A Comparison of Fluorescent Microscopy Methods for the Detection of *Chlamydia trachomatis*

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PLAGIARISM DECLARATION

I, Micaela Lurie, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Acknowledgements

Dedication

My thesis is dedicated to Dr Julius Schachter, past Professor of Microbiology, Laboratory Medicine and Epidemiology at the University of California, San Francisco. His lifetime contribution in the field of Chlamydia research continues to have a significant impact on the lives of many. He also played a major role in the establishment of the culture methods used in my laboratory and without his guidance and expertise, we would not be where we are today. Unfortunately, he passed away in December 2020 at the age of 84 years from Covid-19. May his memory and legacy live on.

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Abbreviations

AH – Aluminium hydroxide

AMR – Antimicrobial resistance

ATCC – American type culture collection

ATP – Adenosine triphosphate

BV – Bacterial vaginosis

C. muridarum – *Chlamydia muridarum*

C. trachomatis – *Chlamydia trachomatis*

CAF01 – Cationic adjuvant formulation

COVID-19 – Corona virus disease 2019

CPAF - Chlamydial protease-like activating factor

CTL – Cytotoxic T lymphocyte

DAPI – 4',6-diamidino-2-phenylindole

DNA – Deoxyribonucleic acid

EB – Elementary body

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

EPT – Expedited partner therapy

eSHC – eSexual Health Clinic System

FDA – Food and Drug Administration

FITC – Fluorescein isothiocyanate

GFP – Green fluorescent protein

HBSS – Hanks basic salt solution

HI-FCS – Heat-Inactivated Foetal Calf Serum

HIV – Human Immunodeficiency Virus

HLA – Human leukocyte antigen

HSP60 – Heat shock protein 60

IDO – 2,3-dioxygenase

IFN – Interferon

IFU – Inclusion forming units

IL – Interleukin

IM – Infection media

Kb – Kilo base pairs

kDa – Kilodalton

LGV – Lymphogranuloma vereneum

LPS – Lipopolysaccharide

Mb – Mega base pairs

MOMP – Major outer membrane protein

***N. Gonorrhoea* –** *Neisseria gonorrhoea*

NAATs – Nucleic acid amplification tests

NICD – National Institute for Communicable Diseases

NPV – Negative predictive value

nvCT – New variant *Chlamydia trachomatis*

OM – Outer membrane

OmcB – Outer membrane complex protein B

OMP2 – Outer membrane protein 2

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

PID – Pelvic inflammatory disease

Pmps – Polymorphic membrane proteins

POC – Point-of-care

PorB – Porin protein B

PPV – Positive predictive value

RB – Reticulate body

rRNA – Ribosomal ribonucleic acid

SPG – Sucrose phosphate glutamine

SSA – Sub-Saharan Africa

STI – Sexually transmitted infection

T3SS – Type III secretion system

TB – Tuberculosis

TFI – Tubal factor infertility

TNF – Tumour necrosis factor

UV – Ultraviolet

VD – Variable domain

WHO – World Health Organization

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Abstract

Chlamydia trachomatis (*C. trachomatis*) is the most common bacterial sexually transmitted pathogen worldwide, especially in low- and middle-income countries, including South Africa. Although frequently asymptomatic, *C. trachomatis* infections in women cause pronounced genital inflammation. Given that genital inflammation increases women's risk for human immunodeficiency virus (HIV) infection, treating and preventing chlamydia is vital. Thus, there is an urgent need for effective interventions to curb chlamydia infection. Although vaccines are currently in development, none are yet approved for use. New drugs should also be developed and tested given the general rise of antimicrobial resistance. To advance such interventions, expertise in the basic microbiology of *C. trachomatis* is required. Techniques have indeed advanced over time; however, the standard methods of culture and quantification of *C. trachomatis in vitro* remain challenging. In South Africa, expertise in *C. trachomatis* culture and *in vitro* manipulation is particularly limited. Therefore, I aimed to establish a method to quantify laboratory-adapted stocks of *C. trachomatis* in *in vitro* cell culture, to develop research capacity and set the stage for the important future research needed to combat this pathogen. In this study, *C. trachomatis* was cultured from existing laboratory stocks and used to optimise and compare microscopy-based quantification methods.

First, representative *C. trachomatis* urogenital serovars (E, H) and lymphogranuloma venereum (LGV) serovars (L1 and L2) were propagated in McCoy cells, using an established centrifugation protocol. These stocks were used for all assays comparing three commercially available reagents: (1) Pathfinder's *C. trachomatis* monoclonal antibody, (2) Invitrogen's *C. trachomatis* major outer membrane protein (MOMP) antibodies, and (3) Trinity Biotech MicroTrak *C. trachomatis* culture confirmation kit.

In the research setting, fluorescent microscopy techniques are widely used for quantification of *C. trachomatis* due to their high sensitivity. However, this study showed the Pathfinder *C. trachomatis* monoclonal antibody kit and Invitrogen's *C. trachomatis* MOMP Monoclonal Antibody kits had poor sensitivity with high background fluorescent signals. Invitrogen's polyclonal antibody yielded inconsistent results, being either very weakly fluorescent or giving extremely bright signals. Thus,

counting bacteria using this polyclonal antibody had limited success and results were not reproducible. MicroTrak's kit, in contrast, allowed for clear visualisation of inclusions and allowed for consistent and successful counting of *C. trachomatis* bacteria.

This study reports inconsistent and/or unreliable results from the kits tested, with two of the three reagents performing poorly. The last, effective kit manufactured by MicroTrak was since discontinued. Thus, molecular methods, particularly qPCR-based methods should be utilised to quantify *C. trachomatis* in future *in vitro* cell culture studies.

Chapter 1: Literature Review

1. Introduction

Today, *Chlamydia trachomatis* (*C. trachomatis*) is one of the most common bacterial sexually transmitted pathogens worldwide (1) and associated with severe reproductive pathologies (2). A meta-analysis conducted in 2020 estimated the pooled prevalence of *C. trachomatis* was 2.9% in the general population (3.1% for females and 2.6% for males) worldwide (3).

Chlamydia is highly prevalent in South Africa, which also has a high incidence of HIV infection, especially in young women (4,5). Although there is limited *C. trachomatis* surveillance data in South Africa, three recent cohort studies have estimated Chlamydia prevalence in South African adolescent girls and young women to be 18-42% depending on geographical location (6–8). Chlamydia prevalence in South African males, ages 15-49 years is estimated to be lower than females, at ~6% (95% CI: 3.8-10.4%) (9). *C. trachomatis* infections are also frequently asymptomatic, leading to many cases being missed and untreated in regions like South Africa where syndromic management of sexually transmitted infections (STIs) is practiced (10). Studies suggest that HIV risk is significantly higher after or during infection with an STI (11) and/or bacterial vaginosis (BV) (12). Mechanisms for increased HIV risk include inflammation associated with the STI or BV in the female genital tract (13) or disrupted mucosal barrier function (14). Due to the significant public health burden of *C. trachomatis*, and its association with HIV infection, there is an urgent need to develop successful and practical interventions to prevent this disease and associated reproductive sequalae.

The field of *C. trachomatis* microbiology has been active since the 1960s (15). Despite this, there is still no vaccine available for use. Clearly, more research is needed that will ultimately lead to effective interventions. This literature review will discuss *C. trachomatis* as a human pathogen, with a focus on laboratory research and diagnostic methods pertinent to this thesis.

1.1 *Chlamydia trachomatis*: the organism

1.1.1 *Chlamydia trachomatis* classification

Bacteria of the genus Chlamydia are obligate intracellular pathogens as they cannot synthesise their own ATP and are therefore dependent on host cells (2). There are nine families of Chlamydia that infect an estimated 400 host species, including humans and animals such as birds, fish, reptiles and amphibians (41,42). Recently, chlamydial lineages have been discovered in deep anoxic marine sediments from the Arctic Mid-ocean ridge (18).

In humans, the two main species that have been found are *C. trachomatis* which targets columnar conjunctival or urogenital epithelial cells (19), and *Chlamydia pneumoniae,* which preferentially infects the epithelial tissue of the respiratory tract (20). Historically, *C. trachomatis* bacteria have been classified into biovars, based on tissue tropism, pathogenesis and biological characteristics in culture (21), or serovars, based on the major outer membrane protein (MOMP) that confers antibody specificity and is determined by serology (22). *C. trachomatis* biovars A-C infect the eye, causing trachoma, biovars D-K are urogenital, infecting the genital epithelium, while biovars L1-L3 cause lymphogranuloma venereum (LGV) (23–25) (**Table 1.1**). Furthermore, there are 15 distinct serovars of *C. trachomatis*, based on MOMP serology, which have been grouped into three serogroups based on serological relatedness (**Table 1.2;** (22,26,27). These include group B serovars (which contain serovars B, Ba, D, E, L1, and L2), the intermediate group (which contain serovars F, G, K and L3), and group C (which contain serovars A, C, H, I and J). The majority of the amino acid sequence of MOMP is conserved between serovars, although there are four variable domains (VD) which confer the differences between the serovars (27).

Table 1.1 *Chlamydia trachomatis* classification by biovar

***C. trachomatis* Biovar Group**	**Included Serovars**	**References**
Trachoma	A-C	(23–25)
Urogenital	D-K	(23–25)
Lymphogranuloma venereum	L1-L2	(23–25)

Table 1.2 *Chlamydia trachomatis* classification by serogroup

***C. trachomatis* Serogroup**	**Included Serovars**	**References**
Group B	B, Ba, D, E, L1, and L2	(22,26,27)
Intermediate Group	F, G, K and L3	(22,26,27)
Group C	A, C, H, I and J	(22,26,27)

1.1.2 Chlamydia lifecycle

Chlamydia undergoes a biphasic developmental cycle within host cells, consisting of a non-metabolically active but infectious elementary body (EB) phase; and a metabolically-active but less infectious reticulate body (RB) phase (28,29) (**Figure 1.1**). The EB is the infectious form of *C. trachomatis* that enters epithelial cells through attachment to host cell receptors, such as heparan sulphate (19).

Internalisation of the EB occurs during the first four hours (30). The *C. trachomatis* EB becomes incorporated into a phagosome where it moves to the distal region of the host Golgi apparatus. The *C. trachomatis* EB, contained within the host phagosome, is resistant to fusion or maturation into fusion with lysosomes by enabling the exit from the host endocytic pathway. This mechanism has similarities to *Mycobacterium tuberculosis* intracellular bacterium's survival strategy (31). As a result of this exit from the normal host endocytic pathway, *C. trachomatis* EBs protect themselves from the low pH, cytolytic and toxic environment of the host lysosome (32). *C. trachomatis*'s ability to survive phagocytosis and the host endocytic pathway may be due to its cysteine and lipopolysaccharide (LPS) rich cell wall which makes up the chlamydial outer membrane complex, protecting the bacteria (2,33). Within 6-12 hours after endocytosis, *C. trachomatis* EBs differentiate into the metabolically-active RB form,

which undergo many rounds of binary fission, and result in the formation of large vacuoles known as the inclusion bodies (26,34) (**Figure 1.1**). Finally, the RBs retro-differentiate into the infectious EB form which is released. When the infected host cell bursts (cytolysis), after 48-72h of infection, the EBs are able to infect neighbouring host cells (35).

During the infection cycle, the *C. trachomatis* EB use their type III secretion system (T3SS) to deliver over 100 bacterial effector proteins into the host cell (36). Once the EB makes contact with the host cell, the pre-packaged T3SS effector proteins are delivered into the phagosome triggering host cell cytoskeletal rearrangement, and promoting EB internalisation (37). *C. trachomatis* infection can become persistent where aberrant bodies (persistent form) may develop (38). Under stressful growth conditions (including nutrient starvation, IFN-γ exposure or antibiotic treatment (48, 49)), EBs convert into enlarged more persistent aberrant RB bodies, disrupting the developmental cycle (39). These bodies contain many inclusions that are viable but culture-negative, and can last for long periods of time in the infected host cells (39,40).

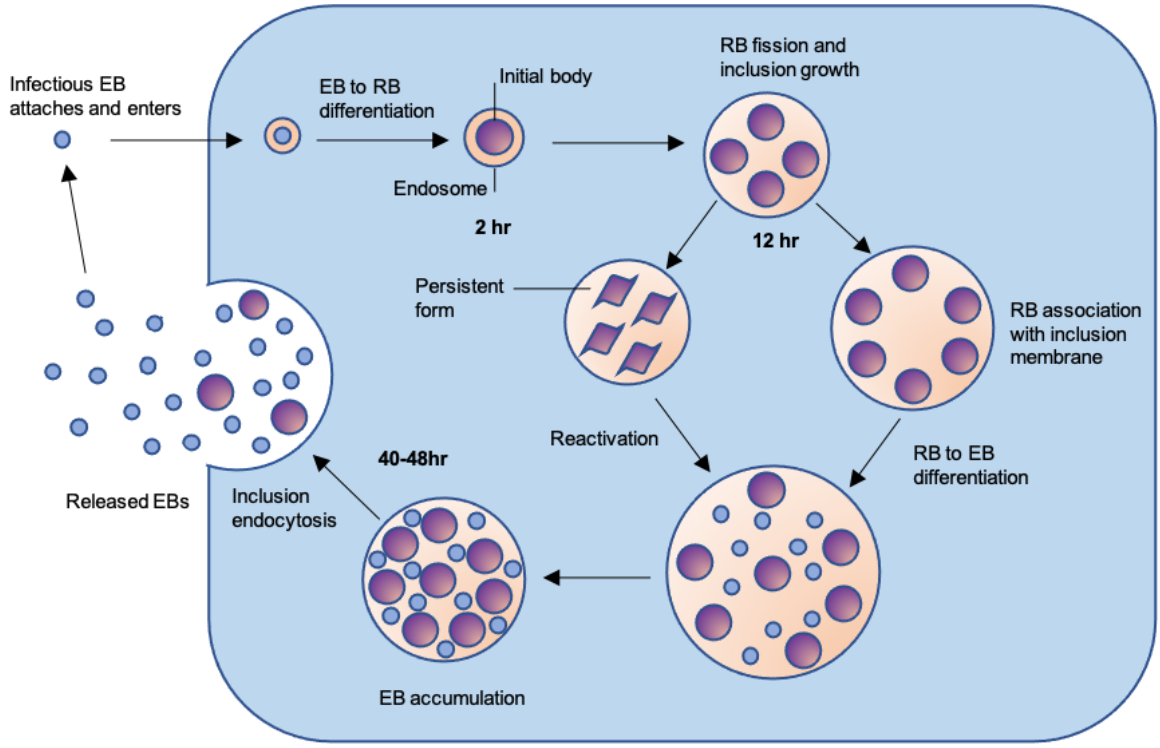

Figure 1.1 The developmental cycle of *Chlamydia trachomatis.* Infectious elementary bodies (EB) attach and enter the host cell. After internalisation, an initial body is formed by the endosomal membrane surrounding the EB. By 6-12 hours an inclusion body forms containing metabolically active reticulate bodies (RB) that differentiated from EBs. The RBs undergo fission and inclusion growth until they retro-differentiate into EBs after 48-72 hours post infection. The infected cell bursts, releasing infectious EBs that attach and enter neighbouring host cells. Under stressful growth conditions, a persistent form, aberrant bodies, may develop and can last long periods of time in the host cell. Figure adapted from **Brunham and Rey-Ladino, 2005** (26).

1.1.3 *Chlamydia trachomatis* genome and major antigens

The highly conserved genome of *C. trachomatis* comprises of 1.0Mb, with a 7.5Kb plasmid (41,42). Despite the genomic conservation of *C. trachomatis* in both the chromosome and plasmid, different serovars of *C. trachomatis* present dissimilar tissue tropism, with different disease outcomes and clinical prevalence (43). The chlamydial EB contains several major antigens including MOMP, a 40-kDa protein, which comprises over 60% of the chlamydial outer membrane complex (**Figure 1.2**) (22). *C. trachomatis* MOMP functions as an adhesin protein in the EB form and as a porin in the RB form (44,45). MOMP has been shown to induce both T and B cell responses making it a promising antigen for vaccine development (46). The polymorphic membrane proteins (Pmps) are type V secretion system autotransporters and are involved in EB attachment to the host cell (47). These proteins are encoded by the nine *pmp* genes. Pmps are thought to aid in immune evasion by the bacteria and may play a role in tissue tropism (30,48). Other important proteins of the chlamydial outer membrane complex include the outer membrane complex protein B (OmcB) and porin protein B (PorB). These highly conserved, cysteine-rich proteins play structural and specific roles during the differentiation of EBs into RBs and during EB infection (49).

