each well was divided into 2 equal aliquots that were pelleted by centrifugation. The RNA was extracted from one cell-pellet and reverse-transcribed to cDNA that was amplified by qPCR using primers against *C. trachomatis* unprocessed *16s rRNA*, *ompA*, *omcB* and *euo*, in order to determine the number of cDNA transcripts of each gene in the sample. The DNA was extracted from the other cell-pellet and amplified by qPCR using the same primers in order to determine the number of DNA copies of each gene in the sample. The number of cDNA transcripts for each gene was divided by the number of DNA copies of each gene to quantify gene expression in the sample. The expression of each gene (cDNA copies/DNA copies) in the presence (red bars) and absence (blue bars) of HIV-1 was plotted graphically (Figure 3.11 H). Co-infection with HIV-1 significantly upregulated the expression of *omcB*, an RB late-gene that encodes the outer membrane protein B, found in EBs. There was, however, no significant alteration in the expression of unprocessed *16S rRNA*, *ompA* (encoding MOMP) or *euo* (involved in the silencing of late-genes) compared to when HIV-1 was absent. These results are not consistent with a persistent phenotype.

At 0, 5, 10, and 15 hours post-co-infection, cells were fixed and subject to TEM (Figure 3.11 I). *C. trachomatis* inclusions containing EBs, intermediate bodies (IBs) and RBs were observed at all time-points in the presence and absence of HIV-1, demonstrating that HIV-1 does not lead to *C. trachomatis* persistence. The number of *C. trachomatis* EBs, IBs and RBs were counted as per the protocol by Timms *et al.*, 2009 in 5 inclusions in co-infected cultures and 5 inclusions in cultures infected with *C. trachomatis* alone. The median number of EBs, RBs, and IBs per inclusion was determined in the presence and absence of HIV-1 at 15 hours post co-infection and tabulated (Table 3.4). The ratio of EBs / RBs was not significantly altered by the presence of HIV-1 (Mann Whitney U $P = 0.310$). As *Chlamydia* persistence is characterised by an alteration in the morphology of intracellular organisms, these observations are not consistent with a persistent phenotype. Moreover, these data suggest that co-infection did not promote the differentiation of RBs to EBs.

An aliquot of supernatant was taken from each well at 0, 5, 10, and 15 hours post-co-infection, and the quantity of HIV-1 assayed by means of an in-house SG-PERT assay (Figure 3.11 J) and TCID₅₀ (Figure 3.11 K). The replication of HIV-1 was not significantly altered in cells that were infected with *C. trachomatis* compared to when the bacteria were absent.

Figure 3.11 A - HIV-1 co-infection of *C. trachomatis*-infected cells

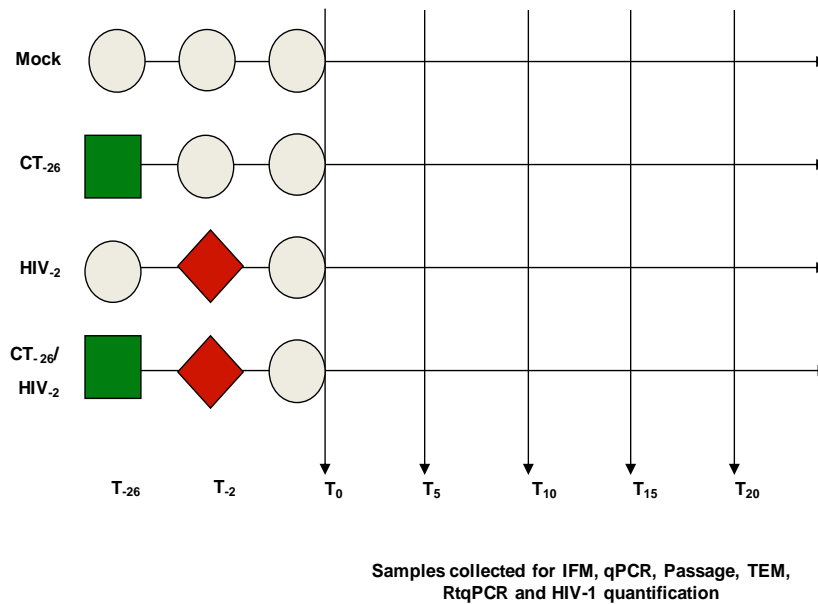


Figure 3.11 A MAGI P4R5 cells were either mock infected with complete medium (grey circle) or infected with *C. trachomatis* (green square) and incubated for 24 hours at 37°C, 5% CO₂, after which cells were either mock infected with complete medium (grey circle), or infected with HIV-1 (red diamond). Two hours post-co-infection, the inoculum was replaced with complete medium (grey circle) (Time point 0) and the cells incubated at 37°C in 5% CO₂. At various times (5, 10, 15 and 20 hours) post co-infection, cells were processed for the following assays: IFM, passage onto BGMK cells to establish the quantity of infectious *C. trachomatis* in the samples (Passage), *Chlamydia* genome copy number quantification (qPCR), *C. trachomatis* gene expression characterisation by RT qPCR, TEM and HIV-1 quantification by the SG-PERT assay and titration onto C8166 cells (HIV-1 quantification).

Figure 3.11 B- The growth of *C. trachomatis* in HIV-1-co-infected cells

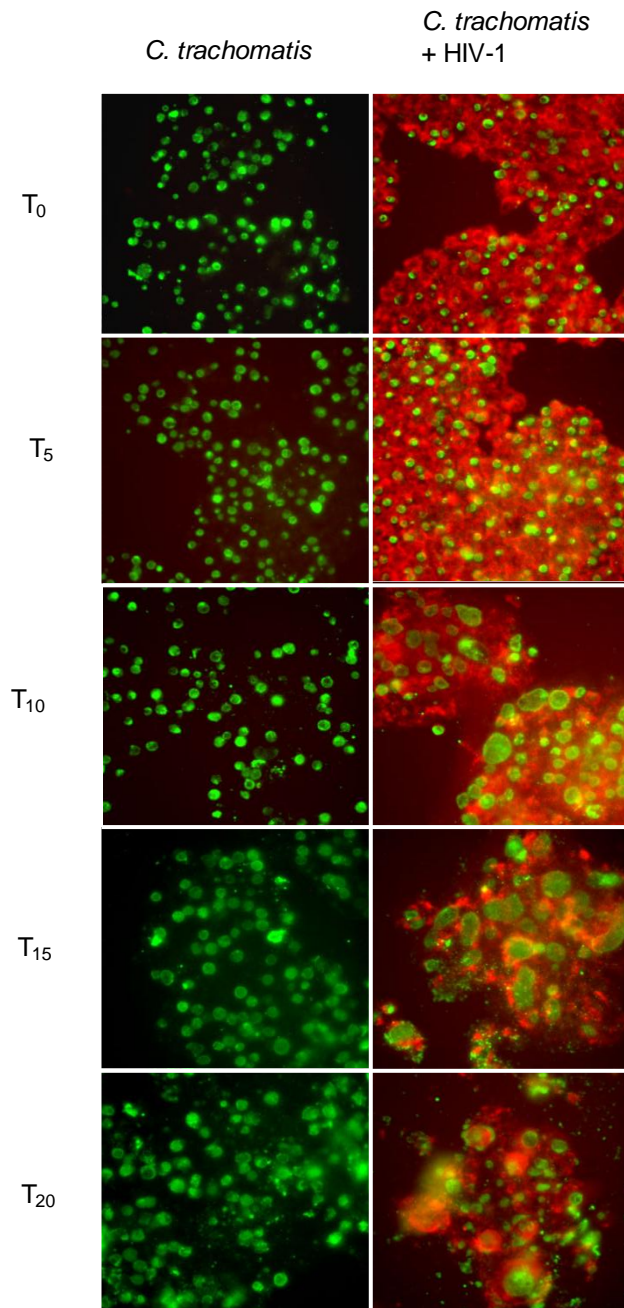


Figure 3.11 B MAGI-P4R5 cells were infected with *C. trachomatis* at an MOI of 10 for 24 hours and mock-infected with complete medium, or infected with HIV-1 at an MOI 10. At various times (0, 5, 10, 15 and 20 hours) post co-infection, cell cultures were fixed, permeabilised subject to IFM with anti-LPS-FITC and anti-P24-PE. Cells were viewed with a fluorescence microscope (x20 objective) and *C. trachomatis* inclusions fluoresced green at excitation/emission wavelengths of 470/505nm and the cytoplasm of cells infected with HIV-1 fluoresced red at excitation/emission wavelengths of 535/565 nm.

Figure 3.11 C and D - Significantly fewer, but larger, *C. trachomatis* inclusions develop in cells co-infected with HIV-1.

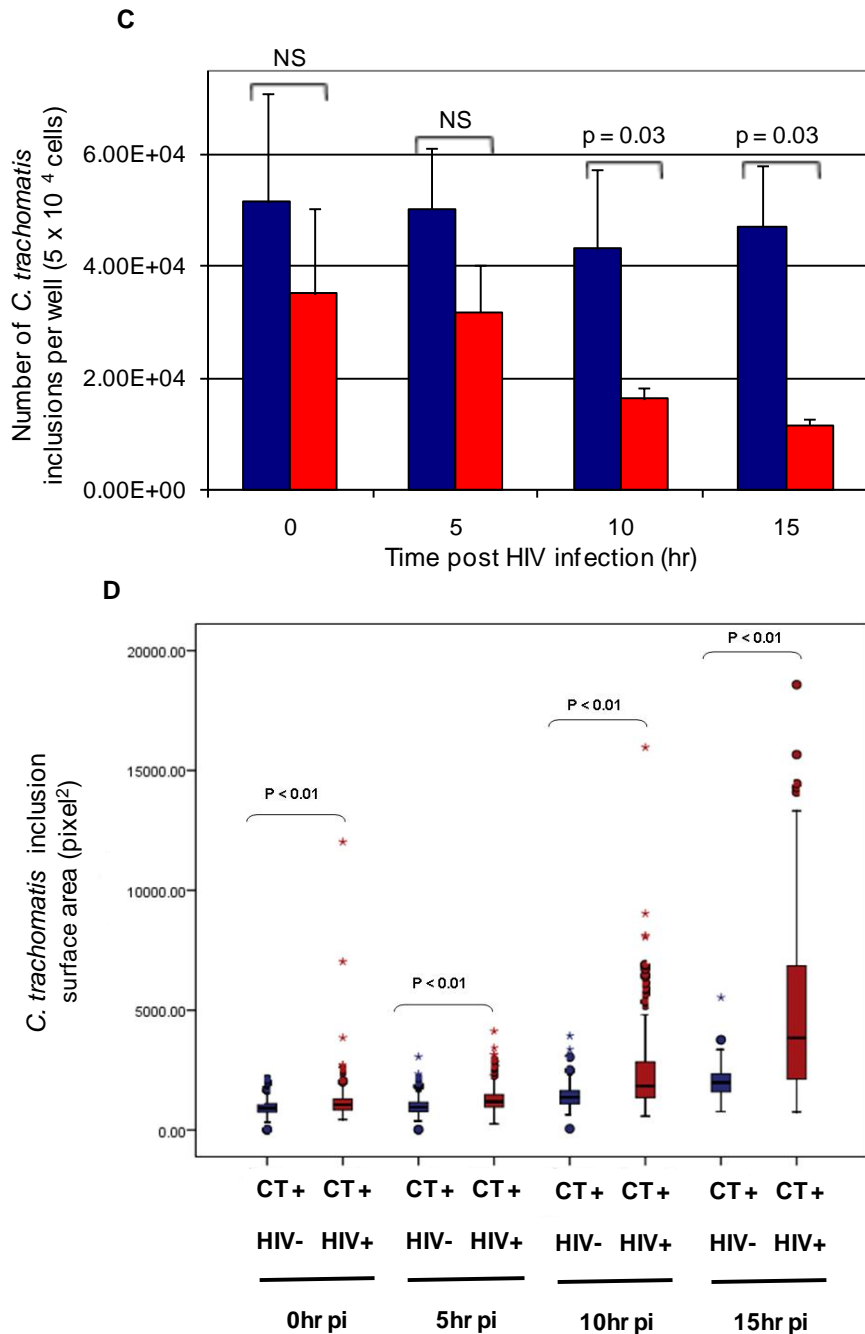


Figure 3.11 C and D MAGI P4R5 cells were infected with *C. trachomatis* and mock-infected with complete medium, or infected with HIV-1. At various times (0, 5, 10 and 15 hours) post-co-infection, cells were subject to IFM and the average number of inclusions per well plotted graphically (\pm SEM) for each time point in the presence (red bars) and absence (blue bars) of HIV-1 (Figure 3.11 C). The median *C. trachomatis* inclusion surface area was also determined for each time point and plotted graphically in the presence (red plots) and absence (blue plots) of HIV-1 (Figure 3.11 D).

Figure 3.11 E, F and G -The replication of *C. trachomatis* genomic copies and the infectivity of *C. trachomatis* progeny remain unchanged by the presence of HIV-1

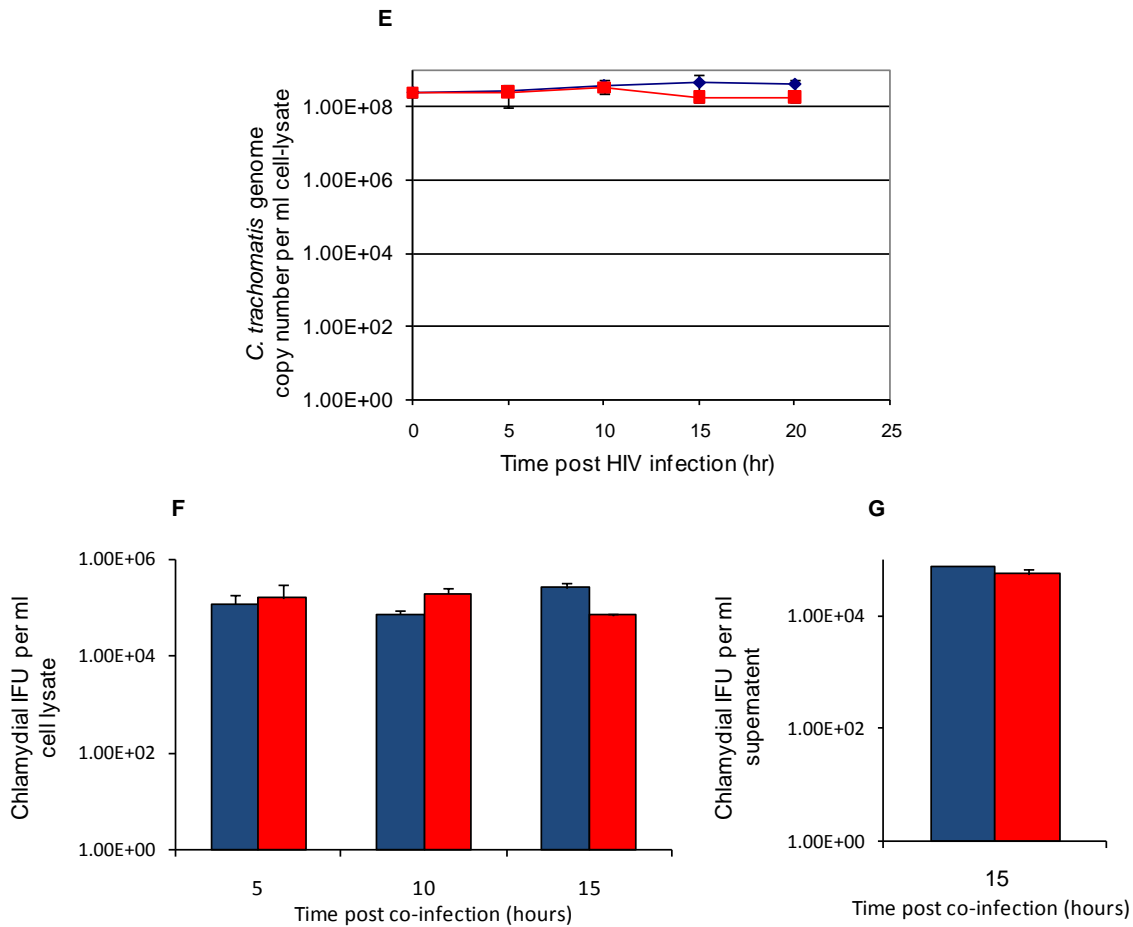


Figure 3.11 E, F and G. MAGI P4R5 cells were infected with *C. trachomatis* and mock infected with complete medium, or infected with HIV-1 in duplicate. At various times (0, 5, 10, 15 and 20 hours), the mean *C. trachomatis* genome copy number was determined by *pmpH* qPCR and plotted graphically (+/- SEM) in the presence (red line) and absence (blue line) of HIV-1 (Figure 3.11 E). Cell-suspensions were also lysed by sonication and BGMK monolayers inoculated with the cell-lysates in order to determine the infectivity of *C. trachomatis* progeny in the presence (red bars) and absence (blue bars) of HIV-1 (Figure 3.11 F). In addition, BGMK cells were inoculated with aliquots of the culture supernatant from 15 hours post-co-infection (Figure 3.11 G).

Figure 3.11 H – *C. trachomatis* gene transcription in HIV-1 co-infected cultures

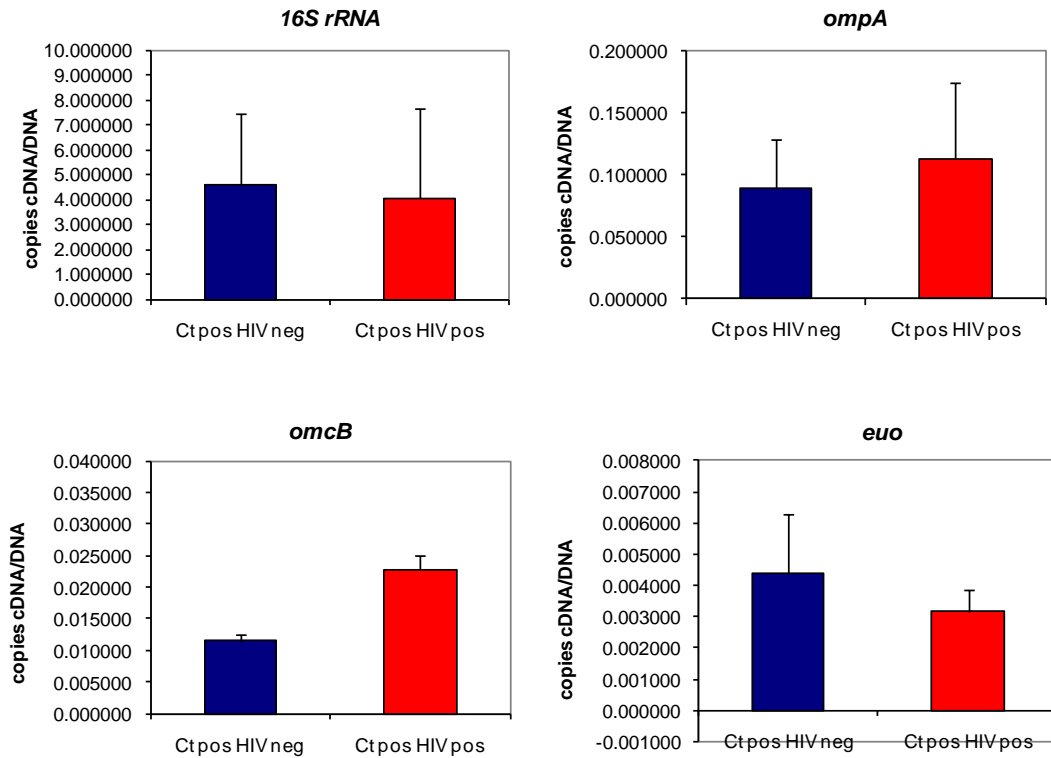


Figure 3.11 H MAGI P4R5 cells were infected with *C. trachomatis* and either mock infected with complete medium, or infected with HIV-1. At various times (0, 5, 10, and 15 hours) post-co-infection, DNA and RNA were extracted and the RNA reverse-transcribed to cDNA in order to quantify the expression of *C. trachomatis* unprocessed *16s rRNA*, *ompA*, *omcB* and *euo* (copies cDNA/DNA.). The expression of each gene in the presence (red bars) and absence (blue bars) of HIV-1 was plotted graphically (Figure 3.11 H).