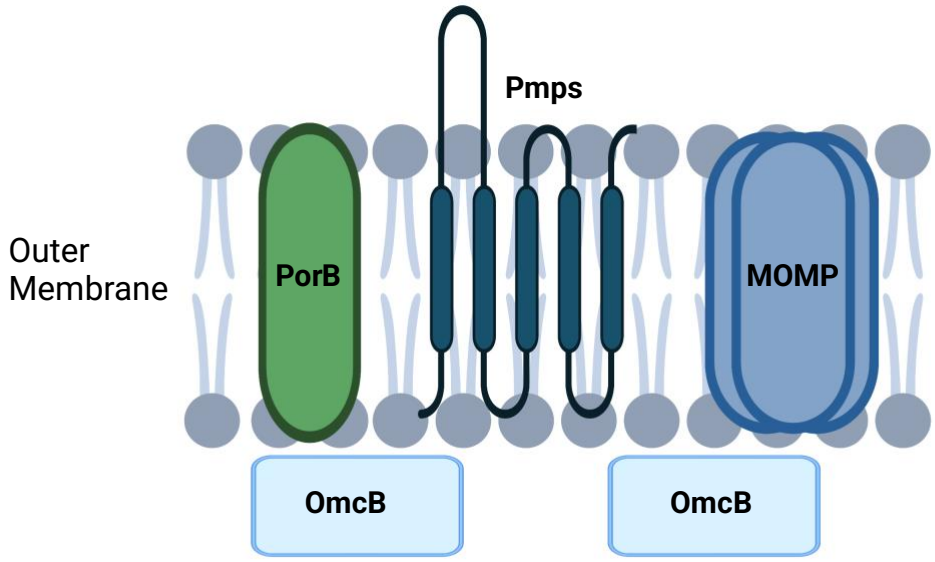

Figure 1.2 Diagram of the chlamydial outer membrane complex. The major outer membrane complex (MOMP) comprises of over 60% of the chlamydial outer membrane complex where it acts as an adhesin protein in the EB form and as a porin in the RB form. The polymorphic membrane proteins (Pmps) are involved in EB attachment to the host cell and are thought to aid in immune evasion by the bacteria and may play a role in tissue tropism. The outer membrane complex protein B (OmcB) and porin protein B (PorB) are highly conserved, cysteine-rich proteins that play structural and specific roles during the differentiation of EBs into RBs and during EB infection**.** Adapted from **Frohlich**, 2014 and **Christensen**, 2019 (209,210). Created with BioRender.com

1.2 *Chlamydia trachomatis* disease and pathology

C. trachomatis infections manifest differently in men versus women. Chlamydia urogenital infections in men occur mainly in those younger than 35 years of age and have been found to be asymptomatic in up to 50% of cases (50). Similarly, women frequently experience asymptomatic infections, or mild symptoms such as dysuria, bleeding and vaginal discharge (51). Although there are major consequences of chlamydial infection, due to inflammation (26), in both males and females, complications in the male genital tract are infrequent and cheaper to treat than in women. As a result, the consequences of infection on sperm quality and infertility in men has been poorly studied and current screening and research efforts are predominantly focused on women (50).

1.2.1 *Chlamydia trachomatis* serovar specific disease

Human biovars of *C. trachomatis* infect ocular, genital tract and respiratory epithelia (19,20). *C. trachomatis* serovars A, B and C are the etiological agent for trachoma, the world's leading cause of infectious blindness (52). Within endemic regions, repeated *C. trachomatis* infection results in chronic inflammation and damage to the conjunctiva leading to trichiasis, scarring and ultimately blindness (53). Sexually transmitted *C. trachomatis* serovars L1-L3 are thought to initially infect mucosal macrophages and monocytes, and pass through epithelial surfaces to regional lymph nodes causing disseminated infection (54). *C. trachomatis* pathology after infection with LGV strains begin as genital ulcers, and may result in blockage of lymphatic drainage and the development of painful buboes (2,55). The urogenital biovars (*C. trachomatis* serovars D-K) are the most prevalent worldwide, specifically serovars D, E and F (56). Infection with these may be asymptomatic or lead to symptomatic, clinically complex syndromes such as pelvic inflammatory disease (PID), tubal factor infertility (TFI) and ectopic pregnancies (57).

1.2.2 Urogenital *Chlamydia trachomatis*-related pathology in men

C. trachomatis EBs enter the penis and begin infection in the single-cell columnar layer of epithelium located in the penile urethra. Once inside the epithelial cells, the bacteria replicate and infect neighbouring cells causing ascending infection. As a result of male genital tract chlamydial infection, urethritis, epididymitis, epididymo-orchitis and even prostatitis can occur (58,59). Thus, *C. trachomatis* infection can affect most of the male reproductive system, including the prostate, seminal vesicles, epididymis, and testis. Studies have indicated that infection also results in increased inflammatory markers such as IL-8, CXCL9 and CXCL1 in semen (60,61). Infertility as a result of chlamydial infection can occur in 5% of men, however the exact cause remains unknown. There is some controversy as some studies suggest *C. trachomatis* infection is associated with poor semen quality and motility, while other reports show no association. This indicates the need for further research into the consequences of *C. trachomatis* infection in men.

1.2.3 Urogenital *Chlamydia trachomatis*-related pathology in women

As complications of infection are more severe in women, and so majority of research efforts have focused on infection in the female genital tract. *C. trachomatis* EBs enter the columnar epithelium of the endocervix where the bacteria begin to replicate and infect neighbouring cells. At the site of infection, inflammation occurs leading to cervicitis (26). Left untreated, the bacteria ascend from the lower genital tract to the upper genital tract (**Figure 1.3**) (62). Consequences of untreated or recurrent chlamydia infections include PID, fallopian tube injury and TFI due to damage and scarring of the upper reproductive tract (63). In fact, chlamydial DNA was detected by polymerase chain reaction (PCR) and in-situ hybridization in the endometrium, fallopian tubes and ovaries in women presenting with ectopic pregnancies or TFI demonstrating ascension from the vagina into the upper reproductive tract when left untreated (64). A meta-analysis showed that infection during pregnancy was associated with a higher risk of preterm birth, stillbirth, preterm premature rupture of membranes and low birthweight babies (65). Women repeatedly exposed to *C. trachomatis* through persistent infection or reinfection could develop severe damage to the upper genital tract induced by cell-mediated immunological reactions (inflammation), leading to the perpetuation of chlamydial sequelae (63). In the United States of America, the Centre for Disease Control estimates that the lifetime medical cost of STIs acquired through sexual contact is $15.9 billion. In sub-Saharan Africa, a costing study conducted in 2017 estimated that this low-to-middle income region covers 44% of the need for STI services (including prevention services, which were costed based on adult population sizes), and 30% of global STI control cost, despite having 40% of global STI burden (66). Clearly, *C. trachomatis* infection, particularly in women, has major medical, social, and economic consequences.

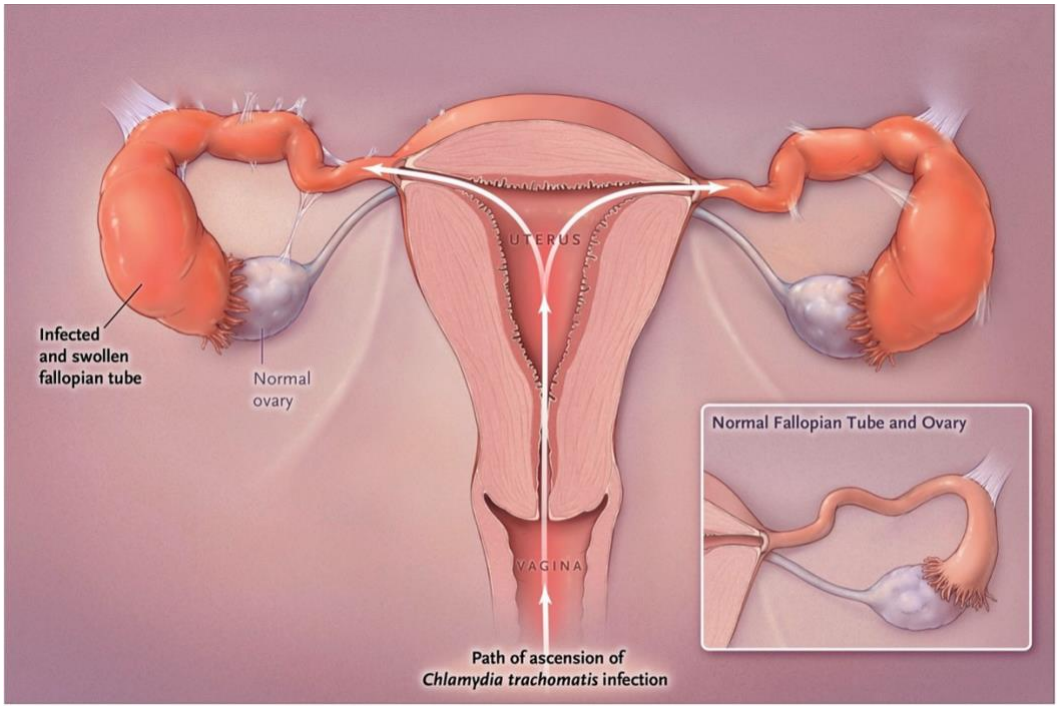

Figure 1.3 *Chlamydia trachomatis* ascension from the lower to upper genital tract in women. The bacteria begin infection in the columnar epithelium cells of the lower genital tract. Left untreated, the bacteria ascend to the uterus and fallopian tubes of the upper genital tract. Consequences of untreated infection include pelvic inflammatory disease (PID), fallopian tube injury, tubal factor infertility (TFI) due to damage and adhesions. Figure taken from **Wiesenfeld, 2017** (67)

1.2.4 Immunity to *Chlamydia trachomatis* infection

Early studies using congenitally athymic nude mice (lacking a thymus) highlighted the importance of a cell-mediated immune responses in control of chlamydia infections, where mice were infected intravaginally with murine *Chlamydia muridarum* (*C. muridarum*). Infection became chronic in nude mice and persisted for as long as 147 days, however heterozygous mice resolved infection in 17-20 days (68), demonstrating the importance of T cells in immunity to Chlamydia.

Both CD4+ and CD8+ T cell subsets have been detected at the site of infection in the genital tract in animal models of *C. trachomatis* infection and in human studies (69,70). In particular, CD4+ T helper (Th) 1 responses, characterised by production of interferon (IFN)-γ, are believed to be important for the generation of a protective

response against chlamydia and considered the best correlate of protection (71). Mice deficient in IFN-γ were confirmed to be more susceptible to *C. trachomatis* infection (72). The mechanism for IFN-γ mediated protection against *C. trachomatis* relates to the role of IFN-γ in inducing host cell indoleamine 2,3-dioxygenase (IDO), an enzyme that catabolizes tryptophan, which is needed for *C. trachomatis* survival (*C. trachomatis* is a tryptophan auxotroph) (73). IFN-γ-mediated induction of IDO is therefore thought to be the primary protective innate response against *C. trachomatis*, whereby intracellular *C. trachomatis* are starved of tryptophan.

Due to the obligate intracellular nature of *C. trachomatis*, there has been an increased focus on CD8+ T cells and their contribution in controlling infection, which is likely driven by the production of IFN-γ (74). CD8+ immune responses were detectable during *C. trachomatis* infection of mice and adoptive transfer experiments of *C. trachomatis*-specific CD8+ T cells into naive mice suggest that these cells can mediate protection (75). CD8+ responses have also been detected during human infection. These include HLA class 1 restricted CD8+ CTLs specific for MOMP as well as non-conventional CTLs (76,77).

Interestingly, there is some evidence that *C. trachomatis* serovar type may influence host-pathogen interactions including immune responses and pathology (78). A study by Verweij *et al*. (79), from the Netherlands, that measured serological responses in 510 women with PCR-confirmed *C. trachomatis* infections, showed that serovar D and E elicited the highest IgG responses, followed by serovar H (79). Other studies have reported an association between different serovars and possibility of *C. trachomatis* infections being asymptomatic (80). Specifically, serovars D, E and I were more likely to be associated with asymptomatic infections, while serovar G were more likely to be associated with symptomatic infections (59). Although correlates of protection are not fully understood, there is some evidence for partial-protective immunity in humans from previous infections, which is thought to be mediated by T-cells (82).

Partial protective immunity to *C. trachomatis* in humans was first suggested in highly exposed sex workers, where younger aged women were at an increased risk of chlamydia infection (11), with prevalence in women declining with age. The probability of reinfection was estimated to be three times more likely in women younger than 25

compared to older women (83). With repeated exposure to *C. trachomatis*, and acquired immunity to infection during each exposure to *C. trachomatis* over time, it has been proposed that protection from *C. trachomatis* infection occurs with age because of the accumulation of acquired immunity (84,85). Further evidence that has been proposed to support this partial protection model comes from the clinical observation that *C. trachomatis* re-infection rates are common after treatment with antibiotics (occurring in about 10-20% of individuals within a 12-month period; (86). The “arrested immunity hypothesis” has suggested that early treatment with antibiotics can blunt the development of adaptive immunity against *C. trachomatis* (87). With early *C. trachomatis* detection and treatment, the duration of infection is reduced. This would be considered a good outcome as the damaging effects to the female genital tract depend on the duration of infection (88). Interestingly, some patients undergo spontaneous resolution where untreated *C. trachomatis* infections resolve spontaneously. However, this observation and the potential contributing host factors are not well defined (83,89).

1.3.1 Chlamydia culture systems for microbiological research and diagnostics

Although diagnostic methods were not a focus of this thesis, there is an overlap between older diagnostic methods using culture, and current microbiological research methods. Historically, chlamydia was isolated and propagated using the yolk sack of embryonated hen eggs, until the introduction of a tissue culture system in 1965 (15). Subsequently, the standard diagnostic approach for *C. trachomatis* was propagation cell culture, whereby monolayers were infected with inoculum prepared from urogenital specimens. Both McCoy and HeLa cell lines were most commonly used during the propagation of *Chlamydia* species (90).

Previous studies have documented the difficulty in obtaining substantial chlamydia yields from propagation in cell lines (91), since chlamydia is cytotoxic (92), resulting in host cell death and consequently lower yields. Throughout the replication cycle, *C. trachomatis* bacteria depend on the viability of host cells, therefore studies have focused on methods to optimize *C. trachomatis* propagation to obtain better yields.

The important role of cycloheximide, a eukaryote protein synthesis inhibitor (93), in large scale culture of chlamydia was first demonstrated by Ripa and Mardh who showed that cycloheximide led to an increase in chlamydia yield by supressing, but not completely inhibiting, host cell metabolism (94).

While culture methods for diagnostics are specific, they have been shown to exhibit low sensitivity with detection rates ranging between 60-80%, even when performed by experienced technicians (95). Culture is also labour intensive and time consuming and has been phased out of use in diagnostic laboratories. However, *C. trachomatis* culture is still required in surveillance laboratories to monitor *C. trachomatis* virulence changes, antimicrobial susceptibility and as a positive control for diagnostic tests (96). Tissue culture systems have allowed researchers to culture, isolate and purify both clinical specimens and laboratory-adapted bacteria, for further investigations (15). Recently, a three-dimensional bead culture system has been developed where *C. trachomatis*-infected cells are polarized on collagen-coated microcarrier beads (31). This novel culture method was shown to yield more infectious *C. trachomatis,* with a higher bacterial titre than the flask culture method (97).

Though rarely used today, Giemsa and iodine stains have been used for detection of *C. trachomatis* cultured for research and diagnostic purposes. However, these staining methods lack sensitivity in comparison to immunofluorescence (98). Stamm *et al*. showed immunofluorescence staining detected eightfold more inclusions per monolayer than iodine staining and exhibited higher overall sensitivity (98% versus 84%) (99). Similarly, other studies have demonstrated direct immunofluorescence staining techniques are more sensitive and specific than the Giemsa staining method (100). Even though these methods are now outdated, they are cheaper than immunofluorescence techniques and are therefore useful to consider in resource-limited settings.

Immunofluorescence staining using anti-MOMP antibodies, is the preferred method for culture confirmation nowadays (101). In addition to MOMP, which is highly conserved, antibodies against Chlamydial LPS are also used. However, due to the uneven distribution of LPS on the surface of EBs, it exhibits inferior staining characteristics when compared to those against MOMP (102).

1.3.2 Molecular diagnostic methods

Although not the focus of this thesis, it is important to evaluate diagnostic methods for *C. trachomatis* infections, since there have been major developments since the late 1990's (103).

1.3.2.1 Nucleic acid amplification tests (NAATs)

Nucleic acid amplification tests (NAATs) have replaced culture as the diagnostic 'gold standard' (103). NAATs are highly sensitive and specific and can be performed on several clinical specimens including cervical, urethral, anorectal, and ocular swabs (96). Non-culture methods such as this offer several advantages over culture, since amplification of nucleic acids require less invasive methods of sample collection, lower starting concentrations of *C. trachomatis* and have higher detection rate success (104). However, NAATs amplify target DNA originating from both viable and non-viable bacteria, resulting in possible overestimation of quantitative test positivity (105).

The near-point of care (POC) a 90-minute real-time PCR-based Cepheid GeneXpert platform is well-established and used for the diagnosis of tuberculosis (TB) (106). In addition to the GeneXpert *C. trachomatis* NAAT (Cepheid, Sunnyvale, California), the US Food and Drug Administration (FDA) has approved 53 microbial tests for the detection of *C. trachomatis* and *Neisseria gonorrhoea* (*N. Gonorrhoea*). These include the commonly used Abbott RealTime CT/NG and Roche AMPLICOR CT/NG test for *Chlamydia trachomatis* test (**Table 1.3**)**.** In 2012, the FDA approved the use of the GeneXpert CT/NG assay for the detection of *C. trachomatis* and *N. gonorrhoea* (107)*.*

Although South Africa utilises syndromic management for the control and treatment of *C. trachomatis* infection, the National Institute for Communicable Diseases (NICD) does offer NAAT testing on request. These tests use PCR or transcription mediated amplification (TMA) methodology and have a 10-day turnaround time (**Table 1.3**). Appropriate samples include urethral, vaginal or endocervical swabs and first-void urine, and must be transported on ice or frozen (108).

1.3.2.2 Point-of-Care (POC) antigen testing for *Chlamydia trachomatis*

POC diagnostic tests (**Table 1.3**) are intended to be performed at primary health care clinics. Test results are intended to be rapidly obtained (10 minutes) for faster patient management and improved clinical management outcomes (109). The World Health Organization (WHO) criteria for POC use in resource-limited settings state that an ideal POC test must be affordable, sensitive, specific and also user-friendly, rapid and equipment-free (110,111). POC tests are generally based on antibody and antigen binding detection and as lateral flow technology, including those used to detect *C. trachomatis*. However, most of these antigen tests have demonstrated significantly lower specificity and sensitivity compared to NAATs (112). This lack of sensitivity in antigen-directed POCs has led to a shift to the more sensitive near-POC testing, that include NAATs (113).