Figure 3.11 I - Transmission Electron Microscopy of *C. trachomatis*-infected cells in the presence and absence of HIV-1

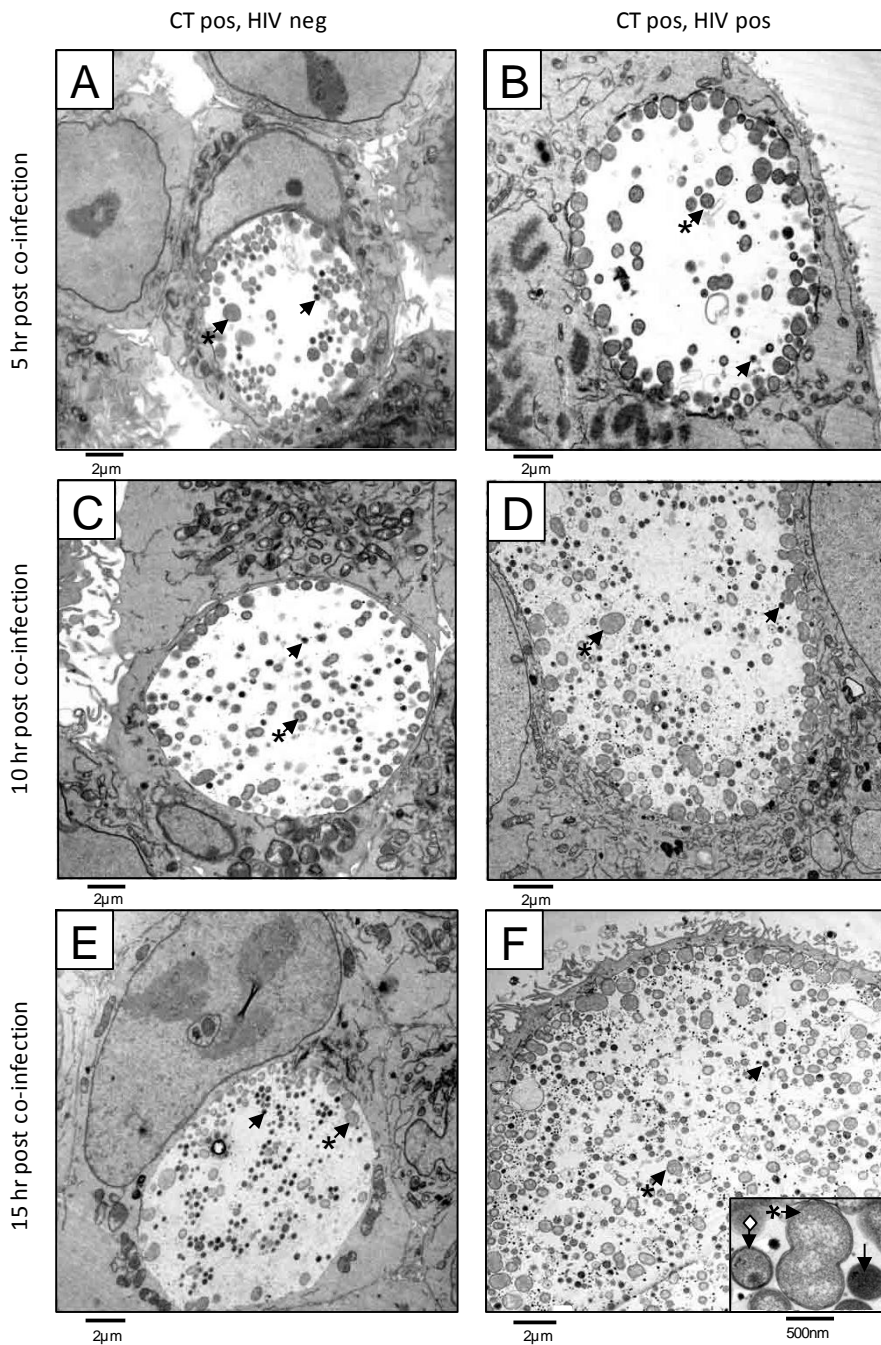


Figure 3.11 I MAGI P4R5 cells were infected with *C. trachomatis* and mock-infected with complete medium (A, C and E) or co-infected with HIV-1 (B, D and F). At 5 (A and B) 10 (C and D) and 15 (E and F) hours post-co-infection, samples were processed and viewed by TEM. EB, elementary body (arrow); RB, reticulate body (arrow with star); IB, intermediate body (arrow with diamond).

Table 3.4 TEM of *C. trachomatis* in the presence and absence of HIV-1

	Ct pos, HIV-1 neg	Ct pos, HIV-1 pos
Number of inclusions imaged	5	5
Median number organisms per inclusion (IR)	112 (108-113)	195 (188-325)
Median number RBs per inclusion (IR)	38 (32-39)	108 (96-131)
Median number EBs per inclusion (IR)	57 (33-59)	71 (59-119)
Median number IBs per inclusion (IR)	17 (14-17)	28 (25-58)
Median EB/RB ratio (interquartile range)	1.55 (0.85-1.78)	0.74 (0.70-0.91)
Mann Whitney U <i>P</i> value	0.310	

Table 3.4 MAGI P4R5 cells infected with *C. trachomatis* were co-infected with HIV-1. At 15 hours post-co-infection, cells were subject to TEM. Five inclusions were imaged in the presence and absence of HIV-1 and the median number of EBs, IBs and RBs determined (IR, interquartile range).

Figure 3.11 J and K - The replication of HIV-1 remains unchanged in cells that are infected with *C. trachomatis*, compared to when the bacteria are absent.

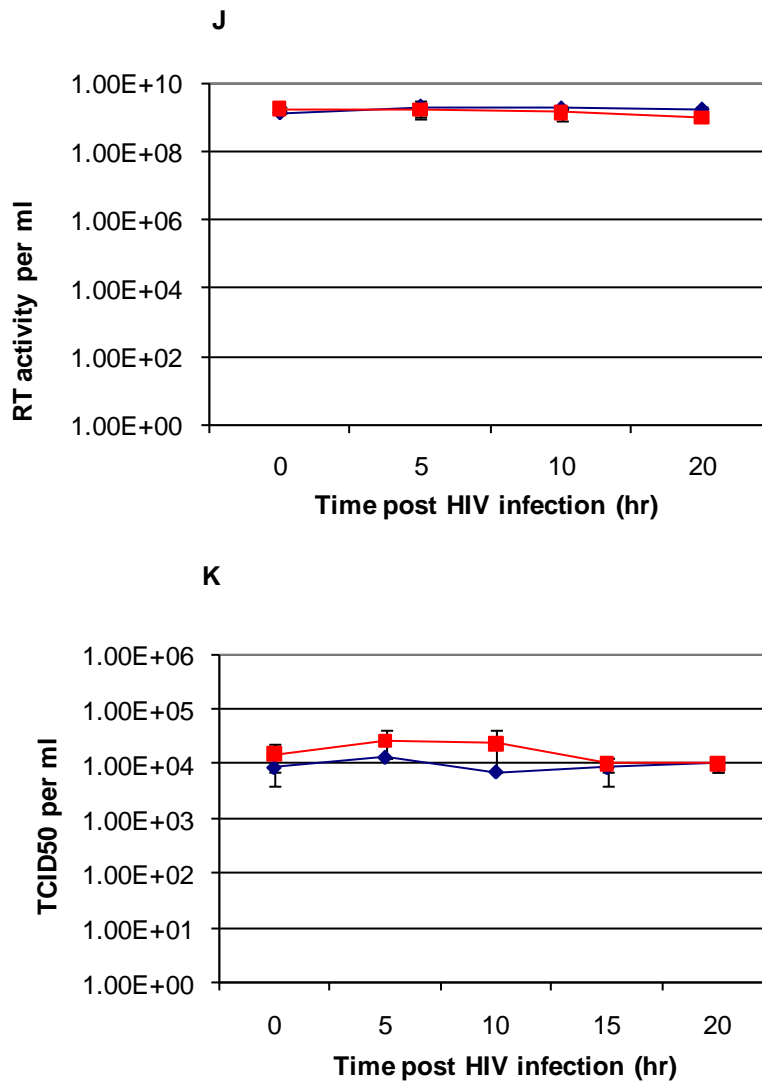


Figure 3.11 J and K. MAGI P4R5 cells were infected with *C. trachomatis* and either mock-infected with complete medium, or infected with HIV-1. At various times (0, 5, 10, 15 and 20 hours) post-co-infection, an aliquot of supernatant was taken from each well and the activity of HIV-1 RT quantified by means of an in-house SG-PERT assay. The TCID₅₀ was also determined by titration onto C8166 cells. Blue lines, absence of *C. trachomatis*; red lines, presence of *C. trachomatis*.

3.3 Discussion

In this investigation we sought to characterise the effect of HIV-1 co-infection on *C. trachomatis* replication by means of several experimental parameters, including chlamydial inclusion size, inclusion number, EB and RB morphology, EB infectivity, genome copy number and the transcription of unprocessed *16S rRNA*, *ompA*, *omcB*, and *euo*. This study represents, to our knowledge, the first characterisation of *C. trachomatis* replication in HIV-1 co-infected cells.

When *Chlamydia* organisms are in a persistent state, the morphology is typically aberrant, infectivity is significantly reduced and gene transcription is altered (Johnson *et al.*, 1977; Matsumoto *et al.*, 1970; Beatty *et al.*, 1993; Beatty *et al.*, 1994b; Raulston *et al.*, 1997; Belland *et al.*, 2003; Hogan *et al.*, 2004; Ouellette *et al.*, 2006; Goellner *et al.*, 2006). As HIV-1 co-infection did not induce significant changes in EB or RB morphology, the infectivity of EBs, or in the transcription of *ompA* (that encodes the EB major outer membrane protein, MOMP) and *euo* (the product of which silences late gene expression), it is reasonable to conclude that the presence of HIV-1 did not induce *C. trachomatis* persistence. Moreover, as there was no significant alteration in *C. trachomatis* genome copy number in the presence of HIV-1, it is also unlikely that HIV-1 accelerated the replication of *C. trachomatis*. In addition, the presence of *C. trachomatis* did not alter the replication of HIV-1 in co-infected cells.

The lack of an observed interaction between *C. trachomatis* and HIV-1 is surprising. Both organisms manipulate the actin cytoskeleton to gain entry to cells (Scidmore, 2006; Carabeo *et al.*, 2007; Fackler *et al.*, 2006; Liu *et al.*, 2009) and *C. trachomatis* proteins can interact

with the cytoplasm (Scidmore, 2006), where HIV-1 structural proteins are located (Freed, 2006). The *Chlamydia* inclusion also intercepts exocytic vesicles budding off the Golgi network (Hackstadt *et al.*, 1996) and, as the HIV-1 gp120 traffics through the Golgi (Stein & Engleman, 1990) it could potentially be re-routed to the inclusion. Interestingly, *C. trachomatis* inclusions have been shown to interact with vesicles derived from multivesicular bodies (Beatty *et al.*, 2006; Beatty, 2008) and, as HIV-1 has been shown to assemble in these structures (Sherer *et al.*, 2003; Joshi *et al.*, 2009), there is additional potential for viral – chlamydial interaction.

Co-infection of *C. trachomatis*-infected HeLa cells with HSV-2 induces *C. trachomatis* persistence (Deka *et al.*, 2006) by initiating intracellular signalling events upon binding to the host-cell plasma membrane (Vanover *et al.*, 2010). Ligation of one such receptor class, the nectins, activates pathways that may be responsible for inducing *C. trachomatis* persistence (Deka *et al.*, 2007). When Nectins 1 and 2 bind their natural ligands, they lead to cytoskeletal rearrangements (Nakanishi *et al.*, 2004) and it has been hypothesised that HSV-2 induction of these pathways induces *Chlamydia* persistence in co-infected cells (Deka *et al.*, 2007). However HIV-1 also leads to actin re-modelling during infection (Fackler *et al.*, 2006; Liu *et al.*, 2009). Why, therefore, does HSV-2 induce *C. trachomatis* persistence but HIV-1 does not?

The answer may lie in differences between the signalling pathways invoked by the two viruses. Intracellular signalling pathways are extraordinarily complex and our knowledge of some remains incomplete. As such, it has not been possible to identify which differences affect *Chlamydia* development, or how. In addition, as MAGI P4R5 cells do not naturally express CD4, CCR5 and CXCR4, they may lack components of the downstream signalling

pathways. It may be that the presence of incomplete signalling pathways in MAGI P4R5 cells is responsible for the lack of persistence observed in this study.

Bianchi *et al.*, 1998 suggest that the presence of HIV-1 accelerated the *C. trachomatis* infection cycle in co-infected cells (Bianchi *et al.*, 1998). This is not consistent with observations made in the present study, however, their conclusions were based on an apparent accelerated rate of cell lysis in HIV-1 co-infected culture, but there was no quantification of *C. trachomatis* inclusions by microscopy, or genomic replication by molecular techniques. Thus, it is not possible to draw conclusions regarding the susceptibility of cells to *C. trachomatis* infection, or the kinetics of *Chlamydia* replication as Bianchi *et al.*, propose. Of note is the cell-line used in the studies Bianchi *et al* were U-937 cells. How permissive these cells are to *C trachomatis* serovar L2 is questionable. While some studies have been able to infect the cells with the organism (Bianchi *et al.*, 1997; Bianchi *et al.*, 1998), others claim that typical inclusions are not observed in these cells and they are not permissive to *Chlamydia* infection (Mpiga *et al.*, 2006).The present study identified punctuate regions of fluorescence when infected U-937 cells were stained with an anti-*Chlamydia* LPS-FITC antibody and viewed by a fluorescence microscope (Figure 3.8 A). Typical inclusions were not observed, consistent with the observations of Mpiga *et al* and it is possible the punctuate fluorescence resembled aggregated *Chlamydia* organisms adhered to the cells. Further analysis with molecular assays to determine whether cells were infected was beyond the scope of this project.

The possibility also remains, however, that *C. trachomatis* and HIV-1 pathogens do not interact at all. The high levels of co-infection observed may reflect high risk sexual behaviour in distinct groups of HIV-1 positive MSM, with HIV-1 positive MSM

preferentially meeting other HIV-positive MSM for whom the imperative to practice safer sex has been diminished. Alternatively, there may be a selection bias, with clinicians selectively testing HIV-1 positive men, and HIV-1 positive men being more aware of LGV as a result of publicity generated through organisations such as the Terrance Higgins Trust (Ward *et al.*, 2007).

This study has also shown that HIV-1 significantly reduces the number, but increases the size, of *C. trachomatis* inclusions in cell culture. When cells were infected with HIV-1, they formed syncytia (multi-nucleated giant cells). A plausible explanation for these observations is that as multiple *C. trachomatis*-infected cells fuse together in an HIV-induced syncytium, multiple inclusions also fuse to form one “giant” inclusion. To our knowledge, this is the first documentation of *C. trachomatis* development within HIV-1 induced syncytia and it has important implications for intracellular chlamydial biology.

Firstly, despite its location within the inclusion, *C. trachomatis* maintains its intracellular niche through the interaction of bacterial proteins with host-cellular proteins in a co-ordinated fashion that can be remarkably complex. The organism can inhibit apoptosis, down-regulate MHC presentation, interact with the NF- κ B pathway, intercept vesicles budding from the Golgi network and multivesicular bodies, while excluding the fusion of vesicles from the lysosomal pathway (reviewed by Cocchiaro *et al.*, 2009). The observation that *C. trachomatis* replication is unaltered in syncytia implies the continued presence and function of these *Chlamydia*-host cell interactions, despite considerable cell-cell fusion and intracellular re-modelling. This observation, although surprising, is consistent with previous studies that demonstrate *C. trachomatis* serovar L2 inclusions fuse to form one large

inclusion in the absence of an intact cytoplasm when cells are treated with compounds that disrupt microtubules and microfilaments (Schramm *et al.*, 1995).

Secondly, the fusion of multiple *Chlamydia* inclusions is thought to be mediated by the inclusion membrane protein A (IncA). Micro-injection of antibodies against IncA results in the development of aberrant, multi-lobed inclusions (Hackstadt *et al.*, 1999) and isolates of *C. trachomatis* that occupy non-fusogenic inclusions lack IncA (Suchland *et al.*, 2000). The IncA protein contains two SNARE-like motifs which mimic members of the eukaryotic SNARE protein family that are involved in intracellular membrane fusion events (Paumet *et al.*, 2009). However, much remains unknown regarding inclusion membrane fusion, and the role of IncA. To our knowledge, fusion on the scale seen in this study has not been previously documented, demonstrating that the *C. trachomatis* fusion machinery is capable of generating structures larger than previously observed. The mature *Chlamydia* inclusion is, however, considered to be fragile (Scidmore, 2008). How can such large structures be maintained without lysis or collapse?

Recently, it was discovered that the *Chlamydia* inclusion is surrounded by a dynamic structural scaffold comprised of actin and intermediate filaments (Kumar & Valdivia, 2008). This scaffolding, or cage, is thought to provide structural support to the inclusion. Interestingly, as the inclusion grows, the *Chlamydia* protease CPAF cleaves the Head domain of the intermediate filaments and essentially ‘nicks’ the scaffolding in order to provide the cage with enough flexibility to permit inclusion expansion. Little else is known about this remarkable structure, but observations from this present study suggesting it has the capacity to stabilise structures larger than previously documented.

Finally, the mechanism of RB differentiation to EB remains unknown. In an intriguing model suggested by Hoare *et al.*, an RB is attached to the inclusion membrane by type 3 secretion (T3S) systems and, as the inclusion grows, the surface area contact between an RB and the membrane decreases, reducing the number of these attachments. The authors hypothesise that as the number of T3S systems attached to the inclusion decrease below a threshold, the RB is triggered to differentiate into an EB (Hoare *et al.*, 2008). In such a model, one might expect an increase in the differentiation of RBs to EBs in giant inclusions. As this is not observed in the present study, additional factors may play a role in RB differentiation.

Chapter 4

Quantification of rectal *Chlamydia trachomatis*

4.1 Introduction

The *C. trachomatis* load in the genital tract is reported to be associated with the presence of clinical symptoms (Geisler *et al.*, 2001; Michel *et al.*, 2007), transmissibility and persistence of infection (Geisler *et al.*, 2008; Rogers *et al.*, 2008) and the risk of developing chronic sequelae (Geisler *et al.*, 2001), however, there has been no study that investigates rectal chlamydial load.

Studies of MSM in the United Kingdom estimate the prevalence of rectal *C. trachomatis* (D-K) infection to be between 6.5 and 8.2% (Anan *et al.*, 2009; Benn *et al.*, 2007; Manavi *et al.*, 2004) and LGV to be ~1.0% (Anan *et al.*, 2009; Ward *et al.*, 2009). The majority (>80%) of individuals infected with LGV have rectal symptoms (Anan *et al.*, 2009; Hamill *et al.*, 2007), whereas the majority (>80%) of patients with serovar D-K infections are asymptomatic (Anan *et al.*, 2009; Kent *et al.*, 2005; Ward *et al.*, 2009).