Table 1.3 Molecular diagnostic methods for the diagnosis of *Chlamydia trachomatis* infection

Test type	Examples of tests	Manufacturers	Estimated turnaround time	Specimen	Sensitivity and Specificity range*	Positive and Negative predictive values (PPV and NPV) range*†
NICD tests (approved for use in SA)	APTIMA Combo 2 Assay *Chlamydia trachomatis* real-TM In-house Multiplex PCR	Hologic, Inc. SACACE Biotechnologies NICD	10 Days	**Male**: Urethral swab, urine, prostatic and seminal liquid **Female**: Endocervical swab, vaginal swab Note: Only *Chlamydia trachomatis* real-TM can be performed on prostatic and seminal liquid.	**Male:** Sensitivity of 95.9%-97.9% Specificity of 97.5%-98.5% **Female**: Sensitivity of 94.2%-94.7% Specificity of 97.6%-98.9%	**Male:** PPV: 92.9%-95.8% NPV: 98.6%-99.3% **Female:** PPV: 87.4%-93.8% NPV: 99.1%-99.0%
Diagnostic laboratory tests (NAATs)	Abbott RealTime CT/NG (114) AMPLICOR CT/NG test for *Chlamydia trachomatis* (115)	Abbott Molecular Inc. Roche Molecular Systems, Inc.	6 hours – 2 days	**Male**: Urethral swab and urine **Female**: Vaginal swab, endocervical and urine	**Male:** Sensitivity of 93.4%-96.6% Specificity of 98.3%-99.3% **Female:** Sensitivity of 91.3%-98.4% Specificity of 94.7%-100%	**Male** and **Female**: PPV: 22.5%-98.3% NPV: 98.0%-100%

Near point of care tests	Xpert CT/NG (116)	Cepheid	90 Minutes	**Male**: Pharyngeal swabs, rectal swabs and male first catch urine **Female**: Patient collected vaginal swabs, endocervical swabs, pharyngeal swabs, rectal swabs, and female first catch urine	**Male**: Sensitivity of 86%-98.5% Specificity of 99.4%-99.8% **Female**: Sensitivity of 86%-99.5% Specificity of 99.1%-99.8%	**Male**: PPV: 57.2%-99.8% NPV: 87.7%-100% **Female**: PPV: 53.6%-99.8% NPV: 87.7%-100%
Point of care (RAPID test)	Chlamydia Rapid Test (117) Chlamydia Rapid Test Device (118)	Creative Diagnostics Abon Biopharm (Hangzhou) Co., Ltd.	10 mins	**Male**: Urethral swab and urine **Female**: Cervical swab Note: Only Chlamydia Rapid Test Device can only be performed on male urine.	**Male**: Sensitivity of 20%-90.9% Specificity of 92.9%-100% **Female**: Sensitivity: 88.5% Specificity: 96.7%	**Male**: PPV: 100% NPV: 91.8% **Female**: PPV: Not given NPV: Not given

***** Specimen-dependent

† Prevalence-rate % dependent

1.3.3 *Chlamydia trachomatis* evolution and diagnostic testing

C. trachomatis is generally considered highly conserved and genetically stable (119). However, new variants of the bacteria have recently emerged with the Swedish new variant (nvCT), named by Ripa and Nilsson (120), being the most well documented after first being reported in 2006. Interestingly, the nvCT appeared to have higher infectivity rates than the non-variants in the Swedish population. The nvCT was shown to carry a 377 bp deletion within its plasmid, including the targets used by the Roche and Abbott diagnostic systems. Wildtype *C. trachomatis* and nvCT appeared to have similar biological fitness *in vitro* which suggests the rapid nation-wide transmission of nvCT was probably due to the favourable diagnostic selective advantage (121). A South African study by Venter *et al*. sequenced 459 *C. trachomatis* positive clinical specimens and found no evidence of nvCT strains, indicating this new variant was not yet present in this South African cohort (122). In 2019, another *C. trachomatis* variant was described in Finland, that had a nucleotide substitution in the 23S rRNA (C1515T mutation) of *C. trachomatis* (123). Importantly, it is widely believed that the emergence of these new *C. trachomatis* variants may be a bigger problem than currently appreciated, since there may be numerous undiscovered variants escaping detection in many populations globally. Such variants will only be detected through widespread sequencing and surveillance.

1.4.1 Syndromic management for the treatment *Chlamydia trachomatis* infections

C. trachomatis infection is readily treatable at the individual level with antibiotics (103), however at the population level, it is more difficult to control the spread of infection due to stigma and behavioral factors (124). Public health intervention approaches include 1) health promotion, access to condoms and education; 2) diagnosis and management services, partner notification and treatment 3) provision of opportunistic testing, especially for at risk populations and 4) provision of population screening. In order for these strategies to have a significant effect, this combination should be employed simultaneously (124).

Due to the difficulties in treating STIs in low resource settings, syndromic management which relies on patient's signs and symptoms (syndromes) to treat infection, was introduced into guidelines of low-income countries, such as South Africa, in the early 1990's upon WHO recommendation (125). The following specific syndromes are outlined in the Department of Health's STI management guidelines for *C. trachomatis* infection: vaginal discharge syndrome, male urethritis syndrome, lower abdominal pain, genital ulcer syndrome and bubo (126). Once specific syndromes have been identified, the appropriate medication is administered, and patients referred for gynecological or surgical assessment if necessary. Syndromic management on a national scale has many advantages such as low cost and only requires a single patient visit, reducing the risk of loss to follow up. Patients receive treatment immediately avoiding potential issues that may be experienced in limited laboratory facilities and limited sensitivity of certain tests (127,128).

1.4.2 Improving *Chlamydia trachomatis* diagnostics and treatment

Although syndromic management can be expected to decrease prevalence of curable STIs that present as symptomatic, this strategy has been shown to be less effective in decreasing the prevalence of asymptomatic infection, such as *C. trachomatis* (6,129–131). On the other hand, some symptomatic women are incorrectly or unnecessarily treated with antibiotics in a syndromic approach. This is a concern as this may lead to a possible increase in antimicrobial resistance over time (132) and is a risk factor for BV (133), a shift in the vaginal microbiome away from being *Lactobacillus*-dominant (134).

A cross-sectional study conducted in South Africa evaluated the performance of three different guidelines for the management of vaginal discharge syndrome. Findings showed that majority of women with *C. trachomatis* or other curable STIs would not receive treatment with the syndromic approach as they presented with asymptomatic infections (135). In fact, a study by Kaida *et al*. comparing genital tract infection prevalence by syndromic-based and laboratory assessment among young sexually-experienced South Africans, reported that 77.8% of females and 100% of males with laboratory-diagnosed genital tract infections were asymptomatic and would not receive treatment in a syndromic approach (136). In an observational cohort study

also conducted in South Africa, pregnant women living with HIV were enrolled to receive either aetilogical testing using Xpert *C. trachomatis*/NG or standard syndromic management. The prevalence of *C. trachomatis* was 30% at baseline, and at postnatal care the prevalence of STIs was lower among those that received aetiological care in comparison to those receiving syndromic management (14% and 23% respectively; aRR 0.61; 95% CI 0.35-1.05) (137). Although syndromic management has been ineffective in decreasing prevalence of *C. trachomatis*, until a vaccine becomes available, other low-cost strategies for enhanced STI care must be considered and implemented, especially in countries like South Africa.

One such study with a different approach to STI care was conducted by Garrett *et al*., among a cohort of young women at high HIV risk in South Africa, using a care model comprising of POC STI testing with immediate treatment, and expedited partner therapy (EPT). The EPT packs included appropriate medication for partners as well as male condoms and an information leaflet. This study showed that this strategy was acceptable to young South African women and larger studies observing feasibility and cost-effectiveness should be conducted in the future (7). If successful, this treatment format could be an effective alternative to syndromic management as partners are also treated for infection.

Chlamydia control programmes predominantly focused on screening sexually active young women, have been implemented across the globe to reduce the burden of *C. trachomatis* infection and related sequalae (138). Early diagnosis and treatment are essential to reduce risk of complications and prevent further disease transmission, however, in low resource settings, there are long delays between testing and treatment. These delays are due to physical distance from laboratories and difficulties with patient follow-up, contact tracing and treatment adherence (139).

Another common issue with screening is low uptake of STI testing by young sexually active individuals (140). Thus, screening in non-medical settings has been proposed as a method to improve uptake (141). A British ‘Test n Treat’ trial was conducted where on-site rapid *C. trachomatis* tests were offered with same day treatment to sexually active students attending further education colleges. Although this pilot study showed

54% acceptance (142), the feasibility trial still had a low uptake of testing (<15%), despite high rates of *C. trachomatis* (6%) at baseline and follow-up (143). Low uptake was attributed to limited knowledge on STIs, stigma and not feeling at risk. Therefore, future studies should include STI education and engagement of peer influencers to increase uptake and acceptance (140).

More recently a study by Estcourt *et al.* employed the eSexual Health Clinic system (eSHC) in England for management and control of STIs such as *C. trachomatis*. Their findings showed that roughly 75% of eligible people chose to access the online chlamydia pathway and about 60% of patients managed their care completely online (144). Studies like these highlight the need for more innovative strategies to tackle programme uptake and adherence.

1.4.3 Behavioral interventions to manage *Chlamydia trachomatis* risk

Behavioral interventions are designed to encourage high-risk individuals to change their actions to improve their health outcomes (145). Behavioural interventions tailored to psychological and behavioural characteristics of individuals have the potential to be more effective than generic interventions to reduce *C. trachomatis* prevalence (146). However, there is conflicting data as to whether such programmes are effective or not.

A community-based intervention trial was conducted in China targeting female sex workers where clinic-based outreach activities, such as awareness-raising, condom promotion, and sexual health care, were developed. As a result of the intervention, an increase in condom use (55.2% at baseline to 67.5% at 12 months) and a decrease in *C. trachomatis* prevalence (41% at baseline to 26% at 12 months) were reported (147). Another trial conducted in the United States targeting minority women used behavioural-cognitive intervention strategies, or enhanced intervention which included weekly support group meetings to the goal of having participants recognize their risk and commit to behaviour change. This study showed those in the intervention arm were significantly less likely to have repeat *C. trachomatis* infections than controls (148). Although successful, other behavioural intervention trials focussing on adolescents in Sub-Saharan Africa (SSA) have been ineffective in decreasing

prevalence of curable STIs (149). As SSA youth bears a disproportionate burden of HIV and curable STIs where four out of five new HIV infections are in young women aged 15-19 years (150), other primary prevention approaches should be investigated until a vaccine has been developed.

1.4.4 Considerations for designing *Chlamydia trachomatis* interventions

Epidemiological trends have been well documented in many countries such as Finland, where case rates have risen by 60% but the population seroprevalence rates have fallen by 50% (151). Some evidence suggests that early treatment may be interfering with the natural development of protective immune responses (87). This idea can also provide a reason for declining sequelae rates as early treatment would result in a shorter duration of infection. As the development of an adequate immune response is hindered, susceptibility to reinfection is increased further reducing herd immunity and resulting in a further increase in prevalence (64,152). The arrested immunity hypothesis, coined by Brunham *et al*. (88), provided a suggestion as to why despite numerous intervention programmes, antimicrobial treatment and contact tracing, case rates continue to rise.

1.4.5 *Chlamydia trachomatis* vaccines

The goal of a successful preventative *C. trachomatis* vaccine is to reduce upper genital tract sequelae in women and reduce the risk of inflammation in this area or to prevent acquisition and transmission of infection. Genital *C. trachomatis* vaccine efforts have been in the preclinical stage of testing for many years and have focused on animal models specifically mice and non-human primates. Live-attenuated, subunit and recombinant protein vaccines have been explored in these well-established animal models (153). One such study showed live-attenuated (plasmid-deficient) *C. muridarum* retained the ability to infect murine genital tracts without causing disease. This model was used to demonstrate that mice previously infected with plasmid-deficient mutants were in fact protected against disease and oviduct pathology upon challenge with wild-type *C. muridarum* (154,155). This favourable result using a live-

attenuated vaccine prompted scientists to evaluate this model in non-human primates (156). However, Qu *et al.* showed that macaques that underwent cervical inoculation with either wildtype *C. trachomatis* serovar D or a plasmid-deficient derivative expressed similar inflammation levels. Notably, both groups also exhibited the same level of pathology after five challenges with *C. trachomatis*. The authors suggested this result may be due to genetic differences between the individual animals (157), but this failure highlighted the shortcomings of live-attenuated chlamydia as a vaccine candidate due to the immunopathology induced.

Subunit vaccines have been shown to be safer than live-attenuated vaccines in numerous vaccine studies that focused on the Chlamydia MOMP (46,158). MOMP has been the most widely researched candidate of subunit vaccines in multiple animal models to date. However, studies show that MOMP-based vaccines alone provide incomplete protection, and effectiveness is highly dependent on the conformational structure of the protein (159). As MOMP is an integral membrane protein it is extremely difficult to stabilise its native conformation (71). This prompted researchers to evaluate other candidate vaccine antigens such as OMP2, HSP60, Pmp2 and CPAF (26,160). A vaccine study, that has recently entered clinical trials, tested a multivalent vaccine containing the variable domain 4 (VD4) region and surrounding constant segments of MOMP from *C. trachomatis* serovars D, E and F adjuvanted with CAF01 (161). The VD4 region contains the highly conserved species-specific epitope “LNPTIAG”, which can elicit a broadly cross-reactive immune response with a high titre antibody response able to neutralize multiple serovars of *C. trachomatis*. Promisingly, the construct induced immune responses in mice, some serovar specific, and neutralized serovars D, E and F. Mice vaccinated with this construct were protected against challenge and exhibited reduced bacteria in the vagina due to a robust T cell response induced by the construct. Pathological changes in the upper genital tract were also prevented after challenge (162,163).

Subunit vaccines generally require the inclusion of adjuvants to improve immunogenicity and replicate the robust immune responses elicited by whole-organism vaccine candidates. Stary et.al (164) utilizing charge-switching synthetic adjuvant nanoparticles conjugated to ultraviolet (UV) light–inactivated *C. trachomatis*

demonstrated the following: uterine exposure to UV-*C. trachomatis* generated tolerogenic *C. trachomatis* -specific regulatory T cells resulted in exacerbated bacterial burden upon rechallenge with *C. trachomatis*. However mucosal immunization with UV- *C. trachomatis* -cSAP elicited long-lived protection with a robust systemic memory T cell response. They also emphasised the need to consider route of injection as their study showed only mucosal vaccination induced a wave of effector T cells that seed the uterine mucosa during week one of vaccination and establish resident memory T cells. This study highlights the importance of a strong adjuvant to induce robust immunological responses (164). A new approach to reduce bacterial or viral load and enhance immunogenicity simultaneously is the use of therapeutic vaccines (165). These vaccines can also be used as alternative treatment to antibiotics. Therapeutic vaccine research targeting *Chlamydia pecorum* in Koalas showed positive therapeutic effects at infected anatomical sites as well as a reduction in infectious load of the bacteria (166). A promising result that can be extended to other species of animal and humans.

As of 2017, vaccine development for Chlamydia has moved into the phase 1 stage of testing, focusing on vaccinations containing rMOMP, with cationic adjuvant formulation 1 (CAF01) (162). This phase 1, first-in-human, double-blind, parallel, placebo-controlled trial was done with healthy women aged 19-45 years of age. The participants were randomly assigned to one of three groups in a 3:3:1 ratio: CTH522 adjuvanted with CAF01, CTH522 adjuvanted with aluminium hydroxide (AH), or placebo (saline). The vaccine schedule comprised of three intramuscular injections followed by two intranasal administrations over a period of 5 months. For this study the primary outcome was safety, and the secondary outcome was humoral immunogenicity measured by anti-CTH522 IgG seroconversion. Both vaccine groups showed an immune response compared with none in the placebo arm and the CTH522:CAF01 vaccine showed a better immunologic profile (5.6-fold higher antibody count) than that of the CTH522:AH candidate. No serious adverse events were recorded in any group. Both candidates appear to be safe and immunogenic (167) and there is indication of additional candidates entering clinical trials in the near future (168).

1.5 Aims and Rationale

The overall objective of this study was to evaluate the performance of the following three fluorescent reagents to quantify *C. trachomatis* in McCoy cells: (1) the Pathfinder® *Chlamydia trachomatis* monoclonal antibody kit, (2) Invitrogen *Chlamydia trachomatis* MOMP polyclonal and monoclonal antibodies and (3) the MicroTrak® *Chlamydia trachomatis* Culture Confirmation Kit.

The specific aims of this project were:

Aim 1: to propagate and store representative *C. trachomatis* serovars E, H, L1 and L2.
Aim 2: to compare the performance of commercial fluorescent reagent kits to detect *C. trachomatis in vitro.*
Aim 3: to determine the concentration of bacteria in *C. trachomatis* stocks generated for **Aim 1**, using the optimal kit determined in **Aim 2**.

Rationale

C. trachomatis is the most common bacterial sexually transmitted pathogen worldwide, especially in Sub-Saharan Africa. Experience and skill in *C. trachomatis* culture methods and *in vitro* manipulation is limited in South Africa, where research into new biological interventions would make the most impact. The ability to culture and manipulate *C. trachomatis* in the laboratory is an essential first step to develop and test new interventions. This study establishes expertise in basic *C. trachomatis* microbiological techniques and considers different methods to confirm the propagation of *C. trachomatis* in *in vitro* cell culture. The ultimate goal of this study was to establish a robust method for the detection and counting of *C. trachomatis* bacteria in cell culture. Serovars E and H were chosen as representative urogenital serovars as E is the second most common serovar in South Africa and we had access to E and H previously isolated stocks in our laboratory. Serovars L1 and L2 were chosen as representative LGV serovars and are less cytotoxic than the urogenital serovars, resulting in less McCoy cell death and ultimately an increased yield of bacteria after propagation.