Asymptomatic individuals are likely to be unaware of their infection, remaining undiagnosed and untreated, thus representing a reservoir of infection in the community. This is particularly important in MSM, as unprotected anal intercourse is not uncommon (Dodds *et al.*, 2007). It is not known, however, how infectious men with rectal *Chlamydia* are, or the extent to which they transmit infection. It has been postulated that individuals with a lower genital tract *C. trachomatis* load may be less infectious than those with a higher load (Wiggins *et al.*, 2009), so it would be of interest to know how the chlamydial load varies between individuals with rectal symptoms and asymptomatic infections, and between those with LGV and non-LGV infections. In addition, as HIV-1 and *C. trachomatis* co-infection is not uncommon, it would

be of interest to determine if rectal *C. trachomatis* load differs between HIV-1 sero-positive and HIV-1 sero-negative individuals.

Hypotheses

We hypothesised that the quantity of *C. trachomatis* shed from individuals with rectal symptoms would be higher than those with asymptomatic infection, the rectal load would be higher in patients infected with LGV serovars than non-LGV serovars and HIV-1 seropositive patients would shed more *C. trachomatis* than HIV-1 negative individuals.

Aims of the project:

This study aimed to develop a qPCR assay to determine *C. trachomatis* load in rectally infected individuals and investigate how load varies between symptomatically and asymptotically infected patients, HIV-1 sero-positive and HIV-1 sero-negative individuals and between those infected with LGV serovars and non-LGV serovars.

4.2 Results

4.2.1 Patient Recruitment

Figure 4.1 illustrates patient recruitment. Ninety-one patients were recruited to the study, however 5 were excluded as research swabs were unavailable. The qPCR assay was conducted on 86 patients (94.6%), 54 from the Bristol Sexual Health Centre (BSHC) and 32 from the Jefferiss Wing, Department of Genito-urinary medicine, St Mary's Hospital, London. Patient recruitment is detailed in Chapter 2, Section 2.26. Seventy-nine (91.9%) of the 86 subjects were male. The mean age was 33 years old (range 16 to 63), and 73 (84.9%) were Caucasian, 10 (11.6%) were non-Caucasian while the ethnicity of 3 (3.5%) was undocumented. Twenty-nine (33.7%) subjects were confirmed as being rectally infected with *C. trachomatis* by a positive NAAT test (Strand Displacement Assay, ProbeTec CT system, Becton Dickinson at St Mary's Hospital and the Aptima Combo 2, Genprobe, at BSHC), whereas 55 (64%) were found to be *C. trachomatis* negative by NAAT. Two patients (2.3%) did not have NAAT performed on rectal swabs, so were excluded from further analysis. Twenty-one (24.4%) of the patients were HIV-1 sero-positive, 13 (61.9%) of whom were also *C. trachomatis*-NAAT-positive.

Of the 29 *C. trachomatis*-NAAT-positive individuals, 28 were male and one was female, 23 were Caucasian, 5 were non-Caucasian and the ethnicity of one was undocumented. Six patients were between 16 and 24 years of age, while 23 were between 25 and 66 years of age. Four (14%) patients were from BSHC and 25 (86%) were from St Mary's Hospital. Ten (34.5%) patients presented with rectal symptoms (bleeding, discharge, discomfort, pain, constipation and tenesmus), but only 2 symptomatic patients were infected with LGV serovars.

A rectal swab was obtained from each patient for quantification of chlamydial load. Samples were collected, processed and stored as described (Chapter 2, Section 2.27). Proctitis was defined as greater than or equal to 5 polymorphonuclear leucocytes per high power (x1000) field of view when a Gram-stained rectal smear was viewed by light microscopy.

Figure 4.2 Patient Recruitment for the qPCR study

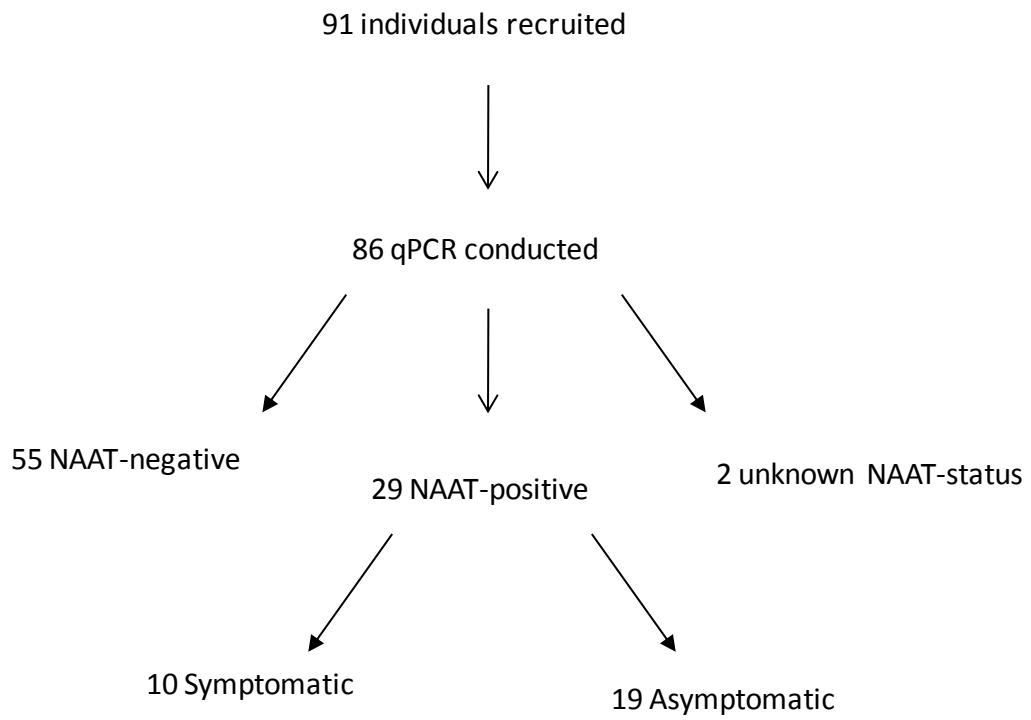


Figure 5.2 Patients were recruited as indicated. The number of patients in each category is shown.

4.2.2 Development of a qPCR assay to enumerate *C. trachomatis* load in patient samples

A qPCR was developed to enumerate *C. trachomatis* organisms in infected cell cultures as described (Chapter 3). The assay amplified a *C. trachomatis*-specific section of the *pmpH* gene and had an analytical sensitivity of 50 organisms per reaction, consistent with other published qPCR assays that target *pmpH* (Chen *et al.*, 2008). The *pmpH* gene, encoded by the chlamydial chromosome, is present in one copy per organism. Many qPCR assays, however, target the *Chlamydia* plasmid in order to improve sensitivity (Mahony *et al.*, 1993), as the plasmid is reported to be present in 7.72 copies per *Chlamydia* organism (+/- 0.68) (Michel *et al.*, 2007).

The majority of qPCR assays that target the chlamydial plasmid are aimed at *orf2* (Pickett *et al.*, 2005; Michel *et al.*, 2007) or *orf5* (Chen *et al.*, 2007; Alexander *et al.*, 2007; Chen *et al.*, 2008). In order to select the most appropriate target in the present study, the ability of primer pairs to amplify *orf2* and 5 regions were compared (Table 4.1). Briefly, DNA was extracted from 10-fold serial dilutions of purified *C. trachomatis* EBs. The DNA in each sample was amplified by qPCR in triplicate, with primers targeting either *orf2* (Pickett *et al.*, 2005), or *orf5* (Chen *et al.*, 2008). Primer sequences are tabulated in Table 2.1. The crossing points of the two targets were compared for each sample, but no significant difference observed (Table 4.1). It was decided that *C. trachomatis* plasmid *orf5* should be the target of choice as it encodes the *C. trachomatis* protein, Pgp3, which is the subject of Chapter 5.

Table 4.1 Selection of a suitable gene target of amplification

	<i>orf2</i> (SD)	<i>orf5</i> (SD)	Student's t-test <i>P</i> value
Sample 1	19.87 (0.09)	20.01 (0.93)	0.800
Sample 2	23.88 (0.03)	23.27 (0.50)	0.103
Sample 3	27.50 (0.30)	27.07 (0.59)	0.323

Table 4.1 The mean crossing points were determined for qPCR assays targeting *C. trachomatis* plasmid *orf2* and *orf5*. These were tabulated, along with the standard deviation (SD) and Student's t-test *P* value. Sample 1, 2 and 3 refer to EBs diluted 1 in 100, 1 in 1,000 and 1 in 10,000 in PBS.

In order to quantify the number of *orf5* copies in a sample, a standard curve was generated using a recombinant plasmid (pCTL11a) consisting of the full-length *C. trachomatis* serovar L1 cryptic plasmid (pLGV440) ligated into an *E. coli* vector (pAT153) (Hatt *et al.*, 1988), supplied by Professor Ian Clarke, Southampton University.

The concentration of pCTL11a (copy number/ml) was determined and the plasmid serially diluted 10-fold in nuclease-free water. Aliquots were added to a reaction mixture containing Taq polymerase, SYBR Green I dye, deoxyribose nucleotides, the *orf5* forward primer and reverse primer, and nuclease-free water. Standards were amplified in a LightCycler 2.0 instrument as described (Chapter 2, Section 2.29). The SYBR Green I dye fluoresced when bound to double-stranded DNA, giving rise to a sigmoidal amplification plot (Figure 4.1 A), described in Chapter 3, Section 3.2.2. As the plasmid was serially diluted, it took an increasing number of amplification cycles for a given fluorescence to be reached, so the sigmoidal curve shifted progressively to the right. The crossing point was determined and plotted against plasmid copy number to give a standard curve (Figure 4.1 B). A melting curve (Figure 4.1 C) and melting peak (Figure 4.1 D) were also constructed to characterise the specificity of the amplified products as described (Chapter 3, Section 3.2.2). The negative control (water) did not give rise to a sigmoidal amplification plot, a melting curve or a melting peak.

The analytical sensitivity of the *orf5* –qPCR assay was determined by diluting the pCTL11a recombinant plasmid to concentrations of 10,000, 1,000, 100, 50, and 10 copies per 2 μ l (the volume added to each lightcycler capillary) and amplifying the target DNA in quadruplicate. The lowest concentration of recombinant plasmid that amplified in all 4 replicates was 100 copies per reaction (Table 4.2) and, as there is an average of 7.72 *orf5* gene copies per *C. trachomatis* organism (+/- 0.68 copies) (Michel *et al.*, 2007), the threshold of detection for

the assay was 13 organisms per reaction. As swabs were placed into 2ml fluid and DNA was extracted from 700µl of this fluid into a total volume of 50µl, the threshold of detection was 929 organisms per swab (Materials and Methods).

Assay reproducibility was determined by amplifying 3 dilutions of DNA extracted from an aliquot of purified *C trachomatis* EBs (Samples 1, 2 and 3) six times in one experiment to determine intra-assay variation, and in 4 separate experiments to determine inter-assay variation. The mean crossing point, standard deviation and coefficient of variation per dilution were determined. Intra-assay variability and inter-assay variability were low, with co-efficient of variations ranging from 1.23-2.01% for intra-assay variation (Table 4.3) and 1.48-3.15% for inter-assay variation (Table 4.4).

Figure 4.1 The *C. trachomatis* plasmid *orf5* Standard curve

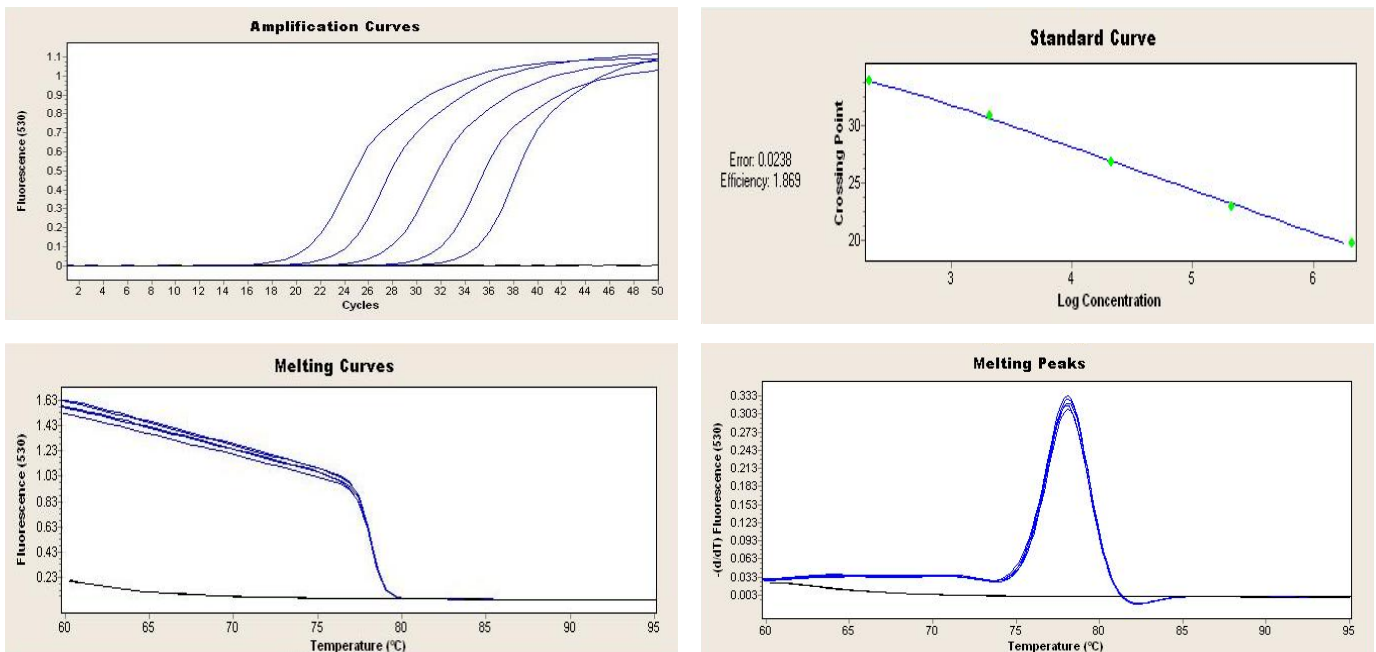


Figure 4.1 The pCTL11a construct was quantified and serially diluted 10-fold in nuclease-free water. For each dilution (10^6 - 10^2 copies per reaction), the amplification plot of fluorescence over cycle number (A) showed a typical sigmoidal curve, from which the crossing point was determined. A standard curve was generated of crossing point against DNA copy number (B). The melting curve (C) and melting peak (D) showed a single peak for the plasmid dilutions, whereas the negative control (H_2O) did not give rise to a sigmoidal amplification plot, a melting curve or melting peak.

Table 4.2 End point serial dilutions of pCTL11a

Number of orf5 copies	Number of <i>C. trachomatis</i> organisms	Repeats			
		1	2	3	4
10,000	1,300	+	+	+	+
1,000	130	+	+	+	+
100	13	+	+	+	+
50	7	+	+	-	-
10	1	+	-	-	-

Sensitivity = 13 organisms per reaction

Table 4.2 The pCTL11a construct was diluted in nuclease- free water to 10,000, 1,000, 200, 100, 50 and 10 copies per 2 μ l reaction and each dilution amplified in quadruplicate. As each *C. trachomatis* organism contains an average of 7.72 plasmids per organism (+/- 0.68), the dilutions contained 1,300, 130, 13, 7 and 1 organism.

Table 4.3 *orf5*-qPCR Intra- assay variation

Sample	Intra-assay variation		
	Mean crossing point	SD	Coefficient of variation (%)
1	19.86	0.30	1.50
2	24.51	0.49	2.01
3	28.08	0.35	1.23

Table 4.3 Three dilutions of DNA extracted from purified *C. trachomatis* EBs (samples 1, 2 and 3) were amplified 6 times using the *orf5* qPCR. The mean crossing point, standard deviation and coefficient of variation for each sample were determined.

Table 4.4 *orf5*-qPCR Inter-assay variation

Sample	Inter-assay variation		
	Mean crossing point	SD	Coefficient of variation (%)
1	19.35	0.42	2.17
2	23.63	0.75	3.15
3	27.57	0.41	1.48

Table 4.4 Three dilutions of DNA extracted from purified *C. trachomatis* EBs (samples 1, 2 and 3) were amplified in duplicate using the *orf5* qPCR in 4 independent experiments. The mean crossing point, standard deviation and coefficient of variation for each sample were determined.

4.2.3 Assay Sensitivity and Specificity

DNA was extracted from patient samples and amplified using a LightCycler 2.0 instrument. A standard curve was simultaneously amplified and used to quantify the number of *orf5* copies per ml of urine or per swab. The sensitivity of the assay was determined from a population of known *C. trachomatis*-NAAT-positive patients. The *orf5* qPCR was positive in 22 out of 29 NAAT-positive rectal swabs, giving rise to a sensitivity of 76% (95% confidence intervals (CI), 56-89%). The specificity of the assay was determined from a population of known *C. trachomatis*-NAAT-negative patients. The *orf5* qPCR was positive in 0 out of 55 NAAT-negative rectal swabs, giving rise to a specificity of 100% (95% CI, 92-100%).

4.2.4 Inhibition Assay

As the DNA extracted from 7 NAAT-positive rectal swabs failed to amplify with the *orf5* qPCR, the existence of PCR inhibitors was investigated by use of a lambda (λ)-phage inhibition assay. Briefly, DNA from the λ -phage was amplified in a qPCR reaction that was spiked with patient samples. The crossing point of the λ -phage PCR-product in the presence of a patient sample was compared buffer alone and no significant difference observed (Student's t-test *P* values ranging from 0.27 to 0.84) (Table 4.5). It is, therefore, unlikely that PCR inhibition is responsible for the failure of these samples to amplify, and more probable that patients were infected with chlamydial loads below the limit of assay detection

Table 4.5 λ -phage Inhibition Assay

Spiked condition	Mean crossing point of lambda phage DNA (SD)	Student's t-test <i>P</i> value
Buffer alone	29.22 (0.23)	
Patient 1	29.01 (0.02)	0.27
Patient 2	29.18 (0.17)	0.84
Patient 3	28.97 (0.06)	0.22
Patient 4	29.27 (0.25)	0.82
Patient 5	29.34 (0.11)	0.53
Patient 6	29.41 (0.26)	0.41
Patient 7	29.32 (0.02)	0.60

Table 4.5 The existence of PCR inhibitors in 7 NAAT-positive patient samples that failed to amplify by *orf5*-qPCR was investigated by use of a λ -phage inhibition assay. DNA from λ -phage was amplified by qPCR and the reaction spiked with patient samples. The crossing point of the λ -phage PCR-product in the presence of a patient sample was compared to the crossing point in the presence of buffer alone.

4.2.6 Statistical Analysis

The number of *C. trachomatis* organisms per swab was determined for each patient and log-transformed. The \log_{10} mean chlamydial load of different patient groups (\pm standard deviation (SD)) was tabulated (Table 4.6) and compared by parametric statistical tests (Student's t-test (2-tailed significance, assuming equal variances)). It is known from previous work that NAAT-positive clinical specimens can contain chlamydial loads below the detection limit of detection of a qPCR assay (Michel *et al.*, 2007; Wiggins *et al.*, 2009). We therefore, assumed that where a patient was negative by the qPCR assay, but NAAT-positive, these individuals were indeed true positives and that the *C. trachomatis* load was below the limit of detection our assay. For the purposes of analysis we assumed this to be half the detection limit (i.e. 470 organisms per swab, or $2.67 \log_{10}$ organisms per swab).