Chapter 2: Materials and Methods

2.1 McCoy cell culture

Cryopreserved McCoy cells (American type culture collection; ATCC®; CRL-1696™) were kindly provided by Dr Leonard Damelin from the NICD (Johannesburg, South Africa). One vial of cryopreserved cells (~2 x 10^6 million) was thawed rapidly at 37°C for 2 minutes and transferred to a 15ml centrifuge tube. Prewarmed E10 media (12ml; **Table 2.1**) was added slowly to avoid shocking the cells. The cell suspension was centrifuged for 7 minutes at 200 x g and the cell pellet was resuspended in fresh 5ml E10 media and transferred to a 75cm^2 tissue culture flask containing 20ml of E10. Cell attachment was checked using an inverted light microscope after 24 hours.

Table 2.1 Components of E10 Media

Component	**Manufacturer**
10% Heat-inactivated foetal calf serum (HI-FCS)	Hyclone, USA
Gentamicin solution (4µg/ml)	Sigma-Aldrich., USA
Fungin (4µg/ml)	Invivogen
L-glutamine (3mM)	Sigma-Aldrich., USA
Eagle's Minimum Essential Medium (EMEM)	Sigma-Aldrich., USA

McCoy cells were subcultured every 72h when they had reached ~90% confluency. To begin the subculturing process, media was removed from the tissue culture flask and the cell monolayer was washed twice with 5ml Hanks Basic Salt Solution (HBSS; HyClone, USA). Prewarmed Trypsin/EDTA (2.5ml; Sigma-Aldrich, USA) solution was incubated with the cell monolayer for 2-3 minutes to detach the cells. The flask was washed with warm E10 media (3ml), and the cell suspension was transferred to a 15ml centrifuge tube. The flask was washed out to collect any remaining cells. The cells were centrifuged for 7 minutes at 200 x g and the cell pellet was thoroughly resuspended in 5ml of E10. To count the cells, 10µl of a 1:1 dilution of cell suspension and Trypan blue (Sigma-Aldrich., USA) was loaded onto an Improved Neubauer Chamber haemocytometer (**Figure 2.1**)**.** Cells in two of the squares (indicated in blue in **Figure 2.1**) were counted under a light microscope, at 40x magnification**.**

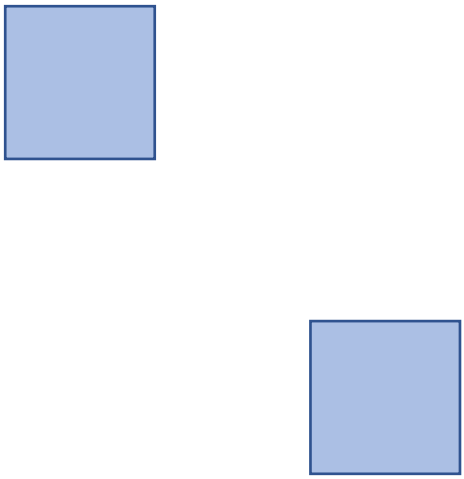

Figure 2.1 An improved Neubauer chamber haemocytometer grid. Cells were counted under a light microscope at 40x magnification. The cells observed in the squares highlighted in blue were counted.

Viability and the total number of cells were determined from these counts. McCoy cells were consistently >99% viable, this was assessed by staining cells with trypan blue. The following formula was used to calculate the total number of cells:

$$\text{Cells/ml} = \frac{total\ no.cells\ counted}{no.squares\ counted} \times\ dilution\ factor \times\ 10^4$$

On average, 7.6 million cells (range of 4.5-11.8 million) were obtained per 75cm^2 flask, and 1 million cells were used to seed a new flask for subculture. McCoy cells were subcultured 24h prior to any chlamydia infection experiment or every 72h for routine maintenance.

2.2 Infection of McCoy cells and preparation of *Chlamydia trachomatis*

C. trachomatis representative serovars E, H (Strain UW-43/Cx ATCC® VR-879™), L1 (strain 440 ATCC VR- 901B) and L2 (US151) were kindly provided by Drs Bronwyn Joubert and Leonard Damelin for this dissertation **(Table 2.2)**. To generate *C.*

trachomatis stocks from previously isolated *C. trachomatis*, infected McCoy cell cultures were scaled up by passage every 72h. Initially, McCoy cells were seeded in 5 wells of a 24-well plate in 500µl E10 media and left for 24 hours for cells to adhere. *C. trachomatis* stocks, previously prepared by Dr Rubina Bunjun (supervisor; UCT) and provided for this study, were thawed at room temperature inside a biosafety cabinet. E10 media (120µl) was removed from the McCoy monolayers and 120µl of *C. trachomatis* stocks of unknown concentration was added to each well. A well with no chlamydia was also included as a negative control. The 24-well plate was centrifuged for 1h at 2500 x g at room temperature to facilitate infection (spinoculation). The plate was immediately rested for 1h at 37°C in a 5% CO_2 incubator, then each well was washed twice with 500µl sucrose-phosphate-glutamate buffer (SPG; Appendix 1). The infected monolayers were incubated at 37°C in a 5% CO_2 incubator for 72h in 1ml of infection media (IM; **Table 2.3**). Infection was monitored by the presence of round intracellular bodies within the host cells under a light microscope (x40) at 24 hours post infection (hpi).

Table 2.2 Origin of *Chlamydia trachomatis* isolates used to prepare *Chlamydia trachomatis* serovar E, L1, L2 and H stocks

***Chlamydia trachomatis* serovar**	**Type**	**Origin of isolate**
E	Urogenital	Maleka *et al.* (169)
L1	LGV	Strain 440 (ATCC VR- 901B) provided by Bronwyn Joubert, University of KwaZulu-Natal
L2	LGV	US151 provided by Bronwyn Joubert and isolated at Prince Cyril Zulu Communicable Diseases Clinic in Durban, South Africa.
H	Urogenital	Strain UW-43/Cx [ATCC® VR-879™] provided by Dr Leonard Damelin from the NICD.

Table 2.3 Components of Infection Media (IM)

Component	**Manufacturer**
E10	Described in Table 2.1
Glucose (330µM)	Sigma-Aldrich., USA
Cycloheximide (0.5µg/ml)	Sigma-Aldrich., USA

At 72 hpi, infected McCoy cells were scraped off the 24 well plate using a P1000 pipette tip (passage 1) and resuspended in the culture media (IM). The infected cell culture media was split between 2 wells of fresh McCoy cell monolayers in a 6-well plate. To ensure the cell monolayer would not dry out, the volume in each well was adjusted to ensure a minimum of 1ml. This plate was then centrifuged at 2500 x g for 1h at room temperature and incubated for 1h at 37°C in a 5% CO_2 incubator. The monolayers were washed twice with 500µl SPG, and IM (3ml) was added to each well and the plate incubated for 72h.

After 72h, the chlamydia culture was passaged again (passage 2), essentially as described above. Briefly, the infected cells were lifted with a cell scraper and resuspended in the culture media. This suspension, now referred to as *C. trachomatis* stocks, was stored in 500µl aliquots in screw cap tubes, to ensure sterility. The stocks were stored at -80°C. The step-by-step process for the preparation of *C. trachomatis* stocks from initial infection to harvest is summarised in **Figure 2.2,** this process takes ten days to complete.

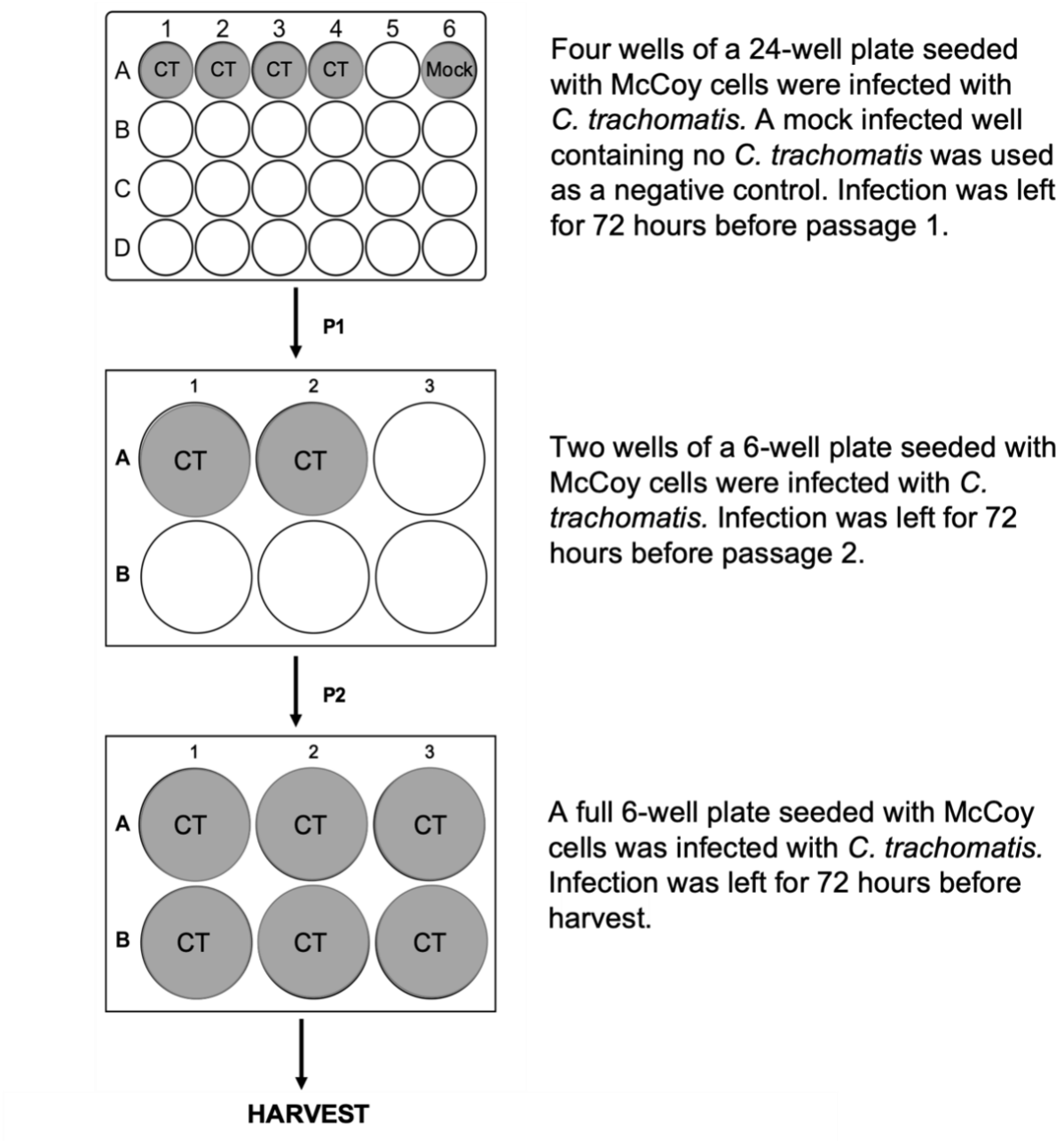

Figure 2.2 Flow diagram for preparation of *Chlamydia trachomatis* stocks from previously isolated *Chlamydia trachomatis* in cell culture plates. The process from initial infection of McCoy cells with previously isolated bacteria to harvest of *C. trachomatis* takes ten days to complete.

2.3 Partial purification of *Chlamydia trachomatis*

Differential centrifugation was used to concentrate the chlamydia preparation. Prior to concentrations, chlamydia stocks were cultured as described in section 2.2, with a third passage of 4 x 6 well plates performed. The cell monolayer was scraped off the plate and the cell suspension transferred to a 50ml centrifuge tube that was then centrifuged at low speed for 10 minutes at 500 x g at 4°C to pellet the large host material. The supernatant containing chlamydia, was then transferred to a 50ml high speed centrifuge tube, and kept at 4°C while the leftover cell pellet of host material was lysed to release any remaining *C. trachomatis* still within the host cell. Physical lysis was performed by resuspending the cell pellet in 475µl SPG and passing it through a 23G hollow needle five times. Chemical lysis was then performed by incubating the cells with 0.25% SDS for 10 minutes at 37°C. HI-FCS (500µl) was added to dilute out the SDS before centrifugation for 10 minutes at 500 x g at 4°C. This supernatant was pooled with the previously collected supernatant and centrifuged for 1 hour at 12000 x g at 4°C to pellet the chlamydia. The chlamydia pellet was then resuspended in 1.5ml SPG, aliquoted and stored at -80°C.

2.4 Quantification of *Chlamydia trachomatis* infected monolayers using microscopy

To determine the concentration of the prepared chlamydia stocks, four commercially available *C. trachomatis*-specific staining kits or reagents were evaluated: (1) the Pathfinder® *Chlamydia trachomatis* monoclonal antibody kit, (2) Invitrogen *Chlamydia trachomatis* MOMP polyclonal antibody, (3) Invitrogen *C. trachomatis* MOMP Monoclonal antibody conjugated to fluorescein isothiocyanate (FITC); and (4) the MicroTrak® *Chlamydia trachomatis* Culture Confirmation Kit. **Table 2.4** summarises details for each of these kits. The Pathfinder and MicroTrak kits contained an Evans blue counter stain whereas the Invitrogen reagents did not.

Table 2.4 *Chlamydia trachomatis* culture confirmation reagents evaluated.

Kit/ Reagent	**Manufacturer**	**Antibody target**	**Antibody type**	**Formulation**
Pathfinder® *Chlamydia trachomatis* monoclonal antibody	Bio-Rad (Hercules, CA, USA).	MOMP	Monoclonal	fluorescein-conjugated murine monoclonal antibody with Evans blue counter stain
Invitrogen *Chlamydia trachomatis* MOMP Polyclonal Antibody, FITC	Invitrogen (Thermo Fischer; Waltham, Massachusetts, USA).	MOMP	Polyclonal	PA1-73073 antibody conjugated with FITC
Invitrogen *Chlamydia trachomatis* MOMP Monoclonal Antibody	Invitrogen (Thermo Fischer; Waltham, Massachusetts, USA).	MOMP	Monoclonal	MA1-10666 antibody conjugated with LYNX kit
MicroTrak® *Chlamydia trachomatis* Culture Confirmation Kit	Trinity Biotech (Ireland).	MOMP	Monoclonal	Monoclonal antibodies labelled with FITC, and counter stained with Evans blue

2.4.1 *Chlamydia trachomatis* infection of McCoy monolayers cultured on coverslips

Sterile Poly-L-Lysine Cellware 12mm round coverslips (Corning, USA) were placed in 24-well cell culture plates. McCoy cells were seeded at $0.1x10^6$ in 1ml E10 per coverslip and each staining condition set up in duplicate. After 24 hours, McCoy cell attachment was confirmed and monolayers were infected with 120µl chlamydia at different dilutions with SPG (1:2, 1:5 or 1:10). The plate was centrifuged for 1 hour at 2500 x g at room temperature and incubated for one hour at 37°C in a 5% CO_2 incubator. The wells were washed with 1ml SPG, and 1ml infection media was added to each well. Coverslips were stained after 48 hours of *C. trachomatis* infection. In all experiments, a mock infected control was always included as a negative control.

2.4.2 Staining chlamydia-infected coverslips

A standard staining protocol was optimised for comparing the four different staining kits and/or reagents. After 48 hpi, coverslips were gently washed twice with room temperature 1ml phosphate-buffered saline (PBS: Sigma-Aldrich., USA). The infected coverslips were fixed by incubation with 1ml of 95% ethanol (Sigma-Aldrich., USA) for 10 minutes at room temperature. The relevant staining reagent (30µl) was pipetted onto parafilm (Bemis Company Inc.; United States). Coverslips were carefully lifted from the culture plate using a needle and forceps and placed cell side down onto the drop of reagent. Coverslips were stained in the dark for 30 minutes at room temperature (25°C) or 37°C. Afterwards, the stained coverslips were rinsed well with PBS, then reverse osmosis water, before mounting (Prolong Diamond mounting reagent, Invitrogen) on a clean microscope slide. Due to the mounting reagent chosen, the slides were left to cure overnight at room temperature in the dark. Slides were visualised within 24h on a Zeiss LMS 510 confocal microscope (ZEISS, Germany) or an Olympus IX71 inverted fluorescent microscope (Olympus Corporation, Japan), where indicated. If it was not possible to image immediately, slides were temporarily stored in the dark at 4°C and were left to equilibrise to room temperature before imaging.

2.4.3 4',6-diamidino-2-phenylindole (DAPI) staining

DAPI, a nuclear DNA stain, was used identify the McCoy cells in the absence of another counterstain. Infected cell monolayers cultured on coverslips were first stained with the *C. trachomatis* culture confirmation reagent as described in section 2.4.1. Coverslips were then washed with PBS and placed cell side down on 100µl of DAPI working stock and left to incubate for 5 minutes at room temperature. The coverslips were washed, then mounted as previously described.

2.4.4 HI-FCS blocking

In some experiments, when troubleshooting and where indicated, HI-FCS was used to block cell monolayers prior to staining in certain experiments to reduce non-specific binding of antibody reagents. After fixing monolayers with ethanol, 1ml of 10% HI-FCS in PBS was added to each well and incubated for 5 minutes at 37°C. The coverslips were then stained, washed, and mounted as previously described in section 2.4.2. For these experiments, *C. trachomatis*-specific staining antibodies were diluted in HI-FCS instead of SPG.