4.2.7 Chlamydial loads in different specimens

The geometric mean *C. trachomatis* rectal load for all NAAT-positive patients was 5.0×10^5 organisms per swab (SD, 1.52). There was no significant difference in the *C. trachomatis* load when patients were grouped according to gender, ethnicity, age, or exposure to *N. gonorrhoeae* (Table 4.6). Patients with proctitis had a significantly higher chlamydial load ($6.43 \log_{10}$ organisms per swab (SD, 1.18) compared to patients with no proctitis ($3.39 \log_{10}$ organisms per swab (SD, 1.25), Student's t-test $P = 0.038$ (Table 4.6 and Figure 4.2). There was, however, no significant difference in the load in patients with rectal symptoms ($4.54 \log_{10}$ organisms per swab (SD, 1.48)) and individuals with an asymptomatic infection ($4.74 \log_{10}$ organisms per swab (SD, 1.72)), Student's t-test $P = 0.761$ (Table 4.6 and Figure 4.3, discussed in Section 4.3).

The *C. trachomatis* load in patients with LGV (6.06 log₁₀ organisms per swab (SD, 0.27)) was higher than non-LGV infection (4.50 log₁₀ organisms per swab (SD, 1.51)), however this was not significant (Student's t-test $P = 0.163$) (Table 4.6 and Figure 4.4). There was also no significant difference in the *C. trachomatis* load in HIV-1 sero-positive individuals (4.20 log₁₀ organisms per swab (SD, 1.43)) compared to HIV-1 seronegative individuals (5.10 log₁₀ organisms per swab (SD, 1.58)), Student's t-test $P = 0.125$ (Table 4.6 and Figure 4.5).

Table 4.6 Mean *C. trachomatis* loads, by patient group

Study group	<i>n</i>	Log ₁₀ mean <i>C. trachomatis</i> load (Log ₁₀ organisms per swab (SD))	Student's t-test <i>P</i> value
Gender			0.318
Men	28	4.50 (1.20)	
Women	1	5.77	
Ethnicity			0.813
Caucasian	23	4.92 (2.11)	
Non-Caucasian	5	4.74 (1.44)	
Age			0.879
16-24 yr	6	4.61 (2.27)	
25-66 yr	23	4.72 (1.39)	
<i>N. gonorrhoeae</i> exposure			0.066
<i>N. gonorrhoeae</i> +	3	5.09 (2.57)	
<i>N. gonorrhoeae</i> –	26	4.65 (1.48)	
Inflammation			0.038
Proctitis	3	6.43 (1.18)	
No Proctitis	3	3.39 (1.25)	
Rectal symptoms			0.761
Symptomatic	10	4.54 (1.48)	
Asymptomatic	17	4.74 (1.72)	
LGV status			0.163
LGV +	2	6.06 (0.27)	
LGV –	26	4.50 (1.51)	
HIV-1 status			0.125
HIV-1 +	13	4.20 (1.43)	
HIV-1 –	16	5.10 (1.58)	

Table 4.6. Patients were grouped according to gender, ethnicity, age, and the presence or absence of *N. gonorrhoeae*, inflammation, symptoms, LGV or HIV-1. The number of patients in each group (*n*) and the log₁₀ mean chlamydial load (Standard deviation (SD)) and the Student's t-test *P* value were tabulated.

Figure 4. 2 Patients with proctitis shed significantly more *C. trachomatis* than patients with no evidence of inflammation

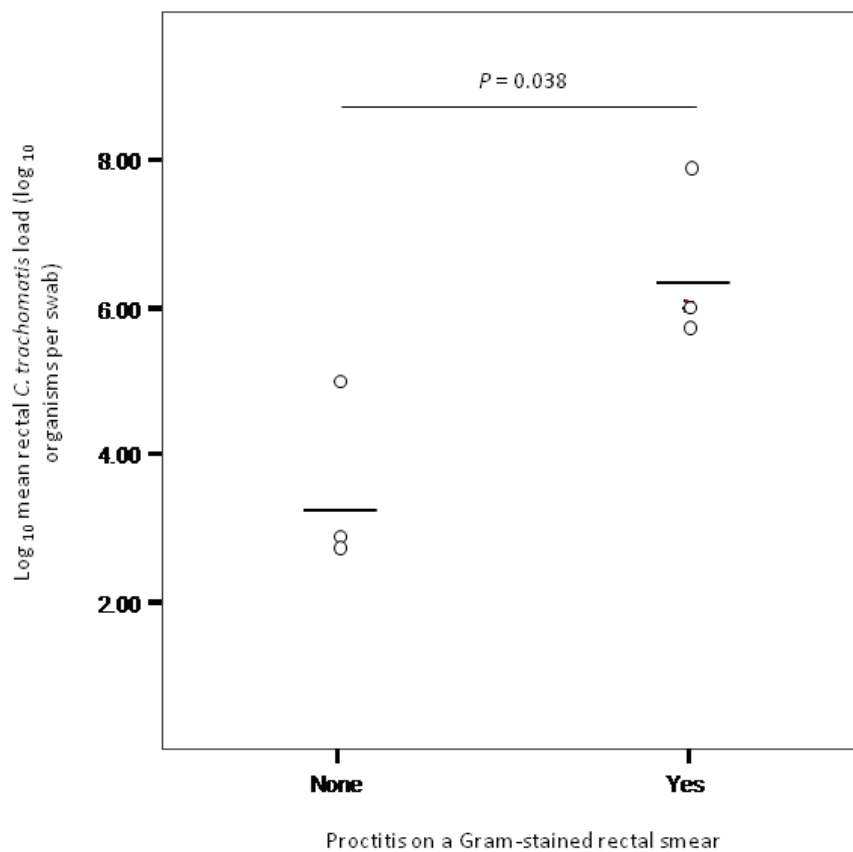


Figure 4.2 The Log₁₀ mean *C. trachomatis* load (Log₁₀ organisms per swab) was determined for patients who had proctitis or no proctitis

Figure 4.3 Patients with rectal symptoms do not shed a significantly different amount of *C. trachomatis* as asymptomatic individuals

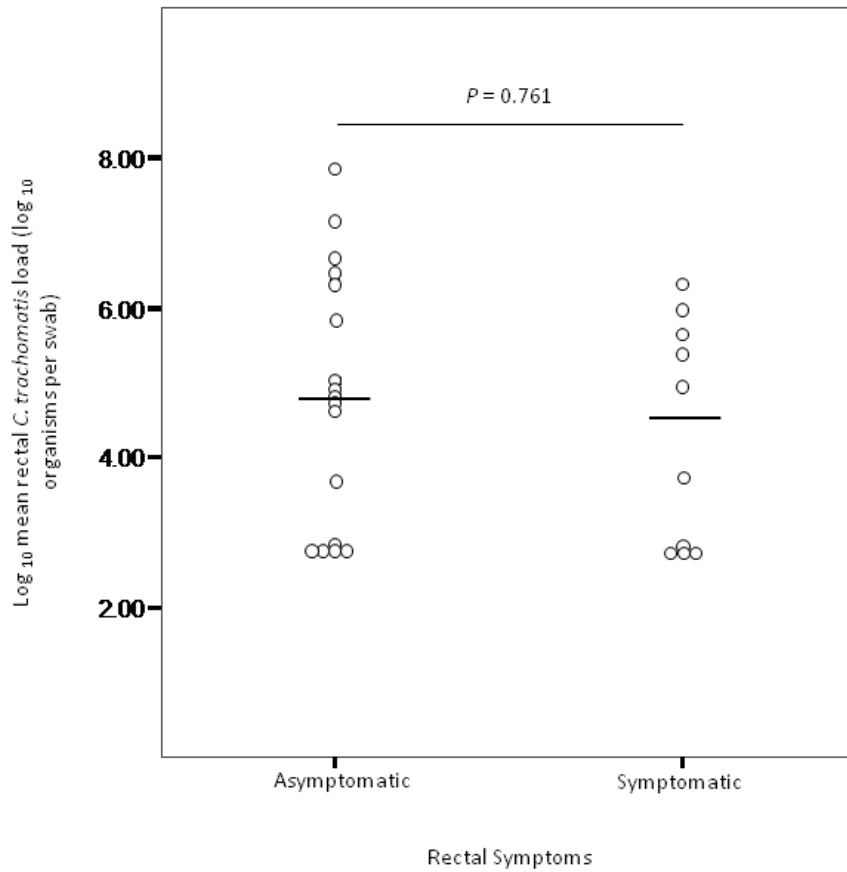


Figure 4.3 The Log₁₀ mean *C. trachomatis* load (Log₁₀ organisms per swab) was determined for patients who had rectal symptoms or no rectal symptoms.

Figure 4.5 There is no significant difference in the *C. trachomatis* load between HIV-1 sero-positive and sero-negative individuals

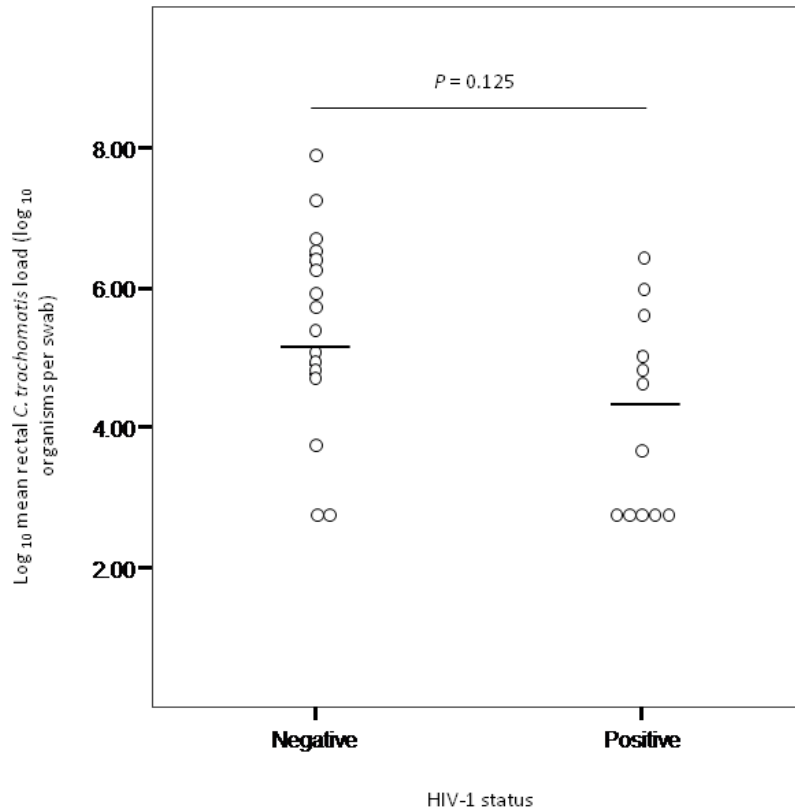


Figure 4.5 The Log₁₀ mean *C. trachomatis* load (Log₁₀ organisms per swab) was determined for patients who were HIV-1 sero-positive and sero-negative.

4.3 Discussion

This study developed a qPCR assay and quantified the rectal *C. trachomatis* load in patients who were *C. trachomatis*-NAAT-positive. The geometric mean rectal load was 5.0×10^5 organisms per swab (Standard Deviation, 1.52) and patients with proctitis had a significantly higher chlamydial load compared to patients who did not have proctitis. There was, however, no significant difference in the load between patients who presented with rectal symptoms and individuals with an asymptomatic infection. Patients with LGV were found to have high *C. trachomatis* loads, but there was no significant difference in the chlamydial load between HIV-1 sero-positive and sero-negative individuals.

The geometric mean rectal *C. trachomatis* load found in this study (5.0×10^5 organisms per swab (SD, 152)) is approximately 50-100 fold higher than the geometric mean *C. trachomatis* load reported in the female genital tract. Michel *et al.*, 2007 found an average of 2.2×10^4 organisms per swab using endocervical swabs (95% CI, 2.5×10^1 - 5.0×10^6), and 3.9×10^3 organisms per swab using self-collected vaginal swabs (95% CI, 1.6×10^1 - 9.7×10^5). Wiggins *et al.*, 2009 also reported a mean chlamydial load of 1.0×10^4 organisms per swab in self-collected vulvovaginal swab samples (95% CI, 5.2×10^3 - 2.1×10^4).

The reason for this difference between rectal and genital tract load is unknown. Different mucosal surfaces may vary in how permissive they are to *C. trachomatis* infection (Miyairi *et al.* 2006; Dessus-Babus *et al.* 2008) which could explain the observation, but there are no published data on growth in rectal mucosal cells compared to cervical cell culture.

At St. Mary's Hospital only male patients were recruited who were at high risk of having rectal *C. trachomatis* infection, whereas at Bristol men and women practising anal intercourse were recruited. It is possible that individuals with higher loads were over represented in this study compared to an unselected population practising anal sex. In addition, as only one *Chlamydia*-positive woman was included in this study, it is also possible that rectal loads are higher in men than women. This is, however, unlikely, as *Chlamydia* load in first voided urine specimens is similar in asymptomatic men and women (Wiggins *et al.*, 2009). A possible implication of this observation may be that patients rectally infected with *C. trachomatis* may be more infectious per episode of sexual intercourse than women with a genital tract infection.

To be confident of these data, the qPCR assay described in this chapter was validated by extracting the DNA from first void urine (FVU) samples from 5 male patients with a urethral *C. trachomatis* infection, identified by a positive-NAAT result. These patients were recruited as part of the study described in Chapter 5. The DNA was amplified in the assay described above and the geometric mean *C. trachomatis* load found to be 2.69×10^4 organisms per ml urine. Michel *et al.*, (2007) report a geometric mean load of 1.2×10^4 organisms per ml urine in men. This figure is similar to our own.

The analytical sensitivity of the assay was only 13 organisms per reaction. This is surprising as the assay utilised published primers, documented as capable of detecting 1-10 copies of the plasmid per reaction (Chen *et al.*, 2008), the qPCR efficiency was adequate (1.866) and there were no evidence of primer dimers. The assay presented by Chen *et al.*, 2008 utilised Taqman^(R) probe technology and a real-time PCR instrument (Rotor-gene 3000; Corbet Research), whereas the assay presented in our study utilised SYBR^(R) green technology and a

Lightcycler 2.0 instrument (Roche). While it is unlikely that these differences gave rise to the observed differences in sensitivity, SYBR^(R) green has been documented to inhibit PCR reactions (Nath *et al.*, 1999; Monis *et al.*, 2005) and may have contributed to the reduced sensitivity. It would be prudent in the future to repeat the quantification of *C. trachomatis* by using a Taqman^(R)- based assay or using an alternative gene target with a higher analytical sensitivity. It is of interest that 7 individuals had undetectable *Chlamydia* load in their rectal specimens. This may be a true observation, or a sampling error as many of the rectal specimens were taken without the use of a proctoscope. As they were *Chlamydia* NAAT-positive we assumed the load was half the cut-off. This enabled us to use parametric statistics by converting the load to logarithms (Wiggins *et al.* 2006).

Patients with proctitis had significantly higher *C. trachomatis* loads than individuals without proctitis in this study. This observation is consistent with reports that chlamydial load is associated with an inflammatory response (Geisler *et al.*, 2001; Horner, 2007; Michel *et al.*, 2007). As LGV is associated with proctitis (Anan *et al.*, 2009; Hamill *et al.*, 2007; Ward *et al.*, 2007), one would expect infected patients to have high *C. trachomatis* loads. Consistent with this observation, both patients with LGV in this study were symptomatic and had high *C. trachomatis* rectal loads (7.5×10^5 and 1.83×10^6 organisms per swab respectively).

In the genital tract, both the degree of inflammation and presence of symptoms are associated with *C. trachomatis* infection (Geisler *et al.*, 2001; Michel 2007 *et al.*, and Wiggins *et al.*, 2009). In men with urethral infection, those with greater inflammation are also more likely to be symptomatic (Wiggins *et al.*, 2006 and Horner, 2007). In this study, however, an increased *C. trachomatis* load was not significantly associated with the presence of rectal symptoms. The observation does, however, demonstrate that asymptomatic individuals shed as much *C.*

trachomatis as patients with symptoms. These individuals could be a reservoir of infection and maintain chlamydial transmission in the community.

Individuals who were HIV-1 sero- positive were found to shed as much *C. trachomatis* as HIV-1 sero-negative individuals. This is consistent with the recent observation of Soni *et al.*, 2010, who failed to observe an association of HIV-1 infection with *C. trachomatis* infection in MSM. It should be noted, however, that the HIV-1 viral load, duration of viral infection, and the CD4 count remain unknown in these patients, nor is it known how many are on anti-retroviral therapy. It is, therefore, not possible to draw conclusions regarding the effect of HIV-1 immunosuppression on chlamydial load.

In conclusion, this study found that patients with rectal *C. trachomatis* infection have high loads relative to what is found in a genital tract infection. Moreover, asymptomatic individuals shed a similar quantity of *C. trachomatis* as patients with rectal symptoms suggesting that they are as infectious and likely to maintain transmission in the community.

Chapter 5

Characterisation of *ex vivo* cellular immune responses to *Chlamydia trachomatis*

5.1 Introduction

Animal models have shown that clearance of a *Chlamydia* infection is dependent on the generation of IFN- γ as part of a cell-mediated immune response (Brunham & Rey-Ladino 2005). Unfortunately, data from mouse- models using *C. muridarum* cannot be directly extrapolated to *C. trachomatis* because of fundamental differences in the biology of the organisms (reviewed by Brunham & Rey-Ladino 2005). Consequently, research has also been conducted using PBMCs or synovial fluid mononuclear cells (SFMCs) from human patients infected with *C. trachomatis*, or from patients with SARA (Brunham & Rey-Ladino 2005). These studies, however, typically use *in vitro* expanded T-cell clones which, not only are less physiologically relevant, but also limit any immunological assessment to the one cell from which the clonal population was derived. In addition, every antigen in which T-cell epitopes have been identified contains a homologue in the common respiratory pathogen, *C. pneumoniae*. Consequently, there have been no studies investigating *ex vivo* human cellular immune responses to *C. trachomatis*-specific antigens.

An IFN- γ ELISpot assay is one means of quantifying *ex vivo* antigen-specific cellular immune responses by enumerating T-cells in the peripheral blood of patients that recognise, and respond to, specific antigens by secreting IFN- γ (Lalvani *et al.*, 1997). Interestingly, *ex vivo* *Mycobacterium tuberculosis*- specific IFN- γ responses can identify a current infection, as once an infection has been cleared, the number of T-cells circulating in the peripheral blood that recognise *M. tuberculosis*-specific antigens decline (Pathan *et al.*, 2001; Millington *et al.*, 2007). Both *M. tuberculosis* and *C. trachomatis* are intracellular pathogens in which IFN- γ is believed to play an important role in the immune response (reviewed by Cooper, 2009 and Brunham Rey-Ladino, 2005). Therefore, quantification of PBMCs that

recognise *C. trachomatis*-specific antigens and secrete IFN- γ could potentially be useful as a biomarker for current *C. trachomatis* infection.

Such a biomarker is desirable as, although *C. trachomatis* at the lower genital tract, rectum or oropharynx can be detected by NAAT with swabs or urine samples, the organism is capable of migrating to the endometrium, salpinges and peritoneal cavity of women, epididymi and testicles of men, and the joints of those afflicted with sexually acquired reactive arthritis (SARA). The extent to which infection at these sites is identified by the use of lower genital tract or rectal samples is unknown (Taylor-Robinson *et al.*, 2009). An *ex vivo* IFN- γ ELISpot assay may be of benefit in detecting such infection.