2.5 Microscopy

For the Zeiss LMS 510 confocal microscope, a 100x magnification was used for scanning the slide to choose which section of the monolayer to image, and images were captured at 250x or 400x magnification. Tile scans (representing consecutive fields) were generated (9x9 panels from the centre of the coverslip) to visualise a larger area. Z-stacks were also created to better visualise inclusions within the monolayer, four or five z-stack images were taken, and a maximum intensity projection was generated from the z-stacks. For all confocal images taken, a scale bar was added after capture. For the Olympus IX71 inverted fluorescent microscope, images were captured at 60x magnification with tile scans also generated (7x7 panels).

2.6 Data analysis

Where staining was determined successful after visualization under the microscope, Image J software (FIJI, developed at the National Institutes of Health, Bethesda, MD) was used to count the number of *C. trachomatis* inclusions from the images. First, the images were split into the red, green, and blue colour channels, resulting in individual black and white images for each channel. Since we were only interested in *C. trachomatis*, the green fluorescent channel image was used for analysis. A threshold was set empirically to count the *C. trachomatis* inclusion bodies and exclude low level background fluorescence. This threshold was kept constant for each experiment (low-end threshold was 60-65 and high-end was 255). Once the threshold was established, the number of particles (i.e., inclusion bodies) was counted using the Image J "analyze particles" algorithm.

The concentration of chlamydia (in infectious units per ml; IFU/ml) was calculated using the following equation adapted from Scidmore (15), where Fields per coverslip corresponds to the area of the tile scan and was calculated using the scale bar to convert pixels to µm.

IFU/ml= average no. inclusions x dilution factor x fields/coverslip volume inoculum (ml)

Where: fields/coverslip= cover slip surface area (mm) area of field/tile scan (mm)

AND: 12mm coverslip surface area = $\pi r^2 = 3.142 \times 6^2 = 113.11 mm^2$

Chapter 3: Results

3.1 Propagation of *Chlamydia trachomatis*

Before comparing the ability of the different kits to detect and count *C. trachomatis* bacteria, laboratory stocks of each *C. trachomatis* representative serovar were prepared and stored at -80°C. All serovars of *C. trachomatis* were cultured in McCoy cells.

3.1.1 Morphological features of CT infected cells

Visible changes were evident in the McCoy monolayers using a light microscope, including changes in the shape of McCoy cells (from spindle-shaped to rounded with visible inclusions present) and lifting of the monolayer on the edges of the well (**Table 3.1**). Compared to L1 and L2, infection with the urogenital *C. trachomatis* strains E and H tended to cause more cytopathicity, with McCoy cells lifting more and the cell membrane no longer intact. *C. trachomatis* serovars E, L1 and L2 were subsequently used for all further experiment to evaluate reagents for the detection and quantification of *C. trachomatis in vitro.*

Table 3.1 Laboratory stocks of *Chlamydia trachomatis* generated from previously isolated serovars (urogenital or LGV) and their effect on the McCoy cell monolayer during infection.

***Chlamydia trachomatis* serovar**	**Type**	**Effect on McCoy cell monolayer**
E	Urogenital	Caused monolayer to lift on edges and middle of the well and cells to become rounded, cell membranes no longer intact.
L1	LGV	Did not cause monolayer to lift and cells were more spindle-shaped than serovar E and H.
L2	LGV	Did not cause monolayer to lift and cells were more spindle-shaped than serovar E and H.

H	Urogenital	Caused monolayer to lift at edges of the well and cells to become rounded, cell membranes no longer intact.

3.2.1 Pathfinder® *Chlamydia trachomatis* direct specimen kit

The Pathfinder® *C. trachomatis* direct specimen kit (Bio-Rad, United States), consisted of a FITC-conjugated murine monoclonal antibody against MOMP, with an Evans Blue counterstain. McCoy cells were infected with *C. trachomatis* serovar E stocks, stained after 48h by confocal microscopy. Using the Pathfinder® *C. trachomatis* direct specimen kit, green fluorescent inclusions typical of *C. trachomatis* growth in culture were evident within McCoy cells infected with *C. trachomatis* serovar E at both 1:20 and 1:50 dilution of the antibodies while mock infected cells exhibited little green fluorescence **(Figure 3.1)**. In addition, morphological differences were evident between infected cells and mock infected cells, with infected cells being rounded with inclusions present and the mock infected cells being spindle shaped. The monolayer was more even in mock infected than *C. trachomatis*-infected McCoy cells. Although *C. trachomatis* was detected using the Pathfinder® kit, monolayers had high backgrounds in the green channel at both 1:20 and 1:50 dilutions, likely due to non-specific binding. Although the 1:20 antibody dilution allowed for better visualization of inclusion bodies when compared to the 1:50 dilution, individual inclusion bodies were difficult to distinguish in this assessment of the reagent.

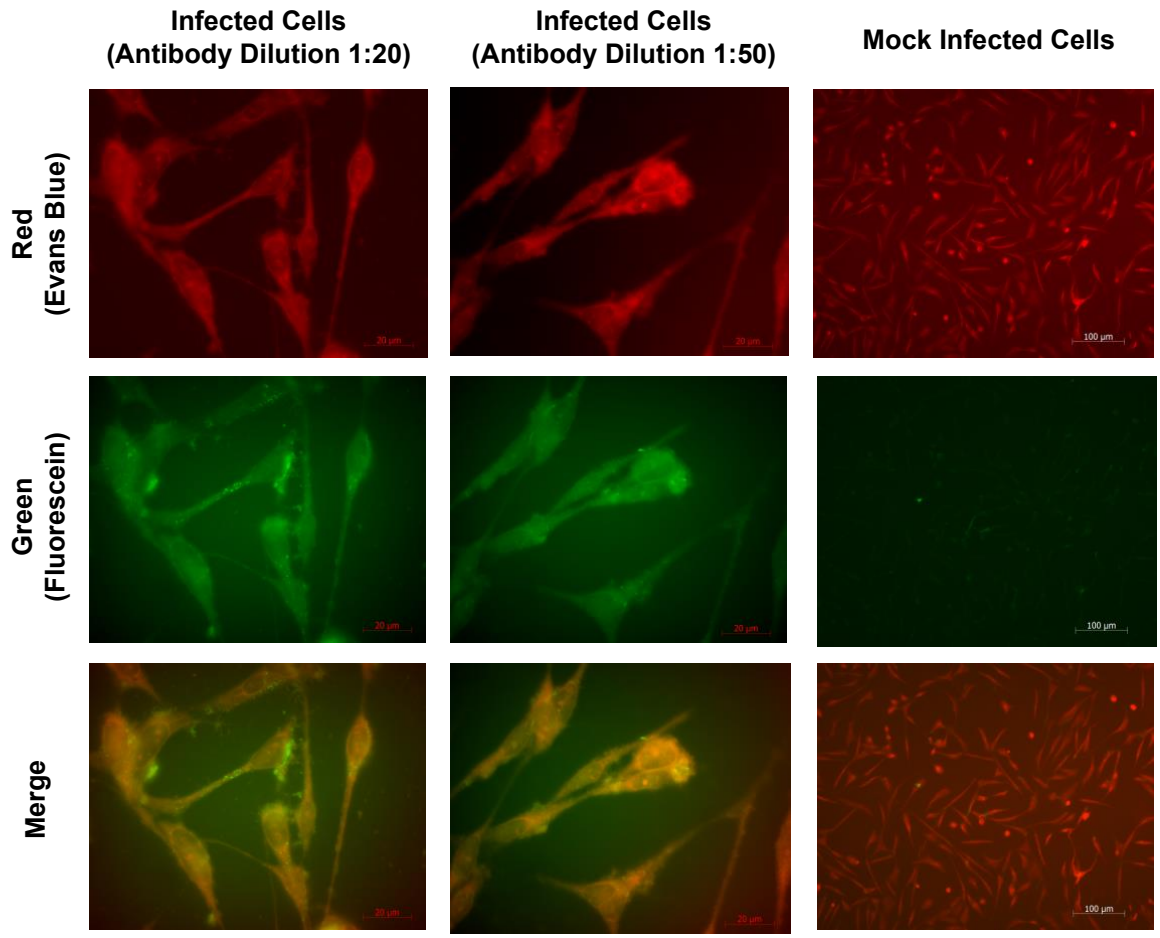

Figure 3.1 Fluorescent microscopy images of *Chlamydia trachomatis* serovar E infected McCoy cells after staining with the Pathfinder kit, indicating high levels of background at 1:20 and 1:50 Ab dilution. Cells are represented in red and *Chlamydia trachomatis* serovar E in green. A 40x objective was used to take these images. Scale bar = 20µm for infected cells and 100µm for uninfected cells.

To reduce the background, temperature of staining was compared (25°C versus 37°C) and green fluorescence was detected at both 25°C and 37°C **(Figure 3.2A and 3.2B)**. However, *C. trachomatis* inclusions were dim and blurry at both temperatures and the contrast between McCoy cells and inclusions were similar at both staining temperatures and the background remained high.

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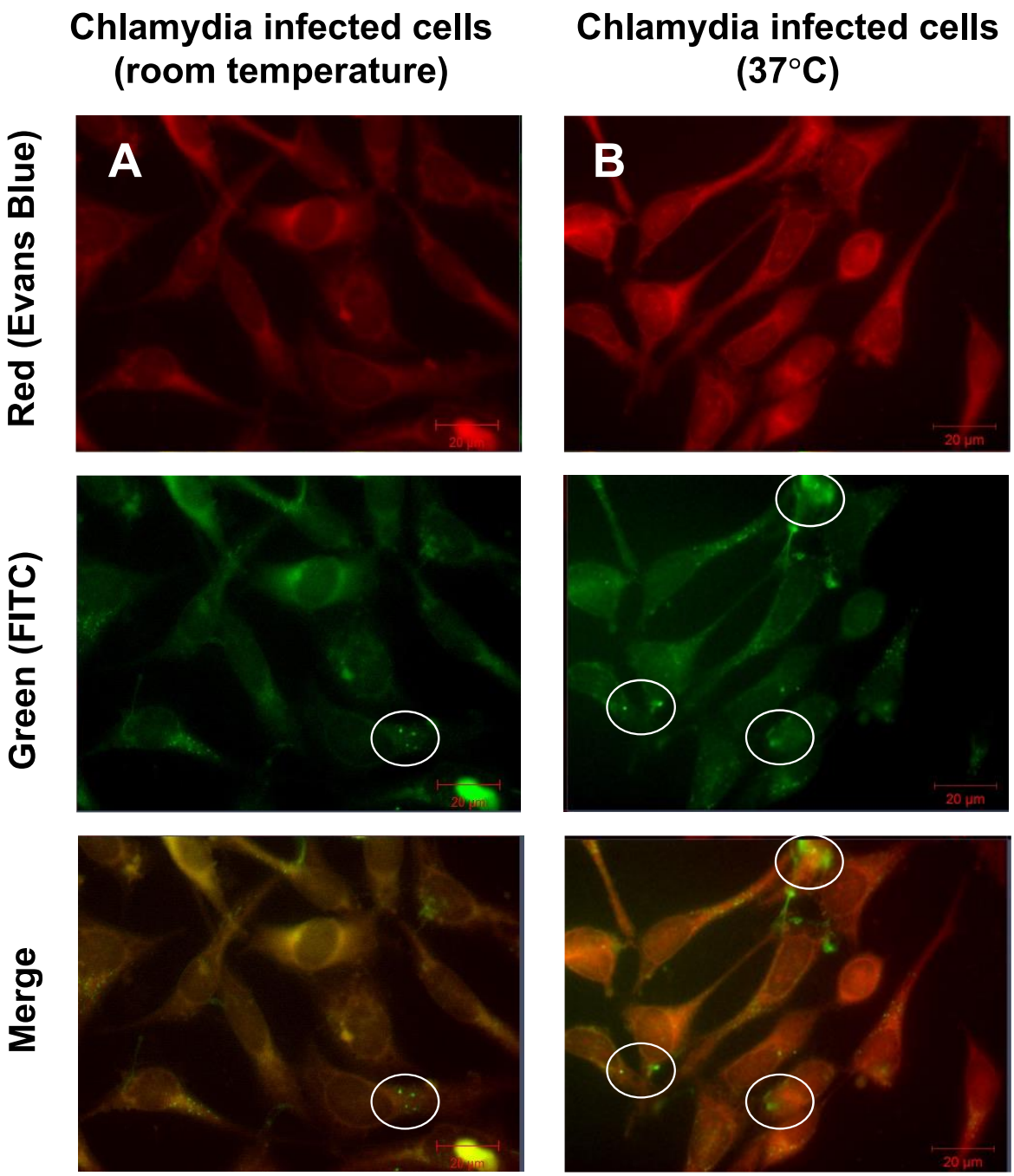

Figure 3.2 Fluorescent microscopy images of *Chlamydia trachomatis* serovar E infected McCoy cells after staining with the Pathfinder kit at different incubation temperatures **(A)** room temperature and at **(B)** 37°C. Images were captured using a confocal microscope. Circles indicate *Chlamydia trachomatis* inclusion bodies within an infected McCoy cell. A 40x objective was used to take these images. Scale bar = 20µm for all conditions.

3.2.2 Invitrogen *Chlamydia trachomatis* MOMP antibodies

The next reagents evaluated were the Invitrogen *C. trachomatis* MOMP polyclonal and monoclonal antibodies, which recognised *C. trachomatis* MOMP. Dilutions of 1:25 and 1:50 were compared to determine the optimal concentration of antibody. Since no counterstain was included, DAPI was used to stain the nuclear DNA of the host McCoy cells. All staining was performed at room temperature. Using these polyclonal antibodies, *C. trachomatis* serovar E inclusion bodies were visible at both 1:25 and 1:50 dilution (**Figure 3.3A and B,** respectively)**.** Although signal was detectable, *C. trachomatis* inclusions were poorly visible and individual inclusions were difficult to detect. Therefore, accurate counting of bacteria was not possible with this approach.

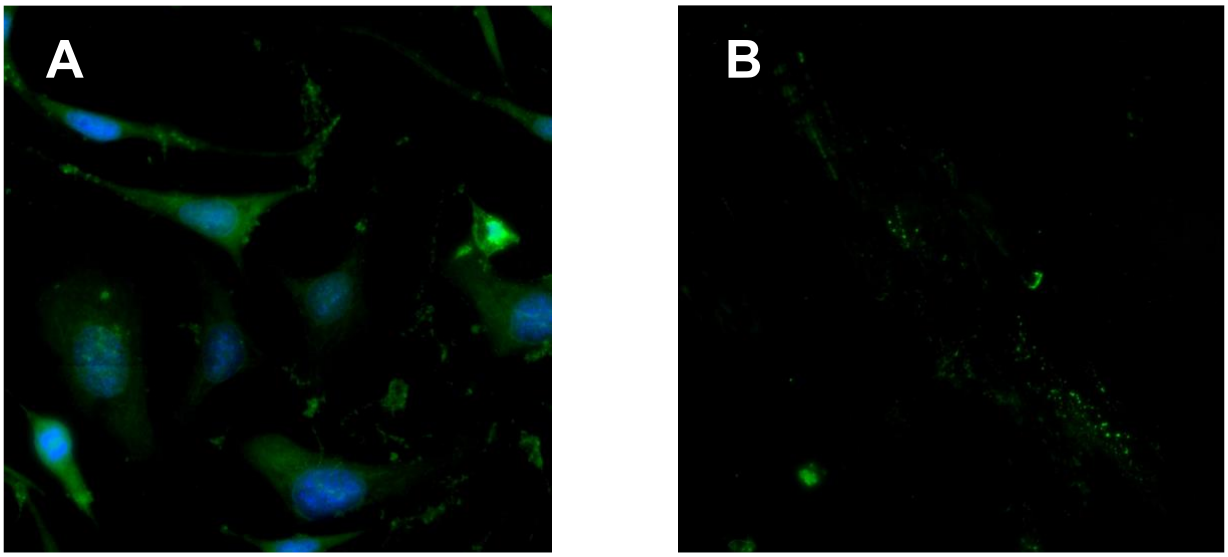

Figure 3.3 Representative fluorescent microscopy images after staining serovar E infected McCoy cells with Invitrogen polyclonal antibody at a dilution of **(A)** 1:25 and **(B)** 1:50 (green fluorescent channel only). *Chlamydia trachomatis* serovar E inclusions are visible in green (FITC) and the nuclear DNA in blue (DAPI). A 40x objective was used to take these images.

With the rationale that cytopathicity of the *C. trachomatis* isolates and McCoy cell death may be contributing to high backgrounds and non-specific binding of the reagents, the less cytotoxic LGV serovars L1 and L2 were next used to infect monolayers using the same staining protocol and antibody dilution **Figure 3.4** shows few inclusions within the cells after infection with serovar L2. However, individual discrete inclusions were difficult to visualize. These images, although clear with low levels of background, could not be counted.