Hypotheses

C. trachomatis-specific T-cells that secrete IFN- γ can be quantified from the peripheral blood of patients during a *C. trachomatis* infection and the *C. trachomatis*- specific IFN- γ is a useful biomarker for infection.

Aims of the Project

This pilot study aimed to identify a *C. trachomatis*-specific antigen recognised by the cellular immune response, recruit *C. trachomatis*-infected patients and uninfected negative controls, and develop an IFN- γ ELISpot assay to quantify *C. trachomatis*-specific IFN- γ in different patient groups. These aims were developed in order to characterise the immunogenicity of the selected antigen, investigate the dynamics of the human T-cell response during a *C. trachomatis* infection, and determine whether *C. trachomatis*-specific IFN- γ is a useful biomarker for infection.

5.2 Results

5.2.1 The IFN- γ ELISpot assay antigen

The Pgp3 antigen (PubMed Accession number: YP_001569038), encoded by *orf 5* of the chlamydial plasmid (Hatt *et al.*, 1988) (PubMed Accession number: NC_010029) was selected for use in the ELISpot assay as the protein is *C. trachomatis*-specific and recognised by acquired immune responses (antibodies) (Chapter 1, Section 1.7).

Fifty one overlapping peptides, spanning the entire length of the Pgp3 protein (Table 5.1) were purchased (GenScript, USA). Overlapping peptides were used to ensure the observed IFN- γ responses were not restricted to, or biased by, certain HLA types. Each peptide was 15 residues in length (termed a “15mer”), with the exception of the last peptide (that was 14 residues in length), and the sequence of each peptide overlapped the previous by 10 residues, with the exception of the first peptide. Peptides were pooled in 4 groups of 10 peptides and one group of 11 peptides.

Table 5.1 Pgp3 overlapping peptide sequences

Pool	Peptide	Residues	Amino acids
1	1	1-16	MGNSGFYLYNTQNCV
	2	6-20	FYLYNTQNCVFADNI
	3	11-25	TQNCVFADNIKVGQM
	4	16-30	FADNIKVGQMTPEPLK
	5	21-35	KVGQMTPEPLKDQQII
	6	26-40	TEPLKDQQIILGTTS
	7	31-45	DQQIILGTTSTPVAA
	8	36-50	LGTTSTPVAAKMTAS
	9	41-55	TPVAAKMTASDGISL
	10	46-60	KMTASDGISLTVSNN
2	11	51-65	DGISLTVSNNPSTNA
	12	56-70	TVSNNPSTNASITIG
	13	61-75	PSTNASITIGLDAEK
	14	66-80	SITIGLDAEKAYQLI
	15	71-85	LDAEKAYQLILEKLG
	16	76-90	AYQLILEKLGQILG
	17	81-95	LEKLGQILGGIADT
	18	86-100	DQILGGIADTIVDST
	19	91-105	GIADTIVDSTVQDIL
	20	96-110	IVDSTVQDILDKITT
3	21	101-115	VQDILDKITTDPSLG
	22	106-120	DKITTDPSLGLLKAF
	23	111-125	DPSLGLLKAFNNFPI
	24	116-130	LLKAFNNFPITNKIQ
	25	121-135	NNFPITNKIQCNGLF
	26	126-140	TNKIQCNGLFTPRNI
	27	131-145	CNGLFTPRNIETLLG
	28	136-150	TPRNIETLLGGTEIG
	29	141-155	ETLLGGTEIGKFTVT
	30	146-160	GTEIGKFTVTPKSSG
4	31	151-165	KFTVTPKSSGSMFLV
	32	156-170	PKSSGSMFLVSADII
	33	161-175	SMFLVSADIIASRME
	34	166-180	SADIIASRMEGGVVL
	35	171-185	ASRMEGGVVLALVRE
	36	176-190	GGVVLALVREGDSKP
	37	181-195	ALVREGDSKPYAISY
	38	186-200	GDSKPYAISYGYSSG
	39	191-205	YAISYGYSSGVPNLC
	40	196-210	GYSSGVPNLCSLRTS
5	41	201-215	VPNLCSLRTRINTG
	42	206-220	SLRTRINTGLTPTT
	43	211-225	IINTGLTPTTYSLRV
	44	216-230	LTPTTYSLRVGGLES
	45	221-235	YSLRVGGLESGVVWV
	46	226-240	GGLESGVVVNALS
	47	231-245	GVVWVNALSNGNDIL
	48	236-250	NALSNGNDILGITNT
	49	241-255	GNDILGITNTSNVSF
	50	246-260	GITNTSNVSFLEVIP
	51	251-264	SNVSFLEVIPQTNA

Table 5.1 Fifty-one overlapping peptide sequences spanning the entire length of the Pgp3 protein (residues 1-246). Peptides were pooled into 4 groups of 10 peptides and 1 group of 11.

5.2.2 Patient Recruitment

Figure 5.1 illustrates patient recruitment. Eighty five patients were successfully recruited from St Mary's Hospital, London. Sixty nine adults were recruited from the Jefferiss Wing, Department of Genito-urinary medicine (GUM) and sixteen children, under the age of 12, were recruited as negative controls from the Paediatric Outpatients "GP bloods" clinic, Imperial College Healthcare NHS Trust.

Initially, patient recruitment was limited to the GUM clinic and negative controls were men who tested *C. trachomatis*-negative by NAAT (GUM negative controls). However, these individuals had a history of sexual intercourse, and may not represent an unexposed population. It was, therefore, necessary to recruit negative controls from a population who were less likely to have been exposed to *C. trachomatis*. Children under the age of 12 were selected as a suitable control population (Paediatric negative controls).

Adults recruited to the study (n = 69) were all male. The mean age was 34 years old (range 18 to 77) and the patients were from ethnically diverse backgrounds: 21 (30.4%) were British, 4 (5.8 %) were African, 4 (5.8 %) were from the Indian Subcontinent, 8 (11.6 %) were from the Caribbean, 27 (39.1 %) were whites of other nationalities, while there were no data on the ethnicity for 5 (7.2 %) patients. Twenty two (32%) of the 69 recruited men were confirmed as being infected with *C. trachomatis* by a positive NAAT test (Strand Displacement Assay, ProbeTec CT system, Becton Dickinson) (NAAT-positive) whereas 47 (68%) were found to be *C. trachomatis* negative by NAAT (NAAT-negative). Eleven (15.9%) of the patients were HIV-1 sero-positive, 6 (55%) of whom were also *C. trachomatis*-NAAT-positive. Men were recruited if they had symptoms and/or signs of

urethritis or proctitis (symptomatic), or if they had no symptoms and no detectable inflammation (asymptomatic). The signs or symptoms of urethritis included urethral discharge and or dysuria, whereas signs or symptoms of proctitis included rectal discomfort, discharge, or bleeding. Ten (45%) of the 22 NAAT-positive individuals were symptomatic (5 with proctitis and 5 with urethritis), while twelve (55%) were asymptomatic. Fifteen (32%) of the 47 NAAT-negative individuals were symptomatic (7 with proctitis and 8 with urethritis), while 32 (68%) were asymptomatic.

Children recruited to the study (n = 16) were all male to match the adult population. The mean age was 6 years old (range 2 to 10) and subjects were from ethnically diverse backgrounds: 2 (12.5 %) were British, 4 (25 %) were African, 1 (6.3 %) was from the Indian Subcontinent, 5 (31.3 %) were whites of other nationalities and 4 (25 %) were non-whites of other nationalities. Children who were born in, or had travelled to, areas where Trachoma is endemic were excluded from the study, as were children with signs or symptoms of acute or chronic inflammatory conditions.

Samples were collected, processed and stored as described (Chapter 2, Section 2.27).

Figure 5.1 ELISpot study Patient Recruitment

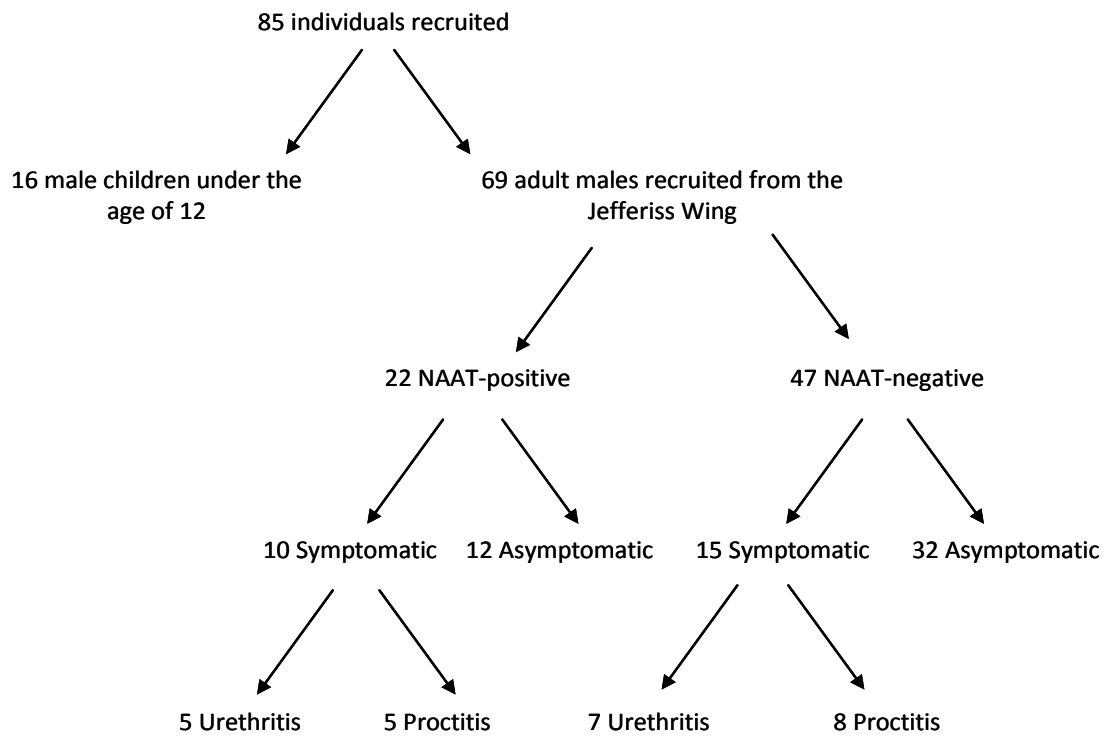


Figure 5.1 Patients were recruited as indicated. The number of patients in each category is shown.

5.2.3 Development of the *C. trachomatis ex vivo* IFN- γ ELISpot Assay

The *ex vivo* IFN- γ ELISpot assay was conducted as described (Chapter 2, Section 2.32), Figure 5.2. Briefly, the assay was conducted in ELISpot plates lined with a membrane that was coated with anti-IFN- γ antibody and blocked with complete medium. Patient PBMCs (2.5×10^5) were added to each well and incubated overnight at 37°C, 5% CO₂, in the presence of Pgp3 peptide pools, complete medium alone (negative control), or the mitogen Phytohaemagglutinin (Pha) (positive control). Where PBMCs were from an infected patient, cells recognised the Pgp3 peptides and secreted IFN- γ that was captured by the antibody coating the well. An additional anti-IFN- γ antibody, conjugated to alkaline phosphatase (ALP), was added to each well followed by ALP substrate (BCIP/NBT). ALP hydrolysed the substrate to a coloured product that formed a spot on the membrane where a cell had produced IFN- γ . For each patient, the number of spots in 14 wells was counted: 5 wells containing one peptide pool each, one well containing unstimulated PBMCs in medium alone and one well containing mitogen-stimulated cells, with the assay being conducted in duplicate (Figure 5.3).

The average number of spots per peptide pool was determined for each patient, and pools were scored as positive if the average of the duplicate test wells was at least 5 spots more than the average of the unstimulated negative control wells, and this number was at least twice that of the negative control well average. This threshold, or “cut-off” is routinely used in *ex vivo* ELISpot studies (Lalvani *et al.*, 2001a and b; Pathan *et al.*, 2001; Chapman *et al.*, 2002; Millington *et al.*, 2007). The average number of spots in each positive pool was summated and expressed as the number of spot-forming cells (SFCs) per million PBMCs that responded to Pgp3 peptides.

Two types of ELISpot plates were used during the course of the study, one that was supplied pre-coated with anti- IFN- γ monoclonal antibody (pre-coated) (Mabtech, SE) and one that was not (non-pre-coated) (Millipore, UK). In the latter, plates were manually coated with anti- IFN- γ monoclonal antibody. The number of SFCs per million PBMCs that responded to Pgp3 peptides was determined for 3 patients using both plates and recorded graphically (Figure 5.4). No significant difference was observed between the number of SFC per million PBMC in the pre-coated and non-pre-coated plates for any of the patients tested. Data from both types of plate could, therefore, be analysed together.

Figure 5.2 A schematic of the ELISpot Assay

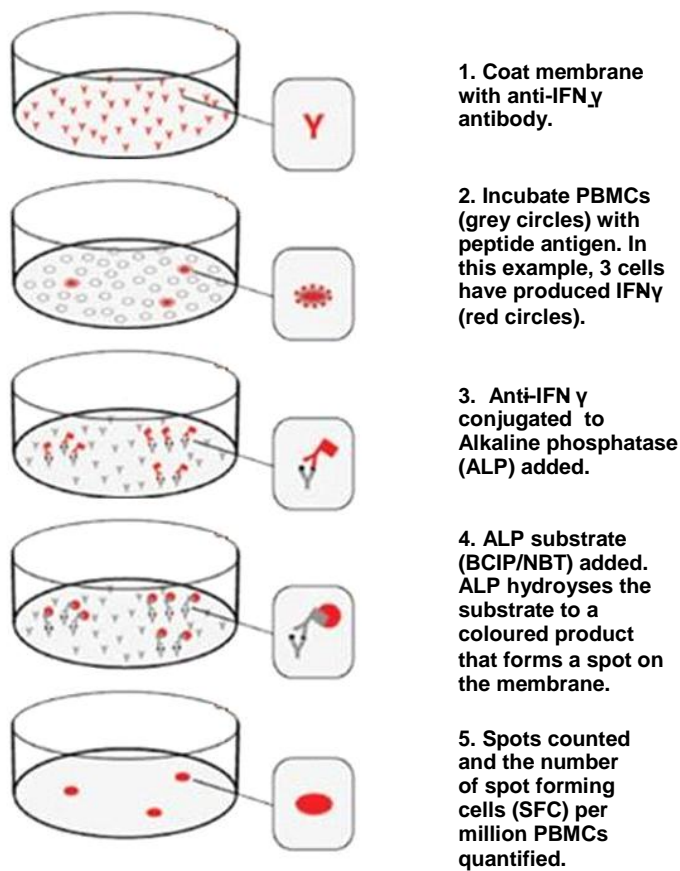
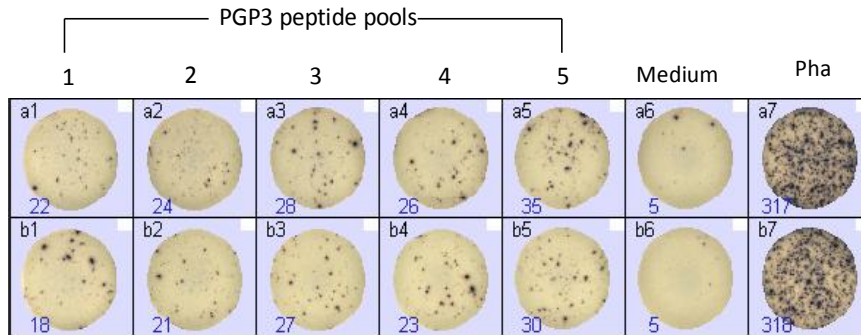


Figure 5.2 A schematic of the ELISpot assay (adapted from Mabtech, SE). (1) ELISpot plates were coated with anti-IFN- γ antibody and blocked with complete RPMI. (2) Patient PBMCs were added to each well in the presence of Pgp3 peptides. (3) Anti-IFN- γ conjugated to ALP was added to each well followed by (4) the addition of ALP substrate (BCIP/NBT). ALP hydrolysed the substrate to a coloured product that formed a spot on the membrane. (5) Spots were counted and the number of spot forming cells (SFC) per million PBMCs determined.

Figure 5.3 Example ELISpot data

Pgp3 ELISpot- positive Patient



Pgp3 ELISpot- negative Patient

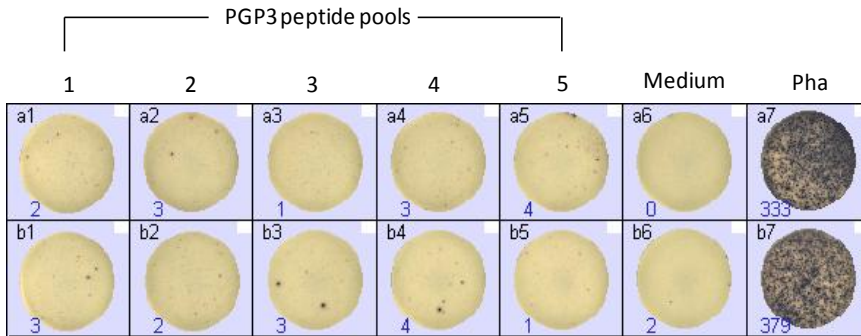


Figure 5.3 Example data for a positive patient (A) and a negative patient (B). Five pools of Pgp3 peptides were added to duplicate wells containing PBMCs (wells 1, 2, 3, 4 and 5). Complete medium was added to one pair of wells (medium) and the mitogen Pha was added to another pair of wells (Pha) as negative and positive controls respectively. Cells were incubated at 37°C overnight, the ELISpot plate was developed and the number of spots counted. For patient A, the average number of SFC per 2.5×10^5 cells was 20.4 (82 SFC per million PBMC). For patient B, no membrane contained more than the cut-off and the average number of SFC per million PBMC was considered to be 0.

Figure 5.4 Pre-coated Vs non-pre-coated plates

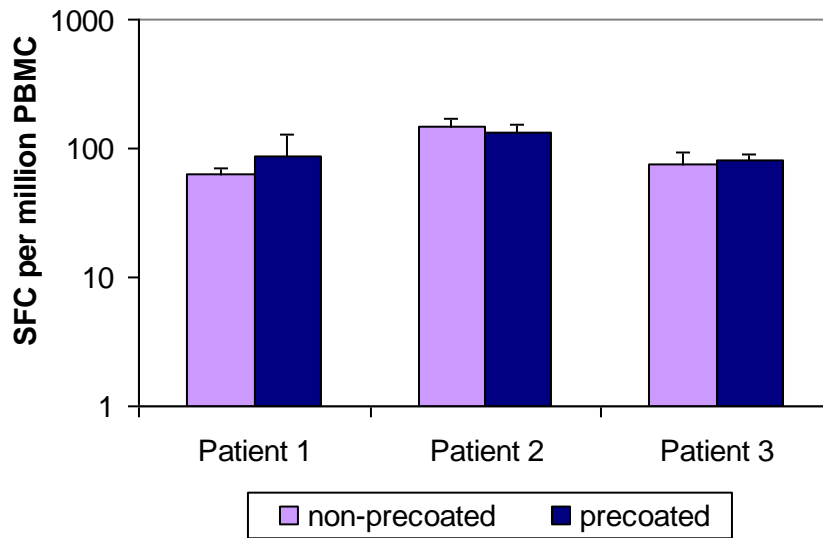


Figure 5.4 The number of SFCs per million PBMCs that responded to Pgp3 peptides was determined for 3 patients using ELISpot plates that were either supplied pre-coated with anti-IFN- γ antibody (blue bars) or were manually coated (non-precoated, pink bars).