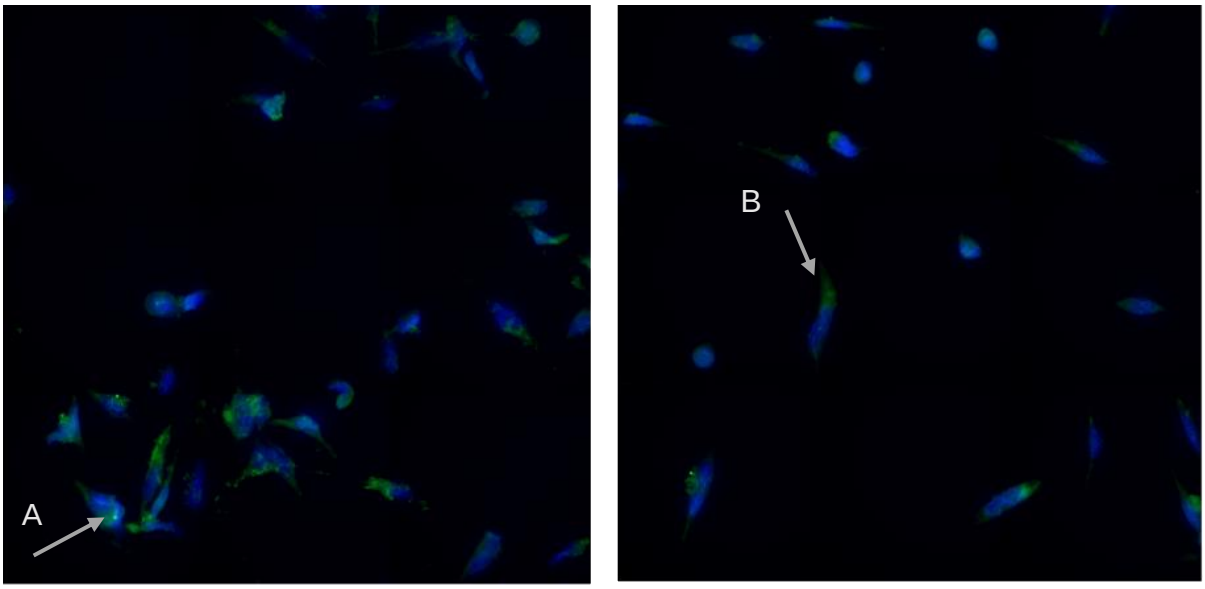

Figure 3.4 Fluorescent microscopy images after staining *Chlamydia trachomatis* infected McCoy cells with Invitrogen polyclonal antibody at a dilution of 1:25. *Chlamydia trachomatis* serovar L2 inclusions are visible in green (FITC) and the nuclear DNA in blue (DAPI). Arrow A representing a distinct inclusion body and arrow B representing diffuse inclusion bodies. A 40x objective was used to take these images.

Because the DAPI counterstain fluorescence signal was bright compared to the fluorescence of the *C. trachomatis* inclusions, the experiment was next repeated using serovars E and L2 but without DAPI. Under these conditions, chlamydia inclusions were more clearly visible and background fluorescence was minimal (**Figure 3.5**). Using the equation adapted from Scidmore (4, **Section 2.6**), the concentration of *C. trachomatis* serovar E were determined to be 6.1×10^5 IFU/ml (for the partially purified batch) and 1.34×10^5 IFU/ml (for the stock) (**Table 3.2**). *C. trachomatis* serovar L2 was determined to have a concentration of 5.9×10^4 IFU/ml. Although the polyclonal antibody method allowed quantification, this could not be reproduced in subsequent experiments, and was considered unreliable (temperamental) as a detection method for further quantification experiments.

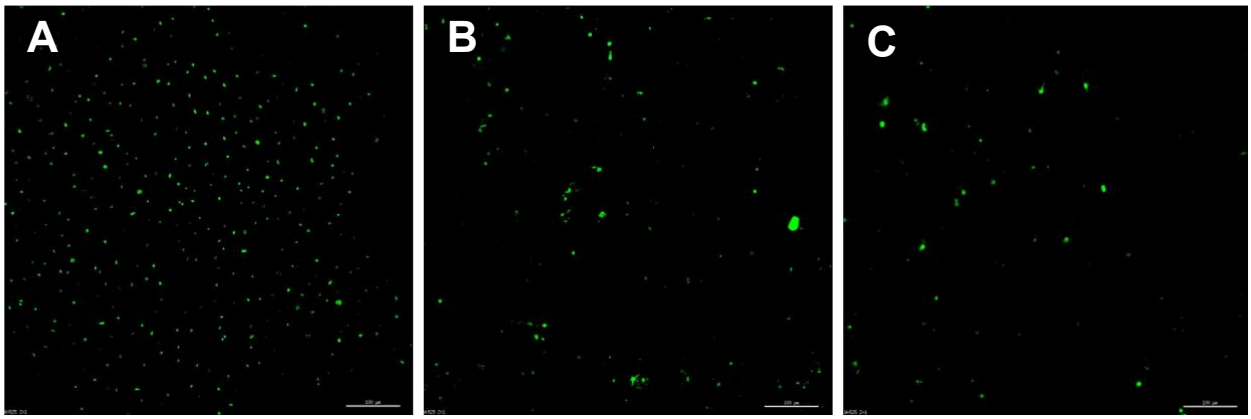

Figure 3.5 Representative fluorescent microscopy images of *Chlamydia trachomatis* infected McCoy cells stained with Invitrogen polyclonal antibody at a dilution of 1:25. **(A)** Partially purified *Chlamydia trachomatis* Serovar E **(B)** *Chlamydia trachomatis* Serovar E stock **(C)** *Chlamydia trachomatis* Serovar L2 stock**.** *Chlamydia trachomatis* is visible in green (FITC)**.** A 40x objective was used to take these images. Scale bar = 100µm.

Table 3.2 Quantification of *Chlamydia trachomatis* serovar E and L2 stocks after staining with Invitrogen polyclonal antibody

Chlamydia trachomatis	No. Inclusions	Dilution factor	Area of Image (Pixels)	Pixel to μm	Fields/coverslip	*Chlamydia trachomatis* Concentration (IFU/ml)
C. trachomatis E partial purification	623	5	956x956	1.25	195	$6.1x10^5$
C. trachomatis E stock	356	2	1004x1004	1.3	188.5	$1.3x10^5$
C. trachomatis L2 stock	144	2	968x968	1.3	205.7	$5.9x10^4$

Next, the Invitrogen monoclonal antibody was tested, with the rationale that a monoclonal would bind more specifically to MOMP as it only recognises one unique epitope. Despite this, high background fluorescence and non-specific binding was observed with this reagent which interfered with counting of the inclusion bodies. Due to high background and non-specific staining, the monoclonal antibody reagent was abandoned as a method of quantification.

3.2.3 MicroTrak® *Chlamydia trachomatis* culture confirmation kit

The final reagent evaluated was the Trinity Biotech MicroTrak *C. trachomatis* culture confirmation kit, which has been the most widely used kit in research settings (170–174). Using the Micro Trak system, *C. trachomatis* serovar L1 and E inclusions were visible as bright green, fluorescent dots in the infected cells (**Figure 3.6 and 3.7, respectively**)**,** which were clearly absent in the mock infected cells. Low levels of background were present and individual inclusions were clear and distinct from one another. There was also a clear distinction between the green of the inclusion bodies and the red counterstain of the McCoy cells.

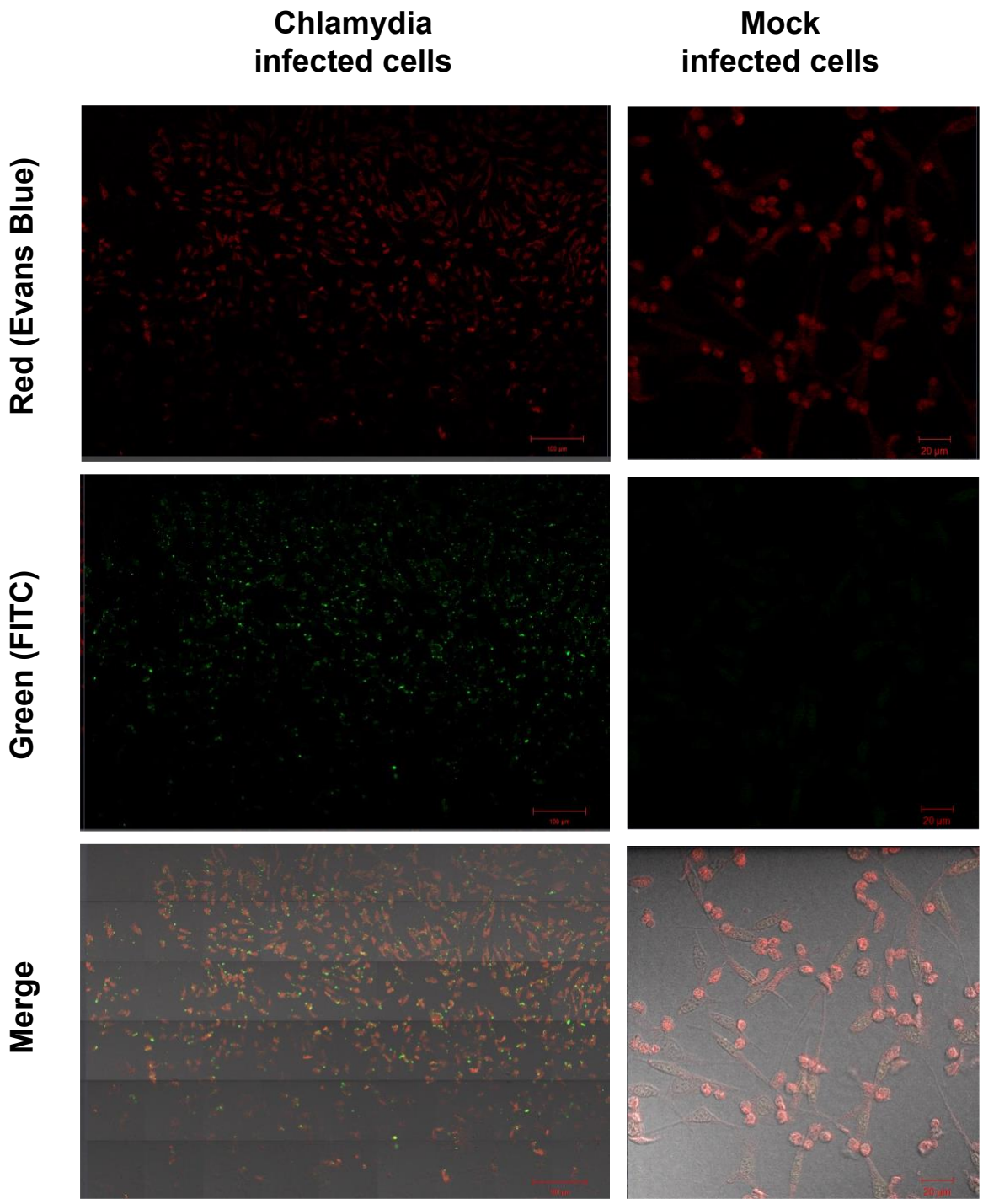

Figure 3.6 Representative confocal microscopy images of *Chlamydia trachomatis* serovar L1 infected cells after staining with the MicroTrak kit. Cells are visible in red (Evans Blue) and *Chlamydia trachomatis* serovar L1 bacteria are visible in green (FITC). The merged panel contains transmitted light. A 40x water objective was used to take these images. Multiple images were captured as tile scans to examine a larger area. The resolution of the tile scans was reduced to count *Chlamydia trachomatis* inclusions. Scale bar = 200µm for infected cells and 20µm for the uninfected cells.

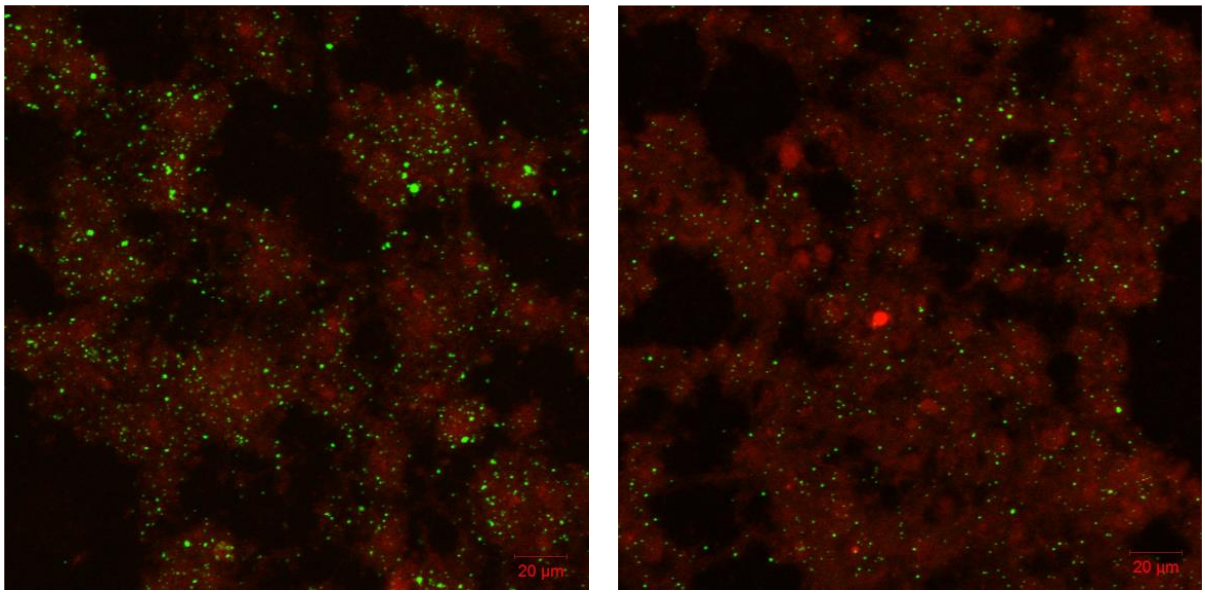

Figure 3.7 Confocal microscopy images of *Chlamydia trachomatis* serovar E infected McCoy cells. Cells are visible in red (Evans Blue) and *Chlamydia trachomatis* serovar E bacteria are visible in green (FITC). A 40x water objective was used to take these images. Scale bar = 20µm

Using the Micro Trak staining system, the concentration of *C. trachomatis* serovar L1 stocks and partially purified serovar E was calculated to be $2.3x10^6$ IFU/ml and 5.1 $x10^6$ IFU/ml, respectively (**Table 3.3**).

Table 3.3 Quantification of *Chlamydia trachomatis* serovar L1 and E using the MicroTrak kit

Chlamydia trachomatis Serovar	No. inclusions	Dilution factor	Area of Field (pixels)	Pixel to µm	Fields/coverslip	IFU/ml
L1	1091	12	795x530	0.8	171.9	$2.3x10^6$
E	1116	2	1143x1143	5	2262.2	$5.1x10^6$

At higher magnification, clear morphological differences between *C. trachomatis* serovar E infected and uninfected cells were observed (**Figure 3.8**). The infected McCoy cells were more rounded in shape when compared to the more intact, spindle-shaped, mock infected cells. *C. trachomatis* inclusions within the McCoy cells were evident. Many *C. trachomatis* bacteria were visible within the inclusion bodies, which were intact at 48hpi. No background fluorescence was seen in the mock control.

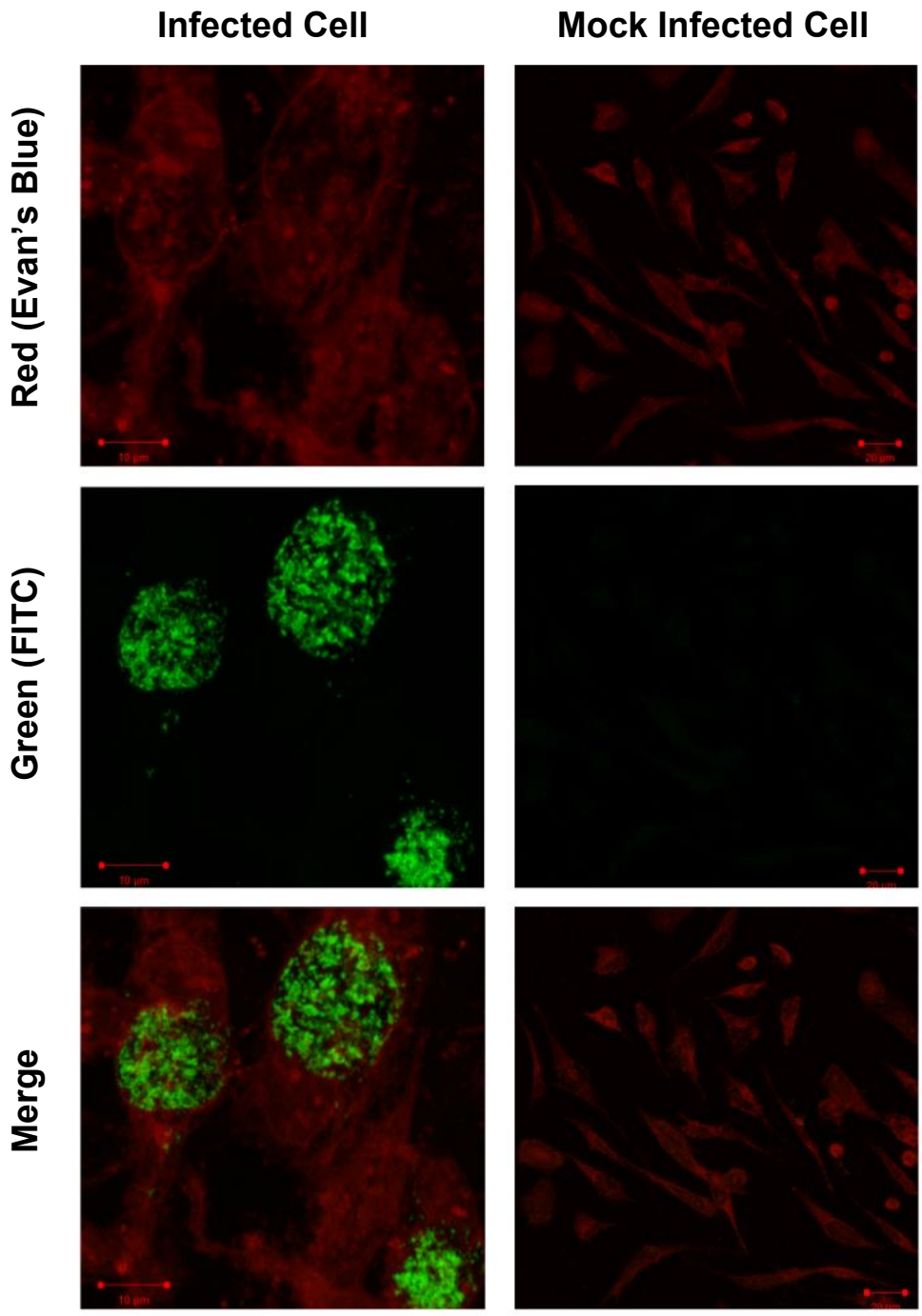

Figure 3.8 Confocal microscopy images of *Chlamydia trachomatis* infected McCoy cells stained with the MicroTrak kit. Cells are visible in red (Evans blue) and *Chlamydia trachomatis* serovar E in green (FITC). A 40x water objective was used to take these images. Scale bar = 10µm for infected cells and 20µm for uninfected cells.