5.2.4 Pgp3 is a target of the human cellular immune response to *C. trachomatis*

The number of SFCs per million PBMCs that recognised Pgp3 peptides was quantified and plotted graphically for *C. trachomatis*-NAAT-positive men, asymptomatic NAAT-negative men (GUM negative controls), and paediatric negative controls (Figure 5.5). The threshold for a positive ELISpot result was 5 SFC per 2.5×10^5 cells (20 SFC per million PBMC).

Thirteen (59%) of 22 *C. trachomatis*-NAAT-positive patients possessed T-cells that produced IFN- γ in response to Pgp3 peptide stimulation, compared with only 8 (25%) of 32 GUM negative controls ($\chi^2 P = 0.01$) and 3 (18.8%) of 16 paediatric negative controls ($\chi^2 P = 0.01$), demonstrating not only that Pgp3 is a target of the cellular immune response during a *C. trachomatis* infection, but also that T-cells recognising a *C. trachomatis*-specific antigen could be enumerated from the peripheral blood of infected humans *ex vivo*. However, as 41% of NAAT-positive individuals were ELISpot-negative and 19% of paediatric negative controls were ELISpot-positive, the assay sensitivity was calculated to be only 59% (95% confidence intervals (CI), 39-77%) and specificity 81% (95% CI, 57-93%).

The frequency of T-cells that recognised and responded to Pgp3 was low, with a median of only 44 SFC per million PBMCs (interquartile range (IR) 26.8-63) in *C. trachomatis*-NAAT-positive, ELISpot-positive individuals. In contrast, IFN- γ responses to *M. tuberculosis*-specific antigens are higher in individuals with TB (median 200 SFC per million PBMCs, interquartile range 105-596 (Lalvani *et al.*, 2001b)). The magnitude of the ELISpot response in *C. trachomatis*-NAAT-positive individuals was not significantly different to GUM negative controls (60.5 SFC per million PBMCs (IR 35.2-78.4), Mann-Whitney U $P = 0.55$) or paediatric negative controls (36 SFC per million PBMCs (IR 34-42), Mann-Whitney U $P = 0.8$).

Figure 5.5 Pgp3 is a target of the human cellular immune response to *C. trachomatis*

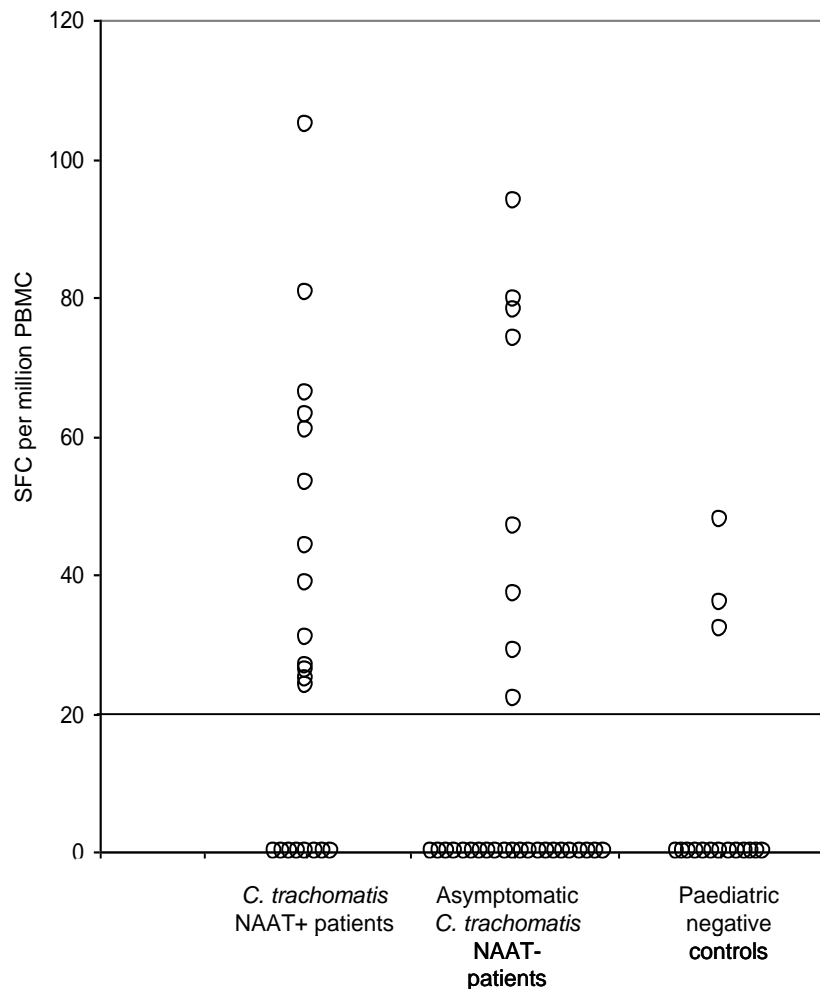


Figure 5.5 The number of SFCs per million PBMCs was quantified and plotted graphically for *C. trachomatis*-NAAT positive men, asymptomatic *C. trachomatis*-negative men (GUM negative controls), and paediatric negative controls. Each circle represents an individual patient. For each individual, the number of T cells that recognised Pgp3 peptides was summated. Circles on the baseline represent individuals with no response to any of the Pgp3 peptide pools. The horizontal line represents the pre-defined cut-off point (20 Pgp3-specific T cells per million PBMC). NAAT, nucleic acid amplification technique; PBMC, peripheral blood mononuclear cells; SFC, spot-forming cell.

5.2.5 Pgp3 contains T-cell epitopes along its entire length

The Pgp3 peptides were divided into 4 pools of 10 and one pool of 11 peptides and the percentage of ELISpot-positive patients who responded to each pool plotted graphically (Figure 5.6). Many of the Pgp3-ELISpot-positive individuals responded to multiple peptide pools spanning the length of Pgp3, indicating that the protein contains T-cell epitopes throughout its length. The amino terminus was preferentially recognised by NAAT-positive patients, compared to NAAT-negative individuals, however peptide pool 2 was the most commonly recognised, with over 80% of Pgp3-ELISpot-positive individuals responding to this pool. The pattern of recognition to the other peptide pools was similar in *C. trachomatis*-NAAT-positive and NAAT-negative individuals who responded.

Figure 5.6 Pgp3 contains T-cell epitopes along its entire length

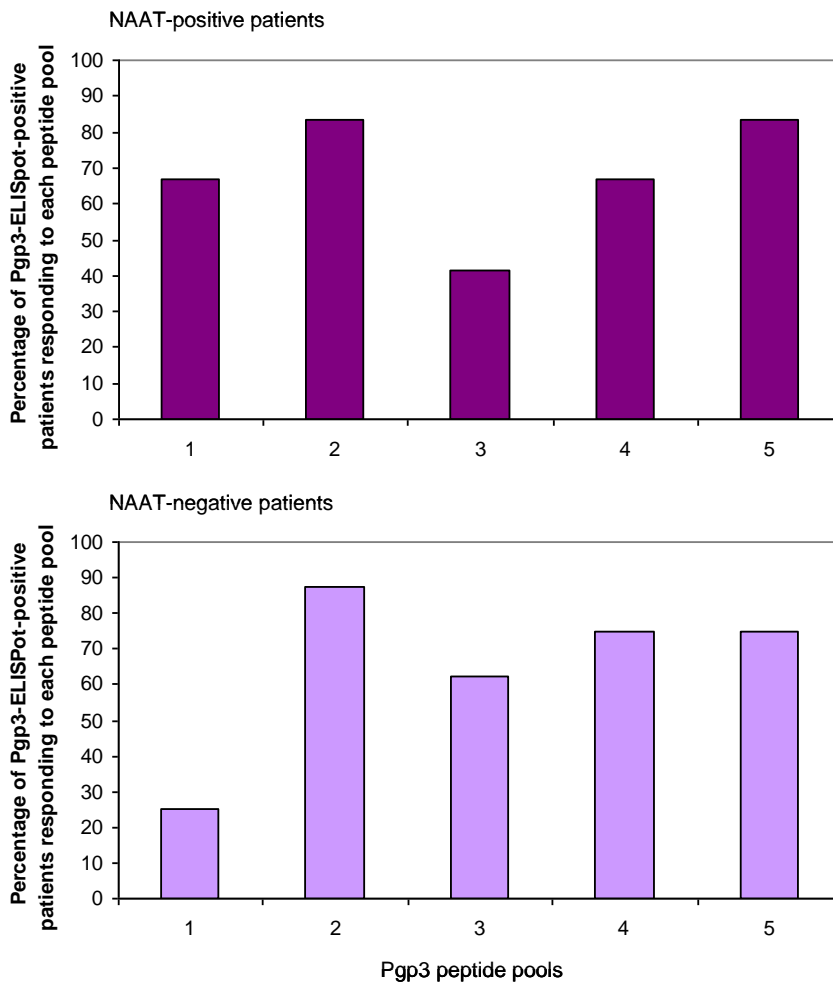


Figure 5.6 The response of T-cells from *C. trachomatis*-NAAT-positive and GUM negative controls to Pgp3 peptide pools. Pgp3 peptides were divided into 4 pools of 10 peptides (1-4) and one pool of 11 peptides (5) and the percentage of Pgp3-ELISpot-positive patients who responded to each pool was plotted.

5.2.6 Pgp3-induced *ex vivo* IFN- γ responses are dependent on *C. trachomatis* exposure

In order to further characterise the relationship between Pgp3-induced IFN- γ and *C. trachomatis* exposure, serum from 69 patients recruited at the GUM clinic was tested for the presence of anti-*C. trachomatis* antibodies by a Pgp3-ELISA developed in this laboratory (Wills *et al.*, 2009). Individuals testing ELISA and NAAT-positive are more likely to have either experienced more severe clinical disease or been exposed to *C. trachomatis* for a longer duration than those testing NAAT-positive alone (Mårdh, 1989b). Antibody-negative, NAAT-positive individuals are likely to have been exposed more recently than those testing serology-positive alone, as antibodies take a number of weeks to develop after infection and persist after the infection has been resolved, but NAATs become negative (Mårdh, 1989b). Individuals testing ELISA and NAAT-negative are less likely to have been exposed to *C. trachomatis* than the other groups, with children being at the lowest risk.

The percentage of patients in each group who were Pgp3-ELISpot-positive was calculated and plotted graphically in addition to the percentage of GUM negative controls and paediatric controls who tested Pgp3-ELISpot-positive (Figure 5.7). Ten (67%) of 15 NAAT-positive, Serology-positive individuals were Pgp3-ELISpot positive, compared with 3 (43%) of 7 NAAT-positive and serology-negative, 5 (42%) of 12 NAAT-negative and serology-positive, and 12 (34%) of 35 NAAT-negative and serology-negative individuals. Five (24%) of 21 GUM negative controls were Pgp3-ELISpotg-positive, as were 3 (19%) of 16 paediatric controls. A trend was apparent in the difference in IFN- γ production between NAAT-positive and serology-positive patients and NAAT-negative and serology-negative individuals, suggesting that Pgp3-induced IFN- γ is dependent on *C. trachomatis* exposure, but the numbers of patients in each group were too small for statistical analysis.

Figure 5.7 Pgp3-induced IFN- γ and *C. trachomatis*-exposure

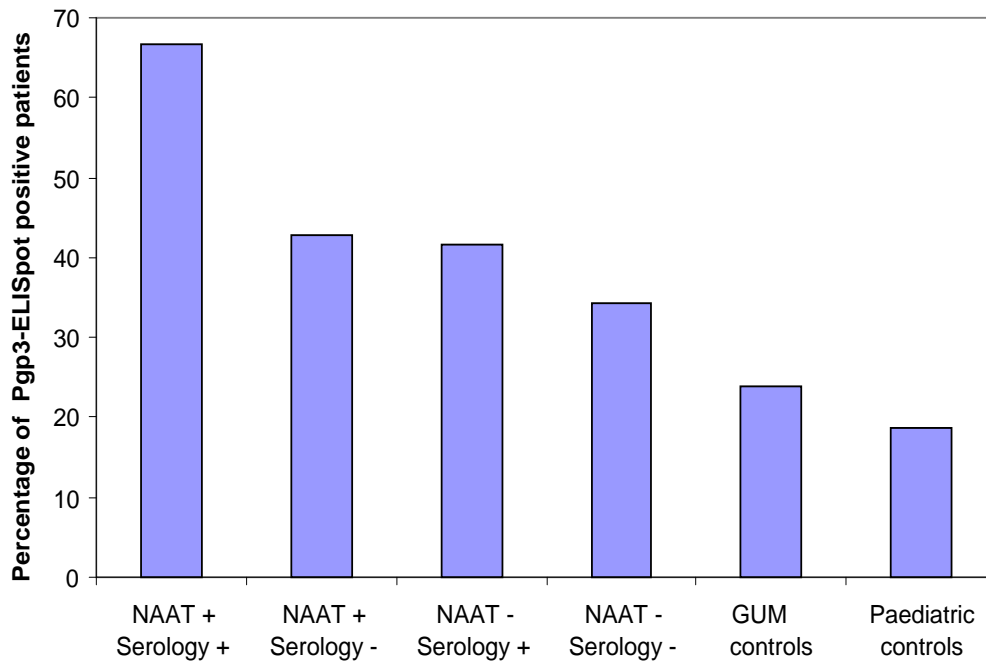


Figure 5.7 Patients were grouped according to whether they were NAAT-positive and serology-positive, NAAT-positive and serology-negative, NAAT-negative and serology-positive, or NAAT-negative and serology-negative. The percentage of patients in each group who were Pgp3-ELISpot-positive was calculated and plotted graphically in addition to the percentage of GUM negative controls and paediatric controls testing Pgp3-ELISpot-positive. NAAT +, NAAT-positive; Serology -, Serology-negative.

5.2.7 Pgp3-induced IFN- γ responses are dynamic

M. tuberculosis-specific IFN- γ responses have been shown to decrease following effective anti-tuberculosis therapy (Pathan *et al.*, 2001; Millington *et al.*, 2007) and we reasoned the same might be true for *C. trachomatis*- specific IFN- γ . Pgp3-induced IFN- γ was quantified prior to, and following, effective treatment in 2 clinically symptomatic patients infected with *C. trachomatis* (Figure 5.8). The first patient, who initially presented with urethritis and tested *C. trachomatis*-NAAT positive, was Pgp3-ELISpot-positive, with 61 SFC per million PBMCs that recognised Pgp3 peptides at the time of presentation. The patient was treated with azithromycin (a single oral administration of 1g), followed up 8 weeks-post treatment and found to be asymptomatic, NAAT-negative and Pgp3-ELISpot-negative, with only 4 SFC per 10^6 PBMCs that recognised Pgp3 peptides. The second patient, who presented with proctitis, was NAAT-positive and Pgp3-ELISpot-positive, with 53 SFC per million PBMC at the time of presentation. This patient was treated with 100mg doxycycline (twice daily oral medication for 12 days), followed up 6 weeks after the initial presentation and found to be asymptomatic, NAAT-negative and Pgp3-ELISpot-negative, with only 1 SFC per 10^6 PBMC.

Figure 5.8 Pgp3 specific IFN- γ responses are dynamic

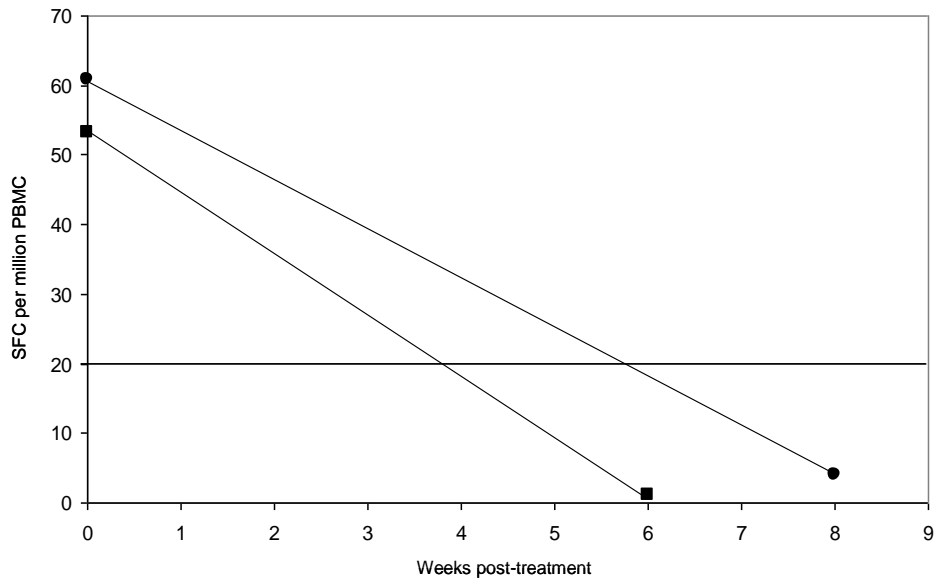


Figure 5.8 Pgp3-induced IFN- γ was quantified prior to, and following, effective treatment in 2 clinically symptomatic patients infected with *C. trachomatis*. The first patient (represented by circles) had 61 SFC per million PBMCs that recognised Pgp3 peptides at initial presentation and only 4 SFC per 10^6 PBMCs on the second presentation, 8 weeks following effective treatment. The second patient (represented by squares) had an average of 53 SFC per million PBMC at initial presentation and only 1 SFC per 10^6 PBMC 6 weeks after effective treatment. The cut-off for a positive ELISpot response was 20 SFC per million PBMC.

5.2.8 Pgp3-induced IFN- γ responses do not correlate with *Chlamydia* load

In order to ascertain whether chlamydial load influenced the anti-*C. trachomatis* IFN- γ response in infected patients, the number of *C. trachomatis* organisms in patient samples was quantified by qPCR and the level of IFN- γ responses (SFC per million PBMC) compared between patients of differing loads.

Of the NAAT-positive individuals, rectal swab samples were available from 10 patients and urine samples available from 4 patients for the quantification of *C. trachomatis* load. DNA was extracted from each sample and the number of *C. trachomatis* organisms quantified by qPCR, with primers targeting *orf5* of the cryptic plasmid (encoding Pgp3) (Chapter 4). The relationship between the IFN- γ response (SFC per million PBMC) and the *Chlamydia* load in rectal samples (number of organisms per swab) and urine samples (organisms per ml) was plotted graphically (Figure 5.9 A & B). There was no significant correlation between the *Chlamydia* load and the magnitude of the IFN- γ response.