Despite the MicroTrak staining kit producing robust and reproducible results (with low background, clear cells and bright, discrete staining of *C. trachomatis* inclusion bodies), the commercial kit was discontinued in South Africa and internationally in 2018.

Although the MicroTrak kit performed most reliably, I conclude that the Invitrogen polyclonal antibody could be explored as an alternative for routine microscopic *C. trachomatis* quantification, with further optimization and standardisation. The most common technical issue that hampered microscopic quantitation of *C. trachomatis* in this study was the high levels of background green fluorescence, seen in both the Pathfinder Kit and Invitrogen monoclonal antibodies.

Chapter 4: Discussion

C. trachomatis remains the most common bacterial STI worldwide, with a high prevalence in SSA women (175). *C. trachomatis* infection is typically characterised by genital inflammation (176), particularly if untreated, which increases HIV risk (13,177). Despite decades of research, many aspects of the pathogenesis of *C. trachomatis* and disease in women are not fully understood about pathogenicity and immune protection. Comprehending the impact of *C. trachomatis* strain variation in pathogenicity and symptoms in women is therefore a major priority. To better understand and compare *C. trachomatis* serovars intracellular pathways within host cells, this dissertation compared several commercially available fluorescent microscopy reagents to visualise and quantify *C. trachomatis* cultured in McCoy cell culture. At the initiation of this project, the objective was to establish a laboratory method to quantitatively evaluate *C. trachomatis* strain-associated differences in intracellular pathogenicity of *C. trachomatis* laboratory strains compared to novel clinical isolates. The COVID-19 pandemic significantly restricted access to core lab infrastructure needed for this project and resulted in modifications to the original aims as it was not possible to expand the project to include novel clinical isolates as initially proposed. Despite these unforeseen restrictions, this dissertation compared four different commercially available fluorescence imaging-based *C. trachomatis* kits for their ability to visualise and quantitate laboratory *C. trachomatis* strains. Findings presented in this study showed significant differences in both the specificity and reproducibility of the commercially available *C. trachomatis* kits. Although the MicroTrak kit performed significantly better than the other three kits, ultimately global access to reagents to enable accurate visualisation of *C. trachomatis* in culture represented a major hurdle that needs to be considered.

In the past, giemsa and iodine stains were routinely used for detection of *C. trachomatis* cultured for diagnostic purposes (102), requiring trained technicians to interpret results as inclusion bodies were difficult to differentiate microscopically. These methods are rarely used today due to lack of sensitivity in comparison to fluorescent staining (98), which has increased sensitivity significantly (99) as inclusion bodies are clearer and easier to distinguish even with an untrained eye. Fluorescence-based methods to estimate *C. trachomatis* concentrations and intracellular trafficking

have increasingly been replaced by NAATs. These are more sensitive and specific than any of the previous microscopic based methods (2), which typically use qPCR primers for the quantification of the ompA gene that codes for MOMP (178,179). Since there is only one copy of ompA in the *C. trachomatis* genome, this specific sequence is considered a good target (180). Although NAATS have significant advantages, this dissertation focused on evaluating fluorescence-based methods only, as an aim was to visualise intracellular inclusions and cytopathogenicity of *C. trachomatis* isolates.

4.1 Only one out of three tested reagents enabled *Chlamydia trachomatis* quantification

To quantify *C. trachomatis* stocks as well as study the pathobiology of *C. trachomatis in vitro*, this dissertation compared several commercially available fluorescent microscopy reagents to visualise and quantify intracellular *C. trachomatis* propagated in tissue culture, including the Pathfinder® *C. trachomatis* direct specimen kit, the Invitrogen *Chlamydia trachomatis* anti-MOMP monoclonal and polyclonal antibodies, and the Trinity Biotech MicroTrak® *Chlamydia trachomatis* culture confirmation kit. Of these, the MicroTrak kit performed consistently and reliably allowing for clear fluorescent visualisation of chlamydia.

Pathfinder and Invitrogen

Staining with the Pathfinder kit and Invitrogen monoclonal antibodies resulted in high background fluorescence, likely due to non-specific binding of the antibodies (181). Individual *C. trachomatis* inclusion bodies within McCoy cells were difficult to distinguish. Non-specific binding of antibodies occurs when an antibody binds to an alternate protein epitope, different to that of the specifically targeted epitope, which creates difficulty in observing the intended protein target due to high background observed. As non-specific binding can be reduced by altering staining temperature, antibody concentrations and addition of exogenous protein, staining with the Pathfinder kit was compared at various temperatures, dilutions, and added protein concentrations. None of these adjustments to antibody staining conditions reduced the background fluorescence or improved resolution of specific *C. trachomatis*

inclusion bodies, so the Pathfinder kit and reagents was not considered appropriate for further testing.

In addition to the monoclonal antibody reagents tested, the Invitrogen polyclonal antibody was also compared. The Invitrogen polyclonal antibody allowed for successful quantification of *C. trachomatis* in initial experiments with serovar E, but these results were not reproducible in subsequent experiments.

MicroTrak *C. trachomatis* kit

C. trachomatis quantification was found to be reproducible with the MicroTrak kit although the kit was discontinued in 2018 by the manufacturers. Images of *C. trachomatis* infected McCoy cells were clear, with low background and distinct inclusions. Compared to the other reagents, the MicroTrak kit for *C. trachomatis* detection was more widely used in previous studies for *C. trachomatis* culture confirmation (Trinity Biotech). Quantification of *C. trachomatis* with the MicroTrak kit was reproducible, regardless of cell condition and infecting serovar.

4.2 Cell culture considerations and pathogenicity associated with *Chlamydia trachomatis* infection

C. trachomatis is known to be cytotoxic in cell culture (92), although it does require survival of the host cell in order to replicate and produce high yields of progeny (185). In the experiments presented here, it was evident that host cells had undergone apoptosis, as they were visibly “fluffy”, without distinct borders. Despite cytotoxicity, individual *C. trachomatis* inclusions remained clear using the MicroTrak system.

Cycloheximide, a protein synthesis inhibitor in eukaryotes (93), is routinely added to *C. trachomatis* culture experiments because it increases *C. trachomatis* yield by partially inhibiting host cell metabolism (94). As cycloheximide has been reported to be toxic to host cells under certain conditions and for prolonged culture (94), the concentration of cycloheximide added to the cultures was considered as a factor contributing to cytopathicity in this study. The concentration of cycloheximide used during cell culture in this study was always adjusted to be less than 1µg/ml. Thus, it

was unlikely that the concentration of cycloheximide played a role in the unsuccessful quantification attempts where high background prevented discrimination of individual bacteria for counting. The intention was to balance the concentration of cycloheximide added and the seeding cell concentration to ensure optimal *C. trachomatis* propagation without high cytotoxicity.

4.3 Limitations and alternative approaches

While microscopy and other immunofluorescence techniques allow for single-cell analysis and quantification of viable chlamydia, there are important limitations to these methods that need to be acknowledged. Microscopic approaches are sometimes difficult to optimise, are reagent dependent and require infrastructure, equipment and well-trained personnel (186). Although this study compared various commercial reagents for microscopic visualisation of *C. trachomatis*, only the MicroTrak was reproducible, unfortunately manufacturers discontinued this kit in 2018. Therefore, access to reproducible and accurate reagents to stain intracellular *C. trachomatis* was one of the biggest challenges identified. Trinity Biotech (the company that supplied the MicroTrak kit), based in Ireland, now only supplies a *C. trachomatis* IgG ELISA and no *C. trachomatis* antigen-based tests. Trinity Biotech was contacted during this study, although the company did not provide more detail as to why the kit was discontinued globally.

Flow cytometry based methods can be used for bacterial quantification (187), and has been successfully used to study larger, rod-shaped bacteria such as *E.coli* [(196) which has a length of ~1-2 μm (190)] and *Mycobacterium tuberculosis* (with a length of 1.5–4.0 μm (191,192)]. Flow cytometric assays are commonly used to study intracellular *M. tuberculosis* where host cells are permeabilised and stained with anti-mycobacteria antibodies (193,194). Similar assays for chlamydia are also in development, using monoclonal antibodies against *C. trachomatis* serovar D LPS, although a relatively high multiplicity of infection (MOI) is required which may cause apoptosis of host cells and consequent high background (195). However, similarly to immunofluorescence microscopy, flow cytometry for *C. trachomatis* requires well-

trained technicians and expensive equipment, making it less accessible in low-middle income settings.

Although this study intentionally compared imaging based reagents for *C. trachomatis* quantification following tissue-culture infection, a method that should be explored in future is qPCR (196). qPCR has been used to diagnose infections, including chlamydia, due to the high specificity and sensitivity. In cell culture, qPCR has been successfully used to amplify and detect nucleic sequences unique to *C. trachomatis* with specificity and high sensitivity (2). In addition to indicating the presence or absence of a pathogen, the absolute or relative quantities of a known sequence in a sample can be determined by qPCR (197).

Another approach is to use genetically modified *C. trachomatis* strains, that are fluorescently tagged with FITC, mCherry or GFP (198,199). These fluorescent *C. trachomatis* strains have also been used in studies to confirm presence of infection, the bacteria's response to cytokines such as IFN-γ, and even allow for real-time monitoring of the infection cycle as well as screen for agents that block *C. trachomatis* replication (198–200). Transfection experiments using laboratory strains could be investigated for quantification purposes. However, this method is limited since clinical strains would have to be modified, which defeats the purpose of studying clinical vs laboratory strains. Similarly, the immunofluorescence In-Cell Western (ICW) assay could also be used to quantify *C. trachomatis* in future experiments. This novel approach quantifies immunofluorescence signals, using specific primary antibodies, from proteins in fixed cultures. The Odyssey CLx imager is then used to quantify the fluorescence signal intensity. This method is sensitive, reproducible and operator independent. The protocol only requires 4 hours work and allows for high-throughput screening, offering a great alternative to fluorescent microscopy (201). This method has already been successfully applied to *C. trachomatis* antimicrobial susceptibility testing and has the potential to be applied to cell proliferation, gene expression and viral titration assay in the future (202).

Lastly, another approach that minimises operator bias, utilising an automated microplate ImmunoSpot reader can be explored for quantification purposes. Wang *et. al* (203) developed this novel approach, which uses anti-*C. trachomatis* LPS

monoclonal antibody and a biotinylated secondary antibody to detect the bacteria. Individual inclusion bodies are counted using the Immunospot Series II Analyzer and are comparable to those determined by traditional microscopic counting methods. Similarly, Keck *et al.* (204) has utilised the Immunospot S6 Universal Analyzer and Immunospot Easy-Count software for quantification of *C. muridarum*, indicating this automated approach's potential for future microbiological research using various chlamydia biovars or serovars.

4.4 Importance of continued laboratory research for chlamydial drug and vaccine development

Although efficacious treatments exist for STI management, antimicrobial resistance (AMR) has become a particular threat to the control of STIs globally (141). AMR specifically to *N. Gonorrhoea* is a huge public health issue, as the bacteria has become a superbug resistant to both previously and currently recommended antimicrobials (205). Currently AMR to *C. trachomatis* is rare, however evidence from clinical treatment failures indicate that some bacterial isolates have demonstrated drug resistance *in vitro,* highlighting the concern with possible AMR and current treatment options (206). Thus, research into antimicrobial susceptibility testing for the detection of possible drug resistance is of importance and *C. trachomatis* propagated in the laboratory can be used for such experiments(207). Laboratory propagated bacteria can also be utilised to determine the therapeutic index of potential drugs for pre-clinical studies (208).

With the rise of AMR and the increasing prevalence of *C. trachomatis* infections despite public health interventions, the development of a *C. trachomatis* vaccine is paramount for the control of disease. Microbiological research will aid in the evaluation of vaccine responses for different antigen preparations *in vitro* before pre-clinical trials can begin (46).

Conclusion

In conclusion, this study clearly demonstrates that the *C. trachomatis* MicroTrak kit was the most reproducible kit for detecting and quantifying *C. trachomatis* in tissue culture, compared to three other commercially available fluorescent detection reagents. The other reagents gave inconsistent and/or unreliable results. Although this dissertation focused exclusively on immunofluorescence staining and microscopy as a method to confirm and quantify *C. trachomatis* propagation in McCoy cells, the inconsistent results from this study suggest that future studies should explore alternative molecular methods to quantify *C. trachomatis*, such as qPCR.

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Appendix I

Reagents for culture of *Chlamydia trachomatis* in McCoy cells.

For 100ml E10 media

80ml EMEM

20ml FCS (filter sterilised)

40µl Gentamicin solution

40µl Fungin

1.5ml L-glutamine

For 100ml IM media

80ml EMEM

20ml FCS (filter sterilised)

40µl Gentamicin solution

40µl Fungin

1.5ml L-glutamine

1ml glucose

50µl Cycloheximide

Cycloheximide (1mg/ml)

Dissolve 10 mg cycloheximide into 10 ml 95% ethanol

Dispense into 0.5ml aliquots and store up to 6 months at −20°C

0.2M dibasic sodium phosphate

35.6g Na_2HPO_4

H_2O to 1L

Autoclave for 20 min

Store at 4°C

0.2M monobasic sodium phosphate

31.2g NaH_2PO_4

H_2O to 1L

Autoclave for 20 min

Store at 4°C

Sucrose-phosphate-glutamate buffer (SPG)

75g sucrose

87ml 0.2M Na_2HPO_4 (dibasic sodium phosphate)

13ml 0.2M NaH_2PO_4 (monobasic sodium phosphate)

0.72g L-glutamic acid

H_2O to 1L

Aliquot to 100-ml bottles and autoclave for 20 min

Store up to 1 year at 4°C

Appendix 2

Turnitin Report

Turnitin Originality Report

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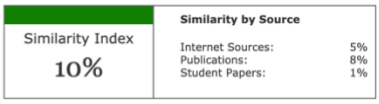


Appendix 3

STAINING SOP

Before staining

- 72h before staining, seed 0.1 million cells per coverslip.
- 24 hours after seeding coverslips, infect confluent monolayers with Chlamydia trachomatis by centrifugation at 2500 x g for 1 hour.

Fixation of coverslips

- After 48h of culture post infection, check cells under an inverted microscope.
- Aspirate culture medium from 24-well plates and gently wash wells twice with 1ml PBS and aspirate.
- Add 1ml absolute ethanol to each well. Be careful not to disrupt the cell monolayer on the coverslip by pipetting down the side of the well. Do not allow the cell monolayers to dry or inclusion bodies may burst and be reduced in number.
- Leave coverslips undisturbed for 10 minutes at RT.
- Aspirate the ethanol from the wells with a Pasteur pipette.

Staining

- Allow the mounting medium to reach room temperature.

- Remove undiluted antibody aliquot from freezer and allow to thaw in the BSL-2 hood. Make up a 1:25 dilution of antibody with PBS with a final of 30 µl per coverslip.

- Place a square of parafilm with circles drawn on where the coverslips will be placed on a piece of firm cardboard. Carefully pipette 30µl of diluted antibody for each coverslip in the centre of the drawn circle.

- Carefully remove the coverslip with needle and forceps and touch the edge to blotting paper.

- Place the coverslip with cell side down on the drop of diluted antibody. Ensure the full coverslip is covered.

- Carefully place the tray in the BSL-2 hood and build a tinfoil tent around it to prevent any light from getting in. Do not allow the antibodies to dry onto the cells; drying will cause nonspecific binding.

- Incubate at room temperature for 30 minutes.

- Place a drop of mounting medium on an appropriately labelled, clean microscope slide.

- Place your thumb directly below the coverslip and use a Pasteur pipette to rinse the coverslip while still on the parafilm that is on the cardboard, first with PBS followed by RO water. Your thumb should prevent the coverslip from washing away.

- Remove the coverslips using forceps and carefully blot edge to get rid of excess liquid.

- Carefully place the coverslip on the drop of mounting reagent. A needle may be used to gently lower the coverslip.

- Allow to cure overnight.

- Store at 4°C.

Appendix 4

invitrogen

Catalog # PA1-73073

Chlamydia trachomatis MOMP Polyclonal Antibody, FITC

Product Details	
Size	1 mL
Species Reactivity	Bacteria
Host/Isotype	Goat / IgG
Class	Polyclonal
Type	Antibody
Conjugate	FITC
Immunogen	Purified MOMP from strain L2.
Form	Liquid
Concentration	4-5 mg/mL
Purification	purified
Storage buffer	PBS with 10mg/mL BSA
Contains	0.1% sodium azide
Storage conditions	-20° C, store in dark
RRID	AB_1016832

Applications	Tested Dilution	Publications
Immunocytochemistry (ICC/IF)	1:10-1:50	1 Publication

Product Specific Information

The PA1-73073 antibody reacts with Chlamydia trachomatis MOMP. Does not react with C. psittacii or C. pneumoniae in MIF.