Figure 5.9 Pgp3-sepcific IFN- γ responses do not correlate with *Chlamydia* load

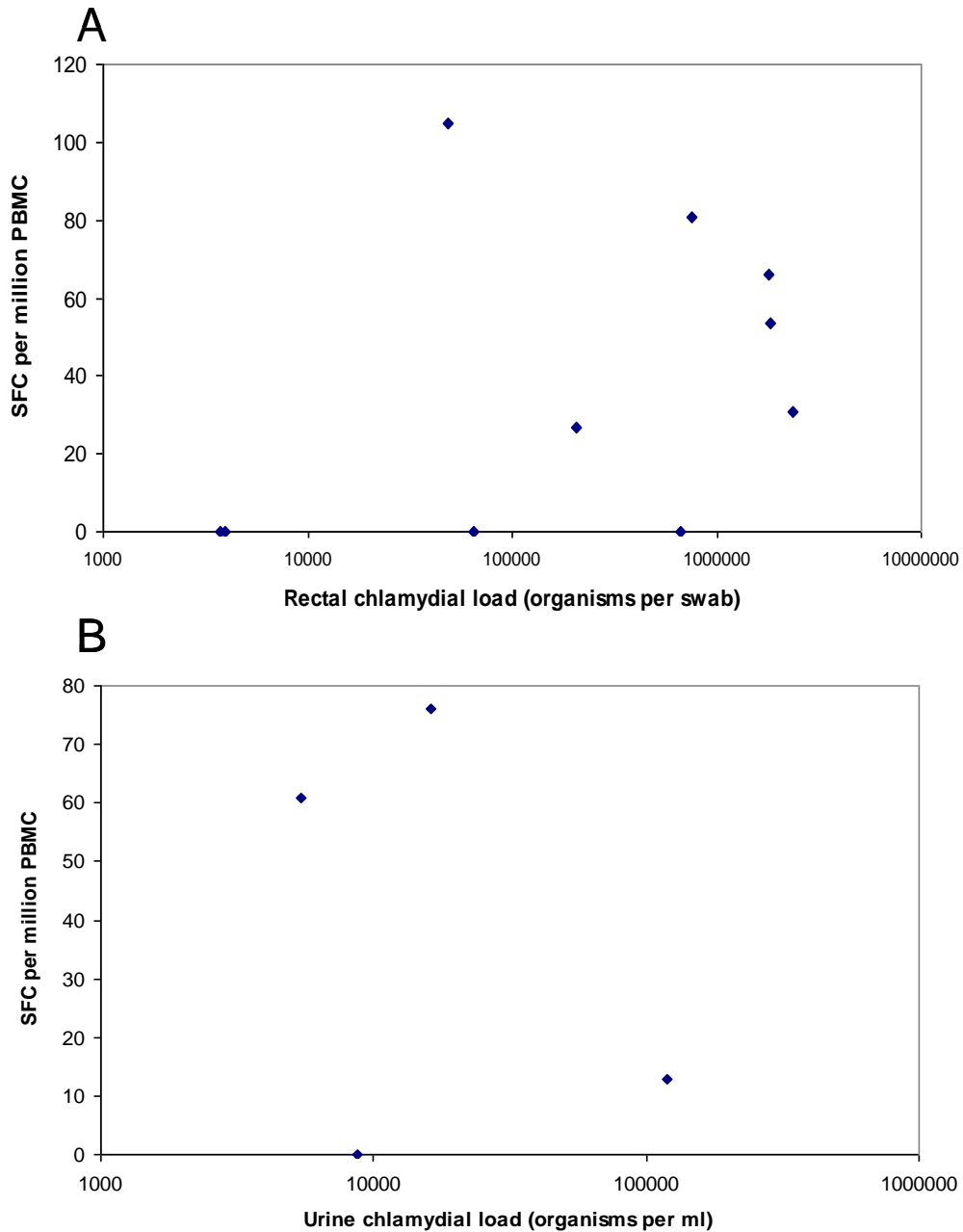


Figure 5.9 The relationship between the IFN- γ response (SFC per million PBMC) and the *Chlamydia* load in rectal samples (organisms per swab) (A) and urine samples (organisms per ml) (B). DNA was extracted from the samples and the number of *C. trachomatis* organisms quantified by qPCR with primers targeting *orf5*.

5.2.9 The HIV-status does not significantly affect Pgp3-induced IFN- γ responses

In order to investigate whether exposure to HIV-1 influenced the number of peripheral blood T-cells that recognised and responded to Pgp3, the percentage of patients who were Pgp3-IFN- γ -ELISpot-positive, and the magnitude of the Pgp3-induced IFN- γ response (SFC per million PBMC), was compared between patients who were HIV-1 sero-positive and HIV-1 sero-negative.

No significant difference in the median IFN- γ responses against Pgp3 was observed between HIV-1 sero-positive and sero-negative individuals in those who were ELISpot-positive. The median response was 39 SFC per million PBMCs (IR 28-55) in HIV-1 sero-positive individuals and 53 SFC per million PBMCs (IR 31-78) in HIV-1 sero-negative individuals (Mann Whitney U $P = 0.302$). As the number of patients in this study was low, these data were interpreted with caution, however the observation is consistent with what is documented for *M. tuberculosis* (Chapman *et al.*, 2002).

There was also no significant difference in the proportion of patients who were Pgp3-ELISpot-positive between HIV-1 sero-positive and sero-negative individuals. Seven (64%) of 11 HIV-1 sero-positive individuals were Pgp3-ELISpot-positive compared to 25 (44%) of 57 HIV-1 sero-negative individuals ($\chi^2 P = 0.23$). The higher percentage of ELISpot-positive individuals amongst HIV-1 sero-positive individuals is consistent with a high level of HIV-1 and *C. trachomatis* co-infection in this study. Thirteen (76%) of 17 HIV-1 sero-positive patients were co-infected with *C. trachomatis* compared to 17 (25%) of 66 HIV-1 sero-negative individuals ($\chi^2 P = 0.001$).

5.2.10 Pgp3-induced IFN- γ responses are independent of *C. pneumoniae* exposure

In order to investigate whether exposure to *C. pneumoniae* influenced the number of peripheral blood T-cells that recognised and responded to Pgp3, the percentage of patients who were Pgp3-IFN- γ -ELISpot-positive, and the magnitude of the Pgp3-induced IFN- γ response (SFC per million PBMC), was compared between patients who had anti-*C. pneumoniae* antibodies in serum and those who did not. The *C. pneumoniae* IgG/IgM microimmunofluorescence (MIF) test kit (AniLabsystems, Finland) was used to detect anti-*C. pneumoniae* antibodies and sera were considered positive if IgG was detected at a dilution of 1:32 as per the manufacturer's instructions.

Ten (56%) of 15 *C. pneumoniae* antibody-positive individuals were Pgp3-ELISpot-positive compared with 20 (39%) of 51 *C. pneumoniae* antibody-negative individuals ($\chi^2 P = 0.06$) and the median IFN- γ response in *C. pneumoniae* antibody-positive individuals was 57 SFC per million PBMCs (IR 46-65) compared with 39 SFC per million PBMCs (IR 27-76) in *C. pneumoniae* antibody-negative individuals (Mann Whitney U $P = 0.253$). These data reinforce sequence homology searches that found no Pgp3 homology with *C. pneumoniae* proteins, however, the number of patients in this study was small and the data should be interpreted with this in mind.

5.2.11 There is no correlation between Pgp3-induced IFN- γ responses and cell viability

Following the recovery of cells from storage in liquid nitrogen, cell viability ranged from 52% to 98%. To investigate the possibility that cell viability influenced the quantity of IFN- γ produced by the cells in a non-specific manner, a graph was plotted of the Pgp3-IFN- γ ELISpot response (SFC per million PBMCs) and PBMC viability (Figure 5.10). There was no significant correlation between cell-viability and the IFN- γ response to Pgp3, indicating that the IFN- γ response was unlikely to be a non-specific result of poor cell viability.

Figure 5.10 Pgp3-induced IFN- γ is independent of cell-viability

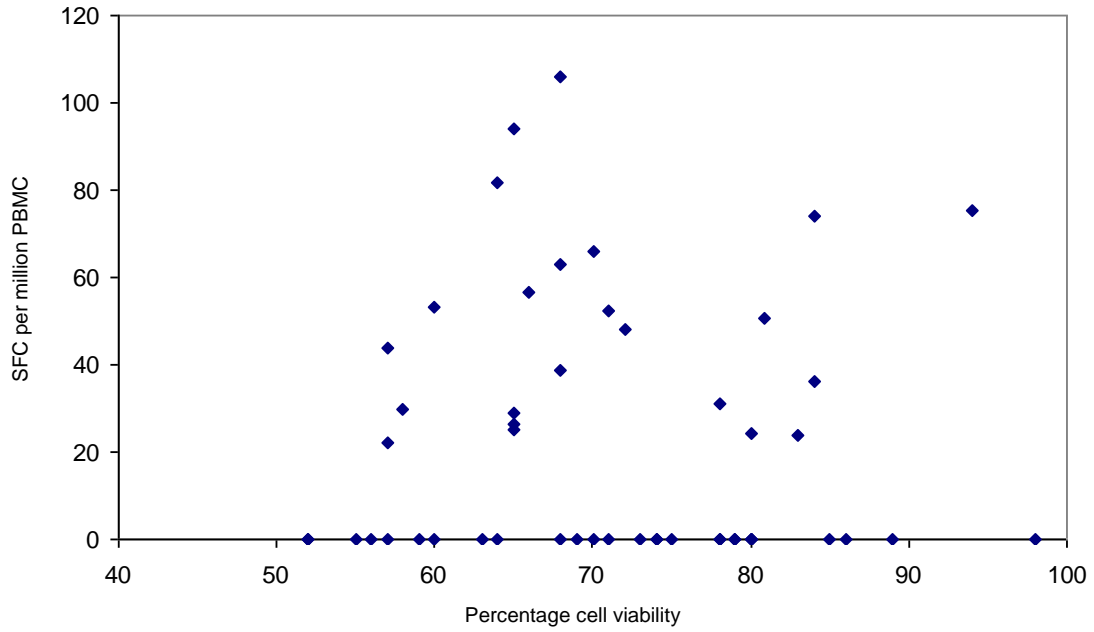


Figure 5.10 To investigate the possibility that cell viability influenced the magnitude of the IFN- γ response in a non-specific manner, a graph was plotted of the Pgp3-IFN- γ ELISpot response (SFC per million PBMCs) Vs PBMC viability.

5.3 Discussion

The work described in this chapter represents the first *ex vivo* characterisation of human cellular immune responses to the *C. trachomatis*-specific protein, Pgp3. The protein is recognised by human T-cells circulating in the peripheral blood of patients infected with *C. trachomatis*, with T-cell epitopes located throughout its length. The Pgp3- induced IFN- γ responses are dependent on *C. trachomatis* exposure, and are dynamic. However, the magnitude of anti-Pgp3 IFN- γ responses in humans is low, and the sensitivity and specificity of the ELISpot assay for the detection of *C. trachomatis* was calculated to be 59% (95% CI, 39-77%) and 81% (95% CI, 57-93%) respectively. Moreover, the Pgp3-induced IFN- γ responses are independent of *C. trachomatis* load, HIV-1 status, *C. pneumoniae* exposure and cell viability.

A cellular immune response is required for the clearance of *C. trachomatis* infection (reviewed by Brunham & Rey-Ladino 2005; Rank, 2006) and targets of the cellular immune response have been identified *ex vivo* using T-cells extracted from the peripheral blood of infected patients (Ortiz *et al.*, 1996; Ortiz *et al.*, 2000; Kim *et al.*, 1999; Kim *et al.*, 2000; Goodall *et al.*, 2001a and b; Gervassi *et al.*, 2004; Deane *et al.*, 1997; Kinnunen *et al.*, 2003; Meoni *et al.*, 2009; Olsen *et al.*, 2006; Olsen *et al.*, 2007). However, only one published study has investigated responses in the absence of *in vitro* clonal expansion (Meoni *et al.*, 2009). As the protein identified (CT043), shares 95% identity with a *C. pneumoniae* protein (Cpn0387), the present study is the first to investigate *ex vivo* human cellular immune responses to a *C. trachomatis*-specific antigen.

Pgp3 is known to be a target of the humoral response in humans with *C. trachomatis* infection (Comanducci *et al.*, 1994; Ratti *et al.*, 1995; Bas *et al.*, 2001; Bas *et al.*, 2002; Ghaem-Maghani *et al.*, 2003; Li *et al.*, 2008a; Donati *et al.*, 2008; Wills *et al.*, 2009), however, no previous study has investigated human cellular immune responses to the protein. Data from this study are consistent with the observation that mice immunised with the gene encoding Pgp3 produce a robust IFN- γ response (Li *et al.*, 2008c) and that Pgp3 has been found both in the inclusion membrane (Comanducci *et al.*, 1994) and in the cytoplasm (Li *et al.*, 2008b) and, therefore, may be presented to either CD4⁺ or CD8⁺ T-cells.

This study demonstrated, for the first time, that T-cell epitopes are present throughout the length of the Pgp3 protein sequence, rather than being restricted to one antigenic “hot-spot”. This phenomenon is similarly documented for *M. tuberculosis*-specific secreted antigens ESAT-6 and CFP-10 (Lalvani *et al.*, 2001a and Chapman *et al.*, 2002). Moreover, several peptide pools were recognised by T-cells from a high percentage of patients from different ethnic backgrounds and are, therefore, not restricted to one HLA haplotype.

These observations suggest that Pgp3 is an immunodominant antigen during a *C. trachomatis* infection in humans, with epitopes recognised by both the humoral and cellular immune responses, however, the median frequency of IFN- γ -secreting T-cells that recognised Pgp3 in infected patients was only 44 per million PBMCs (interquartile range, 26.8-63). In contrast, ESAT-6, an immunodominant protein of *M. tuberculosis*, was recognised by 200 cells per million PBMCs (interquartile range, 105-596) (Lalvani *et al.*, 2001b)).

Studies that have involved the screening of either a library of *Chlamydia* antigens, or fractions of *C. trachomatis* lysates with T-cells reactive to *C. trachomatis* in order to identify

immunodominant antigens (Goodall *et al.*, 2001 a and b; Starnbach *et al.*, 2003; Olsen *et al.*, 2006; Olsen *et al.*, 2009) did not identify Pgp3 as a target of the cellular immune response. These techniques identify the most immunodominant proteins, and it may be that the IFN- γ responses to Pgp3 are too small to be detected by such means. As the *ex vivo* ELISpot assay is able to detect as few as 20 cells that respond to antigen out of 1 million PBMCs, it may have identified T-cell epitopes where other studies have failed.

The sensitivity of the ELISpot assay is 59% (95% confidence intervals (CI), 39-77%). While this is low, it is superior to the average sensitivity of commercial MOMP-peptide ELISAs for the detection of *C. trachomatis*, which range from 44.4-49.2% (Wills *et al.*, 2009). Either *C. trachomatis* does not induce a systemic immune response in some patients, or the immune responses are directed against different antigens.

The specificity of the ELISpot assay is 81% (95% CI, 57-93%) compared to that of the Pgp3 ELISA, 97.6% (95% CI, 96.2-98.6%). Sequence homology searches of the SwissProt and translated GenBank databases of all known protein sequences were conducted with a basic local alignment search tool (BLAST) for each Pgp3-peptide. Two peptides (21 and 46) had homology with members of the *Aspergillus* genus that may cause infection in humans (9 out of 15 residues identical) and one peptide (44) had homology with *Roseburia inulinivorans*, a human colonic commensal (Duncan *et al.*, 2006) (10 out of 15 residues identical). In addition, 24 peptides had homology with the zoonotic pathogen, *C. psittaci*. While it is possible that exposure to this pathogen contributed to the poor specificity, this is unlikely, as the prevalence of *C. psittaci* infection is low (around 100 cases reported in the US annually (Vanrompay *et al.*, 2007)).

Of note is that 6 (31%) of the paediatric patients were from Africa. It is possible they could have been exposed to *C. trachomatis* serovars A-C (Trachoma), thus reducing assay specificity. It is also possible that some of the clinically asymptomatic, NAAT-negative, Pgp3-ELISA-negative men had previously been exposed to *C. trachomatis* as the number of new sex-partners in the 3 months preceding presentation was not significantly different from NAAT-positive individuals.

Peptides were dissolved in DMSO which can increase the permeability of cell membranes. It is, therefore, possible that DMSO increased the release of intracellular IFN- γ non-specifically, leading to the background observed in the paediatric controls. Never-the-less, if this were the case, the same background IFN- γ would be expected in all patient groups investigated. The fact that, significantly more *C. trachomatis*-NAAT-positive individuals are Pgp3-ELISpot-positive compared with NAAT-negative individuals indicates that the IFN- γ response observed is specific to a *C. trachomatis* antigen.

A positive correlation was observed between ELISpot positivity and *C. trachomatis* exposure. Pgp3-induced IFN- γ may, therefore, have potential as a biomarker for *C. trachomatis* infection. Chlamydial exposure was based on NAAT and ELISA results: Individuals testing ELISA and NAAT-positive are more likely to have either been exposed to *C. trachomatis* for a longer duration than those testing NAAT-positive alone, or experienced more severe clinical disease (Mårdh, 1989b). Antibody-negative, NAAT-positive individuals are likely to have been exposed more recently than those testing serology-positive alone, as antibodies take a number of weeks to develop after infection and persist after the infection has been resolved, but NAATs become negative (Mårdh, 1989b). Individuals testing ELISA

and NAAT-negative are less likely to have been exposed to *C. trachomatis* than the other groups, with children being at the lowest risk.

In this study, cells secreted IFN- γ in the absence of *in vitro* stimulation and, were therefore, capable of rapid effector function. High frequencies of circulating effector T-cells are likely to be maintained following a recent encounter with antigen *in vivo* (Zinkernagel *et al.*, 1996) and, as continued Pgp3 expression by reticulate bodies would require metabolically active and viable *C. trachomatis* organisms, it was reasoned that the presence of circulating IFN- γ secreting effector T-cells that recognise Pgp3-peptides is more likely to indicate a recent *C. trachomatis* infection, as opposed to prior exposure to the organism. In support of this hypothesis, two *C. trachomatis*-infected patients with positive Pgp3-ELISpot responses were followed post-treatment and found to be NAAT-negative and Pgp3-ELISpot-negative, demonstrating that Pgp3-induced IFN- γ responses were dynamic. This suggests that an *ex vivo* IFN- γ ELISpot is capable of distinguishing between a recent *C. trachomatis* infection and prior exposure to the organism not related to the current episode. A larger longitudinal study following patients before and after treatment is necessary to confirm these findings. In contrast, anti-*C. trachomatis* IgG antibodies persist after resolution of infection (Ghaem-Maghani *et al.*, 2003; Mårdh, 1989b) and, therefore, titres do not permit temporal analysis of transient immune responses (although rising IgM antibody titres can be used to identify recent exposure (Mårdh, 1989b). An *ex vivo* IFN- γ ELISpot assay using a *C. trachomatis*-specific antigen may, therefore, prove a useful additional tool for studying the burden of acute disease caused by *C. trachomatis* infection. (Horner, 2007).

As Pgp3-induced IFN- γ responses declined after effective treatment, it is possible that the frequency of Pgp3-specific T cells was related to antigenic load. In order to address this

hypothesis, a qPCR was developed to quantify *C. trachomatis* plasmid *orf5* copies (encoding Pgp3) in patient samples and the magnitude of Pgp3-induced IFN- γ responses was compared to the *Chlamydia* load. No significant correlation was found.

It is possible that a positive correlation between the *Chlamydia* load and the frequency of Pgp3-specific T-cells was not observed as some patients may have acquired a recent infection and not yet mounted an immune response, giving rise to high *Chlamydia* loads and low IFN- γ responses. Other patients may have, however, generated an immune response that cleared the infection, giving rise to high IFN- γ responses but low *Chlamydia* loads. A longitudinal study would enable a more detailed investigation into the kinetics of the anti-*Chlamydia* immune response, and how it relates to *Chlamydia* load in humans.

Neither the magnitude of the Pgp3-induced IFN- γ response, nor the proportion of people who responded were significantly influenced by exposure to HIV-1. Although it is possible that the low number of samples in this study accounted for the lack of statistical significance, these preliminary data imply the assay may be valuable in an HIV-1 sero-positive population.

There was a slight reduction in the frequency of T-cells that responded to Pgp3 peptides in HIV-1 sero-positive patients compared to HIV-1 sero-negative individuals. This has been also been reported for *M. tuberculosis* (Chapman *et al.*, 2002) and may be due to an impaired ability of T-cells to mount an IFN- γ response in HIV-1 infected individuals. In contrast, the proportion of individuals who responded to Pgp3 peptides was slightly higher in HIV-1 sero-positive individuals than HIV-1 sero-negative. This may be due, in part, to the high level of HIV-1 and *C. trachomatis* co-infection seen in this study and is consistent with observations

by Ratti *et al.*, 1995 who reported that a higher proportion of HIV-1 seropositive individuals have anti-Pgp3-antibodies, compared with HIV-1 negative individuals.

Exposure to *C. pneumoniae* does not significantly influence either the magnitude of the Pgp3-induced IFN- γ response, or the proportion of patients responding. While the small number of patients in this study may account for this observation, it is consistent with Pgp3 being absent from human *C. pneumoniae* isolates, which lack the cryptic plasmid (Campbell *et al.*, 1987; Lusher *et al.*, 1989).

This study has shown that Pgp3 is a target of the human cellular immune response during *C. trachomatis* infection and suggests that Pgp3-specific IFN- γ may be a useful biomarker for *C. trachomatis*- specific inflammation.

Chapter 6

Discussion

More than a hundred years after the first identification of intra-cytoplasmic chlamydial inclusions, *Chlamydia trachomatis* remains the most common curable sexually transmitted bacterial infection in young people, with approximately 5-10% of sexually active women under the age of 24 and men between 20-24 years old currently infected (Horner, 2008).

Clinically, there is no non-invasive way of identifying infected individuals who are not shedding organisms from the lower genital tract or rectum, nor is there a licensed vaccine against *Chlamydia*. There is a paucity of information on the load shed by individuals with a rectal *C. trachomatis* infection and the recent LGV outbreaks have highlighted our lack of understanding on the association between *C. trachomatis* and HIV-1 co-infection.

This thesis addresses three aspects of chlamydial biology that have clinical implications. Firstly, as over 75% of patients with LGV are HIV-1 sero-positive (Mayaud, 2006) for reasons that remain unknown, a cell-culture model was established to investigate whether HIV-1 altered the replication of *C. trachomatis* serovar L2. This was the first investigation of *C. trachomatis* growth in HIV-1 co-infected cells and we found that HIV-1 does not induce chlamydial persistence, or accelerate the lytic cycle, suggesting that the association of LGV with HIV-1 infection is not due to induction of chlamydial persistence by HIV-1 *in vivo*.

The high levels of co-infection observed may reflect high risk sexual behaviour in distinct groups of individuals, with HIV-1 positive MSM preferentially meeting other HIV-positive MSM for whom the imperative to practice safer sex has been diminished. Alternatively, there may be a selection bias, with clinicians selectively testing HIV-1 positive men, or HIV-1 positive men being more aware of LGV as a result of publicity generated through organisations such as the Terrance Higgins Trust (Ward *et al.*, 2007).

It also remains possible that HIV-1 and *C. trachomatis* interact indirectly in a co-infected host. In models of HIV-1 and *Leishmania* co-infection, HIV-1 infection leads to a decrease in Th-1 cytokines such as IFN- γ and IL-12 and an increase in the Th2 cytokine IL-4 and the inhibitory cytokine IL-10. As Th1 responses are required for clearance of *Leishmania* infection, the HIV-1 induced shift to Th2 responses could explain the increased severity of disease seen in co-infected patients (Alvar *et al.*, 2008). A similar scenario is documented in HIV-1 and *M. tuberculosis* co-infection models, with a shift from Th1 to Th2 dominance impairing the ability of the immune response to contain the bacilli in a latent form, with subsequent reactivation of disease in co-infected individuals (Siawaya *et al.*, 2007). As *C. trachomatis* also requires a protective Th1 cellular response to clear the infection (reviewed by Brunham & Rey-Ladino, 2005) it is possible that an HIV-1- mediated shift toward Th2 responses affects the pathogenesis of the bacteria.

Our findings could potentially be of use in defining pathways that lead to chlamydial persistence. Currently, although HSV-2 is known to induce chlamydial persistence, the mechanism of this remains unknown (Vanover *et al.*, 2010). As HIV-1 does not induce persistence, it might be possible to identify where the two viruses differ in the cellular pathways they activate, in order to narrow down those that induce persistence.

We found that HIV-1 co-infection leads to a significant enlargement of chlamydial inclusions. This is most likely due to the fusion of multiple inclusions in an HIV-1 induced syncytium to form one “giant” inclusion, something not previously reported. This observation demonstrates that the chlamydial fusogenic machinery is capable of producing structures far larger than previously documented and that the protective mesh or scaffold that surrounds the

inclusion (Kumar & Valdivia, 2008) is capable of supporting such structures. Moreover, as no alteration in chlamydial growth was observed in co-infected cultures, the *Chlamydia* organisms must be maintaining their complex intracellular niche despite considerable cytoplasmic rearrangement.

In order to complete the investigation of *C. trachomatis* and HIV-1 co-infection *in vitro*, future studies should be conducted in reticuloendothelial cell-lines and *ex vivo* macrophages. These cells are more representative of the likely site of co-infection *in vivo* and there are notable differences in the growth of *C. trachomatis* in these cells compared to epithelial cells (Köhler *et al.*, 1997). If co-infection is found to influence the replication of either organism, the effect on the sensitivity of antibiotics and antiretroviral drugs could then be investigated *in vitro*.

The second aim of the thesis was to develop a qPCR and use it to determine the number of *C. trachomatis* organisms per rectal swab in NAAT-positive patients with a rectal infection. This was the first evaluation of the quantity of bacteria shed during a rectal *C. trachomatis* infection. As rectal *C. trachomatis* is not uncommon in MSM, with prevalence estimated between 6.5 and 8.2% (Annan *et al.*, 2009; Benn *et al.*, 2007; Manavi *et al.*, 2004) and little is known of how infectious such patients are, this information is clinically relevant.

We found the average rectal load to be considerably higher than the average load reported in the female genital tract on the basis of endocervical or vulvo-vaginal swabs (Michel *et al.*, 2007; Wiggins *et al.*, 2009). While this could be due to a bias in patient recruitment (favouring those with higher chlamydial loads), it may reflect a difference in how permissive the rectal mucosa is to *C. trachomatis* infection, compared with the mucosa of the genital

tract, and may mean that patients with a rectal infection are more infectious per episode of unprotected intercourse than women with a genital tract infection.

We found that a higher chlamydial load was associated with proctitis, but not the presence of rectal symptoms or HIV-1 infection. As chlamydial load is associated with inflammation in the genital tract (Geisler *et al.*, 2001; Wiggins *et al.*, 2006; Horner, 2007; Michel *et al.*, 2007), the former observation is to be expected. The lack of association between load and rectal symptoms or HIV-1 infection may be due a lack of statistical power of the study. Nevertheless, we found that asymptomatic individuals shed as much *C. trachomatis* as patients with rectal symptoms. Asymptomatic individuals are likely to be unaware of their infection, remaining undiagnosed and untreated, thus representing a reservoir of infection in the community. This is particularly important in MSM, as unprotected anal intercourse is not uncommon (Dodds *et al.*, 2007) and where the majority (>80%) of serovar D-K infections are asymptomatic (Annan *et al.*, 2009; Kent *et al.*, 2005; Ward *et al.*, 2009a).

Although the qPCR developed in this study was adequate for the investigations described above, the analytical sensitivity was low. It would, therefore, be wise to utilise a confirmatory assay, such as a validated in house qPCR or commercial NAAT platform capable of quantifying infection, such as the RealArt *C. trachomatis* PCR kit (Qiagen).

Finally, an *ex vivo* IFN- γ ELISpot assay was developed to characterise human cellular immune responses to the *C. trachomatis*-specific protein, Pgp3. This not only represents the first characterisation of T-cell immune responses to Pgp3, but is also the first investigation into *ex vivo* human cellular immune responses to a *C. trachomatis*-specific antigen.

There is currently a drive in the *Chlamydia* field toward developing a sub-unit vaccine comprised of chlamydial antigens. We found that Pgp3 is a target of human cellular immune responses and, although we found the magnitude of these responses to be low, this study highlights the potential of an *ex vivo* IFN- γ ELISpot assay in identifying additional proteins recognised by the human immune response during a natural chlamydial infection that could then be tested in vaccine studies.

The Pgp3- induced IFN- γ response correlated with *C. trachomatis* exposure and was dynamic, decreasing after effective treatment. As *M. tuberculosis*- specific IFN- γ is known to be a marker for current TB infection (Lalvani *et al.*, 2001a and b; Millington *et al.*, 2007), it may be that *C. trachomatis*-specific IFN- γ also signifies the presence of an active infection. If this is so, the ELISpot assay may represent a non-invasive way of identifying individuals with a chlamydial infection who are not shedding organism from the lower genital tract. This may prove to be a more satisfactory approach than serological screening to identify patients at risk of TFI. However, the specificity and sensitivity of the assay requires to be improved before this can be put to the test.

To improve assay sensitivity, additional *C. trachomatis*-specific antigens that are more immunodominant could be investigated. The antigen CT694 has been identified as *C. trachomatis*-specific (Griffiths *et al.*, 2006), is located within the cytosol (Hower *et al.*, 2009), and found to be recognised by antibodies from infected patients (Sharma *et al.*, 2006; Frikha-Gargouri *et al.*, 2009). This protein may also contain T-cell epitopes and be of use in an IFN- γ ELISpot assay for the detection of *C. trachomatis*.

It may be possible to refine the response to Pgp3 by enumerating the frequency of T-cells that recognise and respond to individual Pgp3-peptides rather than peptide pools. This would enable an “epitope map” to be constructed of the whole antigen, in the same manner as has been done for the *M. tuberculosis* antigens, ESAT-6 and CFP-10 (Lalvani *et al.*, 2001b; Pathan *et al.*, 2001; Chapman *et al.*, 2002) and enable peptides that contribute to the poor specificity to be identified and omitted from the assay.

Murine studies have implicated CD4⁺ Th1 cells to be of major importance for the clearance of *Chlamydia* infection. However, the contribution of CD4⁺ and CD8⁺ T-cells to the human anti-*Chlamydia* immune response remains unknown (reviewed in Wizel *et al.*, 2008). As this study considered human cellular immune responses to Pgp3 in the context of a mix population of PBMCs, it would be of benefit to deplete patient PBMCs of either CD4⁺ or CD8⁺ T-cells and enumerate cytokine responses from each cell population to characterise patient CD4⁺ and CD8⁺ T-cell responses to *C. trachomatis*-specific antigens. This would enable more information to be obtained on the nature of human cellular immune responses to *C. trachomatis*.

A longitudinal study could be conducted in order to determine the kinetics of anti-*C. trachomatis*-IFN- γ and antibody responses. However, once a patient presents at the clinic with suspected *C. trachomatis* infection, they would be treated with antibiotics. A longitudinal study would collect patient samples at initial presentation and periodically during and after treatment to determine how quickly IFN- γ responses declined and if *C. trachomatis*-antibody responses develop and persist. This would enable increased information on the natural history and the host response to infection.

By culturing PBMCs in the presence of antigen *in vitro*, memory T-cell responses can be evaluated (Todryk *et al.*, 2009). As vaccines aim to stimulate immunological memory, it would be of benefit to establish if Pgp3 elicits memory T-cell responses in humans. If memory responses are detected in response to this protein, it could explain why murine models of Pgp3 vaccination show protective immune responses (Donati *et al.*, 2003; Li *et al.*, 2008c) whereas the magnitude of the effector IFN- γ response to Pgp3-peptides is low.

In conclusion, this thesis has examined three novel aspects of *C. trachomatis* translational research and characterises, for the first time, *C. trachomatis* growth in HIV-1 co-infected cells, rectal chlamydial load in different patient groups, and *ex vivo* human T-cell responses to a *C. trachomatis*-specific antigen. The information gained from these studies will be of benefit to the *Chlamydia* research field.

Chapter 7

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Appendices

Figure Appendix 1. Bristol Sexual Health Centre Ethics Approval

Frenchay Research Ethics Committee

C/o North Bristol NHS Trust
Pembroke Room
Beaufort House
Southmead Hospital
Westbury-on-Trym
Bristol
BS10 5NB

Telephone: 0117 323 5211
Facsimile: 0117 323 2832

08 April 2009

Dr. P.J. Horner
Consultant Senior Lecturer in Genitourinary Medicine
University of Bristol
Bristol Sexual Health Centre
Tower Hill
Bristol
BS2 0JD

Dear Dr. Horner

Full title of study:	A study to assess rectal <i>Chlamydia trachomatis</i> using quantitative PCR in individuals having receptive anal sexual intercourse.
REC reference number:	09/H0107/6

Thank you for your letter of 19 March 2009, responding to the Committee's request for further information on the above research [and submitting revised documentation](#).

The further information has been considered on behalf of the Committee by the [Chair](#).

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation [as revised](#), subject to the conditions specified below.

Ethical review of research sites

The Committee has designated this study as exempt from site-specific assessment (SSA). The favourable opinion for the study applies to all sites involved in the research. There is no requirement for other Local Research Ethics Committees to be informed or SSA to be carried out at each site.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the

start of the study at the site concerned.

Management permission at NHS sites (“R&D approval”) should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at <http://www.rdforum.nhs.uk>.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

<i>Document</i>	<i>Version</i>	<i>Date</i>
Questionnaire	1	27 January 2009
Covering Letter		
Investigator CV		28 January 2009
Application		
Referee report Graham Taylor		01 March 2009
Referee report Alan Winston		13 February 2009
Response to Request for Further Information		19 March 2009
Participant Consent Form	2	12 March 2009
Participant Information Sheet	2	12 March 2009
Covering Letter		12 March 2009
Protocol	2	12 March 2009

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document “After ethical review –guidance for researchers” gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

09/H0107/6

Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely

Dr Mike Shere
Chair

Email: Anthony.Sack@nbt.nhs.uk


Enclosures:

"After ethical review – guidance for researchers", [SL- AR2](#)

Copy to:

[R&D office](#)

Figure Appendix 2. St. Mary's Hospital Ethics Approval for Recruitment of Adults


National Research Ethics Service
Hounslow & Hillingdon Local Research Ethics Committee
50 Eastbourne Terrace
2nd Floor A Block
London
W2 6LG
Telephone: 020 7725 5672
Facsimile: 020 725 5317

10 August 2007

Dr Alan Winston
Consultant Senior Lecturer
Imperial College, London and St. Mary's NHS Trust
Clinical Trials, Ground Floor
Winston Churchill Wing
Praed Street, London
W2 1NY

Dear Dr Winston

Full title of study: A preliminary investigation of Chlamydia trachomatis specific antibody secreting cells (ASCs) and interferon-gamma producing T cells in patients. Identification of potential proteins for use in an ELISpot Assay

REC reference number: 07/H0709/55

Thank you for your letter of 08 August 2007, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered at the meeting of the Committee held on 03 July 2007. A list of the members who were present at the meeting is attached.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

This Research Ethics Committee is an advisory committee to London Strategic Health Authority
The National Research Ethics Service (NRES) represents the NRES Directorate within
the National Patient Safety Agency and Research Ethics Committees in England

Document	Version	Date
Application		22 May 2007
Investigator CV		
Protocol	1.0	22 May 2007
Covering Letter		23 May 2007
Covering Letter		08 August 2007
Letter from Sponsor		23 May 2007
Participant Information Sheet: Patient Information Sheet	2	01 August 2007
Participant Consent Form: Patient Consent Form	2	01 August 2007
Response to Request for Further Information		08 August 2007

R&D approval

All researchers and research collaborators who will be participating in the research at NHS sites should apply for R&D approval from the relevant care organisation, if they have not yet done so. R&D approval is required, whether or not the study is exempt from SSA. You should advise researchers and local collaborators accordingly.

Guidance on applying for R&D approval is available from <http://www.rdforum.nhs.uk/rdform.htm>.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

Feedback on the application process

Now that you have completed the application process you are invited to give your view of the service you received from the National Research Ethics Service. If you wish to make your views known please use the feedback form available on the NRES website at:

<https://www.nresform.org.uk/AppForm/Modules/Feedback/EthicalReview.aspx>

We value your views and comments and will use them to inform the operational process and further improve our service.

07/H0709/55

Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely



PP Dr Jan Downer
Chair

Email: irene.gordon@nationalres.org.uk

Hounslow & Hillingdon Local Research Ethics Committee

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

REC reference number:	07/H0709/55	Issue number:	1	Date of issue:	10 August 2007
Chief Investigator:	Dr Alan Winston				
Full title of study:	A preliminary investigation of Chlamydia trachomatis specific antibody secreting cells (ASCs) and interferon-gamma producing T cells in patients. Identification of potential proteins for use in an ELISpot Assay				
This study was given a favourable ethical opinion by Hounslow & Hillingdon Local Research Ethics Committee on 03 July 2007. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.					
Principal Investigator	Post	Research site	Site assessor	Date of favourable opinion for this site	Notes ⁽¹⁾
Dr Alan Winston	Consultant Physician and Clinical Senior Lecturer	St Mary's Hospital, Praed Street, London	St Mary's REC	10/08/2007	

Approved by the Chair on behalf of the REC:

() (Signature of Acting Coordinator)

.....Irene Gordon..... (Name)

Figure Appendix 3. St. Mary's Hospital Ethics Approval for Recruitment of Children

St Mary's REC
Research Ethics Committees
Room 4W/12, 4th Floor West
Charing Cross Hospital
Fulham Palace Road
London
W6 8RF

Telephone: 020 8846 7251
Facsimile: 020 8846 7280

17 July 2009

Prof Michael Levin
Professor of Paediatrics and International Child Health
Imperial College London
Department of Paediatrics
Wright-Fleming Institute,
Imperial College, Norfolk Place
W2 1PG

Dear Professor Levin

Study Title: A comprehensive, long-term integrated programme to study the aetiology and immunopathology of childhood infectious, inflammatory and allergic disease, using the large patient base of children attending St Mary's Hospital, London

REC ref number: 09/H0712/58

Protocol number: 1

Thank you for your letter of 13 July 2009, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered in correspondence by a sub-committee of the REC at a meeting held on 16 July 2009. The sub-committee members involved were Barrie Newton and Vassilios Papalois.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a **favourable ethical opinion** for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at <http://www.rdforum.nhs.uk>. Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

<i>Document</i>	<i>Version</i>	<i>Date</i>
Covering Letter		12 May 2009
Investigator CV		15 April 2009
Application	2.2	11 May 2009
Compensation Arrangements	CRO1330	08 May 2009
Peer Review	Dr M Walters	22 April 2009
Letter from Sponsor	CRO1330	08 May 2009
Participant Consent Form: Children under 16	1.0 Assent Form	15 April 2009
Participant Information Sheet: 6-10 Years Old	1.0	15 April 2009
Participant Information Sheet: Under 5 Years Old	1.0	15 April 2009
Response to Request for Further Information	letter	13 July 2009
Participant Consent Form: parents or guardians	2.0	13 July 2009
Participant Consent Form: 16 year olds	2.0	13 July 2009
Protocol	2	12 July 2009
Participant Information Sheet: 11-15 Years Old	2.0	13 July 2009
Participant Information Sheet: 16 Years Old	2.0	13 July 2009
Participant Information Sheet: Parents or Guardians	2.0	13 July 2009

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "*After ethical review – guidance for researchers*" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

09/H0712/58

Please quote this number on all correspondence

Yours sincerely

**pp. Barrie Newton
Chairman**

Email: Rosalind.Cooke@imperial.nhs.uk

Enclosures: "After ethical review – guidance for researchers"

Copy to: Mr Gary Roper, Imperial College London