PA1-73073 has been successfully used in Immunofluorescence applications.

The immunogen for PA1-73073 is purified MOMP from strain L2.

1 Reference

Immunocytochemistry (1)

Journal of bacteriology

Impact of Active Metabolism on Chlamydia trachomatis Elementary Body Transcript Profile and Infectivity.

"PA1-73073 was used in Immunocytochemistry to supports the idea that the chlamydial elementary body (EB) is an actively maintained cell form that responds to nutrients and environmental signals to maintain infectivity in multiple environments, thus aiding in chlamydial pathogenesis."

Authors: Grieshaber S,Grieshaber N,Yang H,Baxter B,Hackstadt T,Omsland A

Species
Not Applicable

Dilution
Not Cited

Year
2018

Chlamydia trachomatis MOMP Monoclonal Antibody (CL12-712.3)

Product Details	
Size	500 µg
Species Reactivity	Bacteria
Host/Isotype	Mouse / IgG2a
Class	Monoclonal
Type	Antibody
Clone	CL12-712.3
Conjugate	Unconjugated
Immunogen	C. trachomatis elementary bodies.
Form	Liquid
Concentration	2.2 mg/mL
Purification	purified
Storage buffer	PBS, pH 7.4
Contains	no preservative
Storage conditions	-20° C, Avoid Freeze/Thaw Cycles
RRID	AB_1073634

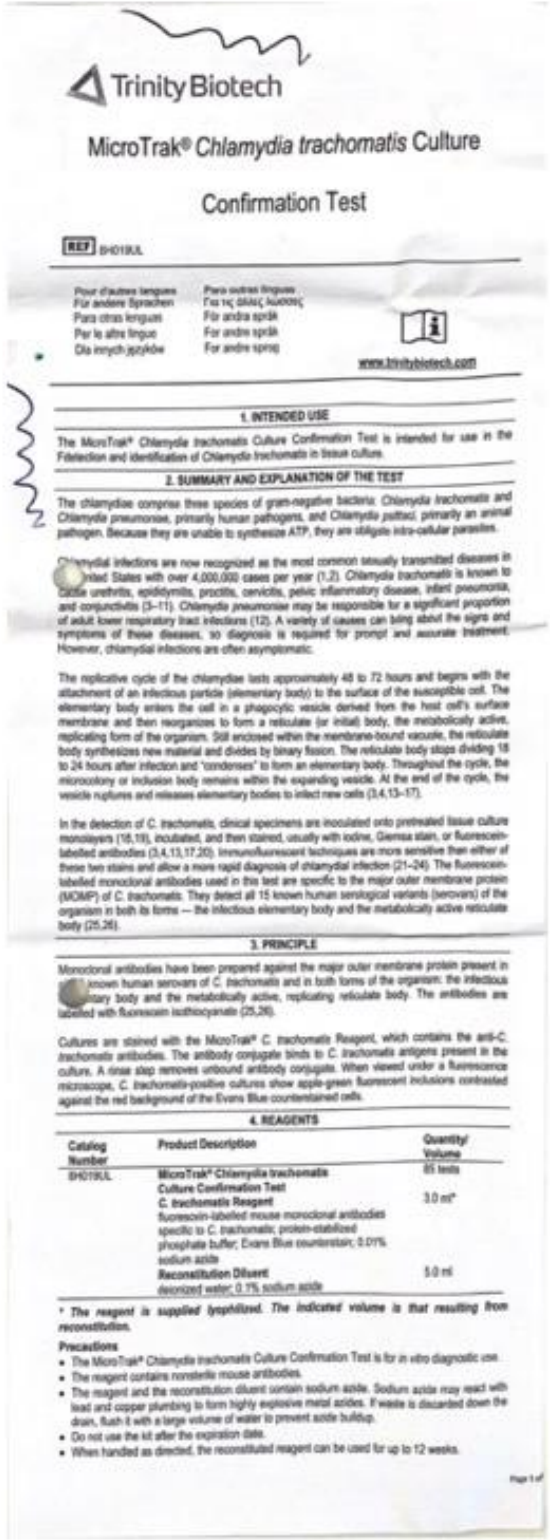
Applications	Tested Dilution	Publications
ELISA (ELISA)	1-10 µg/mL	-

Product Specific Information

MA1-10666 detects Chlamydia trachomatis MOMP from C. trachomatis serovars A, B, Ba, C, D, E, F, G, H, I, J, K, L1, L2, and L3 samples.

MA1-10666 has been successfully used in ELISA applications.

The MA1-10666 immunogen is C. trachomatis elementary bodies.



Trinity Biotech

MicroTrak® *Chlamydia trachomatis* Culture

Confirmation Test

REF BH019UL

Pour d'autres langues	Para outras línguas	
Für andere Sprachen	Για τις άλλες λωσσες	
Para otras lenguas	För andra språk	
Per le altre lingue	For andre språk	
Dla innych języków	For andre sprog	

www.trinitybiotech.com

1. INTENDED USE

The MicroTrak® *Chlamydia trachomatis* Culture Confirmation Test is intended for use in the detection and identification of *Chlamydia trachomatis* in tissue culture.

2. SUMMARY AND EXPLANATION OF THE TEST

The chlamydiae comprise three species of gram-negative bacteria: *Chlamydia trachomatis* and *Chlamydia pneumoniae*, primarily human pathogens, and *Chlamydia psittaci*, primarily an animal pathogen. Because they are unable to synthesize ATP, they are obligate intra-cellular parasites.

Chlamydial infections are now recognized as the most common sexually transmitted diseases in the United States with over 4,000,000 cases per year (1,2). *Chlamydia trachomatis* is known to cause urethritis, epididymitis, proctitis, cervicitis, pelvic inflammatory disease, infant pneumonia, and conjunctivitis (3–11). *Chlamydia pneumoniae* may be responsible for a significant proportion of adult lower respiratory tract infections (12). A variety of causes can bring about the signs and symptoms of these diseases, so diagnosis is required for prompt and accurate treatment. However, chlamydial infections are often asymptomatic.

The replicative cycle of the chlamydiae lasts approximately 48 to 72 hours and begins with the attachment of an infectious particle (elementary body) to the surface of the susceptible cell. The elementary body enters the cell in a phagocytic vesicle derived from the host cell's surface membrane and then reorganizes to form a reticulate (or initial) body, the metabolically active, replicating form of the organism. Still enclosed within the membrane-bound vacuole, the reticulate body synthesizes new material and divides by binary fission. The reticulate body stops dividing 18 to 24 hours after infection and "condenses" to form an elementary body. Throughout the cycle, the microcolony or inclusion body remains within the expanding vesicle. At the end of the cycle, the vesicle ruptures and releases elementary bodies to infect new cells (3,4,13–17).

In the detection of *C. trachomatis*, clinical specimens are inoculated onto pretreated tissue culture monolayers (18,19), incubated, and then stained, usually with iodine, Giemsa stain, or fluorescein-labelled antibodies (3,4,13,17,20). Immunofluorescent techniques are more sensitive than either of these two stains and allow a more rapid diagnosis of chlamydial infection (21–24). The fluorescein-labelled monoclonal antibodies used in this test are specific to the major outer membrane protein (MOMP) of *C. trachomatis*. They detect all 15 known human serological variants (serovars) of the organism in both its forms — the infectious elementary body and the metabolically active reticulate body (25,26).

3. PRINCIPLE

Monoclonal antibodies have been prepared against the major outer membrane protein present in all known human serovars of *C. trachomatis* and in both forms of the organism: the infectious elementary body and the metabolically active, replicating reticulate body. The antibodies are labelled with fluorescein isothiocyanate (25,26).

Cultures are stained with the MicroTrak® *C. trachomatis* Reagent, which contains the anti-*C. trachomatis* antibodies. The antibody conjugate binds to *C. trachomatis* antigens present in the culture. A rinse step removes unbound antibody conjugate. When viewed under a fluorescence microscope, *C. trachomatis*-positive cultures show apple-green fluorescent inclusions contrasted against the red background of the Evans Blue counterstained cells.

4. REAGENTS

Catalog Number	Product Description	Quantity/ Volume
BH019UL	**MicroTrak® Chlamydia trachomatis Culture Confirmation Test**	65 tests
	C. trachomatis Reagent fluorescein-labelled mouse monoclonal antibodies specific to *C. trachomatis*; protein-stabilized phosphate buffer; Evans Blue counterstain; 0.01% sodium azide	3.0 ml*
	Reconstitution Diluent deionized water; 0.1% sodium azide	5.0 ml

* ***The reagent is supplied lyophilized. The indicated volume is that resulting from reconstitution.***

Precautions

- The MicroTrak® *Chlamydia trachomatis* Culture Confirmation Test is for *in vitro* diagnostic use.
- The reagent contains nonsterile mouse antibodies.
- The reagent and the reconstitution diluent contain sodium azide. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. If waste is discarded down the drain, flush it with a large volume of water to prevent azide buildup.
- Do not use the kit after the expiration date.
- When handled as directed, the reconstituted reagent can be used for up to 12 weeks.

Page 1 of

The following instruction should be adhered to when opening the silver flipseal cap as it has a sharp edge after opening:

- A tweezers, needle-nose pliers, forceps, de-cappers, spatula or similar type of object should be used to open and peel off the flip-seal from the vial. When doing this action, ensure it is done outwards, away from the body.
- Latex gloves should also be worn to provide further protection to the user.

Reagent Preparation and Storage

To reconstitute the reagent, remove the metal seal and rubber stopper from the reagent vial; add 3.0 ml reconstitution diluent and then discard the remaining diluent. Replace the reagent stopper on the vial and gently swirl the vial to dissolve the contents. Record the reconstitution date on the reagent vial label. After reconstitution, allow the reagent to remain at a room temperature of 20–25°C for 15 minutes before use.

Store the reconstituted reagent at 2–8°C when not in use. Do not freeze or expose to temperatures above 32°C. When handled as directed, reagent can be used for up to 12 weeks.

Discard the reagent and reconstitution diluent after the expiration dates printed on the vial labels.

5. SPECIMEN COLLECTION AND PREPARATION

Collection of a proper specimen is critical to the detection of *Chlamydia trachomatis*. Personnel collecting specimens should be well trained so as to minimize the possibility of false-negative results caused by inappropriate or inadequate specimens.

Transport clinical specimens to the laboratory in appropriate transport medium. Process the specimens in an established and uniform manner. Use McCoy or HeLa 229 cells for isolation, and incubate tissue cultures for 48 hours (17, 27–31).

Inoculate each patient specimen, suspended in transport medium, onto a pretreated monolayer of cells. For details of *Chlamydia trachomatis* cell culture technique, please consult references 17,24,27, and 30.

Positive and negative controls should be run with every batch of shell vials or with every microtiter plate, and should be treated in the same manner as patient specimens. Positive control stock material can be prepared by inoculating cell cultures with known *Chlamydia trachomatis*-positive samples (eg, ATCC stocks or previously identified laboratory isolates). After maximal infection of the monolayer, the cells can be scraped into additional medium, aliquoted, and stored frozen. Uninfected cell monolayers treated the same way can be used as negative control stock material.

Caution: During the collection and processing of specimens, users are advised to observe the same safety precautions as employed when handling or disposing of other potentially infectious materials (32).

6. PROCEDURE

Materials Provided

MicroTrak® *Chlamydia trachomatis* Culture Confirmation Test, containing:
C. trachomatis Reagent (3.0 ml)
Reconstitution Diluent (5.0 ml)

Materials Required But Not Provided

McCoy or HeLa 229 cells seeded into shell vials or microtiter plate.
Inoculated cultures (48-hour incubation)

- Patient samples
- Positive and negative controls (recommended)

Fixative solution — 90% ethanol
Rinse — distilled or deionized water
PBS (KCl 0.2 g/l, KH_2PO_4 0.2 g/l, Na_2HPO_4 7 H_2O 2.16 g/l, NaCl 8 g/l, pH 7.4)
Mounting Medium — Glycerol/Tris (glycerol plus 0.1M Tris, pH 8.5, 1:1)
— Glycerol/0.2M K_2HPO_4 (pH 9.0, 1:1 or 9:1)
Coverslips (Procedure A: Microtiter Plate Technique)
Microscope slides (Procedure B: Vial/Coverslip Technique)
Micropipette (30 µl)
Pasteur pipette
Forceps (Procedure B: Vial/Coverslip Technique)
Moist incubator (37°C ± 2°C)
Fluorescence microscope with filter system for fluorescein isothiocyanate (FITC), ie, maximum excitation wavelength = 490 nm, mean emission wavelength = 520 nm; 200–250x and 400–630x magnification (dry objective).

Note: A w ell-functioning fluorescence microscope is crucial. Variations in quality of objectives, bulb wattage, intensity and alignment, type of illumination, and filters may affect test performance. Use a p ositive control to verify adequate functioning of the reagent, culture system, staining procedure, and microscope.

Setup

1. Prepare the reagent according to directions in Section 4, Product Description, Preparation, and Storage.
2. Allow the reagent to reach room temperature before use.
3. Immediately before use, swirl the reagent vial to mix the contents thoroughly.
4. Test positive and negative controls alongside the patient samples to verify the performance of the culture system, reagent, staining procedure, and microscope.

A: Microtiter Plate Technique

Fixing of Monolayers

1. After incubating for 48 hours, carefully aspirate the medium from each well. Avoid disturbing the monolayers. Do not allow the cell monolayers to dry or the inclusion bodies may burst and be reduced in number.
2. Immediately cover the monolayers with ethanol for 1–10 minutes.
3. Remove the ethanol by aspirating or by inverting the plate onto absorbent paper.
4. For best results, stain the monolayers immediately after fixation.*

** If the monolayers cannot be stained immediately, they may be refrigerated for no more than 24 hours. If the monolayers will not be stained within 24 hours after fixation, they may be stored at -70°C in a closed protective container.*
The monolayers must be brought to room temperature and remoistened prior to staining.

Staining and Mounting

1. Allow the *C. trachomatis* reagent and microtiter plates to reach room temperature before staining. Gently swirl the reagent vial to mix the contents.
2. Add 30 µl of reagent to each well, making sure the entire monolayer is covered. (If the monolayer is dry, first moisten with deionized water or PBS. Remove excess water or PBS by aspirating or by inverting the plate onto absorbent paper. See Section 11, Critical Parameters.)
3. Incubate at 37°C for 30 minutes in a moist chamber. Do not allow the reagent to dry in the wells; drying can cause nonspecific binding, resulting in dense staining around the perimeter of the wells. If wells have this staining pattern, repeat the test. (See Section 12, Problem Solving.)
4. Aspirate the excess reagent.
5. Rinse each well with deionized or distilled water. Wait 10 seconds.
6. Aspirate the excess water.
7. Allow the mounting medium to reach room temperature before use.
8. Place a drop of mounting medium in each well.
9. Place a coverslip on the drop in the well. To do this step, attach a Pasteur pipette to an aspirator. Use the suction of the aspirator to pick up a coverslip. Place the coverslip at the base of the well. Excess medium is concurrently aspirated. Withdraw the pipette, releasing the coverslip.
10. Invert the microtiter plate to read it unless you are using an inverted microscope.

Vial/Coverslip Technique

Fixing of Monolayers

1. After incubation of the specimens in the shell vials, carefully aspirate the medium from the vials, being careful not to disrupt the cell monolayer on the coverslip inside each vial. Do not allow the cell monolayers to dry or inclusion bodies may burst and be reduced in number.
2. Immediately add ethanol to the vials and leave the vials undisturbed for 1–10 minutes.
3. Aspirate the ethanol from the vials.
4. For best results, stain the monolayers immediately after fixation.*

** If the monolayers cannot be stained immediately, they may be refrigerated for no more than 24 hours. If the monolayers will not be stained within 24 hours after fixation, they may be stored at -70°C in a closed protective container.*
The monolayers must be brought to room temperature and remoistened prior to staining.

Staining and Mounting

1. Allow the *C. trachomatis* reagent and the fixed coverslip to reach room temperature before use. Gently swirl the reagent vial to mix the contents.
2. Remove the coverslip with forceps and touch the edge to blotting paper. Place the coverslip with cell side up on a clean, labelled microscope slide.
3. Add 30 µl reagent to each monolayer, making sure the entire area is covered. (If the cells are dry, this volume will not cover the entire area. First moisten the cells with deionized water or PBS and blot the excess. See Section 11, Clinical Parameters.)
4. Incubate at 37°C for 30 minutes in a moist chamber. Do not allow the antibodies to dry onto the cells; drying will cause nonspecific binding.
5. Aspirate the excess reagent.
6. Remove the coverslip from the slide using forceps, and rinse it in deionized water for 10 seconds.
7. Allow the mounting medium to reach room temperature before use.
8. Place a drop of mounting medium onto an appropriately labelled, clean microscope slide.
9. Place the coverslip, cell side down, on top of the drop.

Reading

Immediately after staining,* examine the monolayers using a suitable fluorescence microscope with a dry objective. For optimum clarity, use 100x magnification for scanning and 250x or 400x magnification for confirmation of morphology. For Procedure A, Microtiter Plate Technique, the objective should have a long focal length for reading through the thickness of the plastic plate.

** If plates or slides will not be read immediately, store them in the dark at -20°C and read them as soon as possible (within 24 hours for best results). Plates and slides should be stored in closed protective containers. Allow the plates to reach room temperature before reading. Otherwise, condensation may occur, preventing accurate interpretation of results.*

7. INTERPRETATION OF RESULTS

Patient Specimens

Scan the entire well or coverslip for inclusion bodies displaying fluorescent apple-green staining. Positive diagnosis is made when the fixed stained cultures contain morphologically typical inclusion bodies showing apple-green fluorescence against the red background of the

counterstained cells. The size of the inclusion bodies is dependent on the culture conditions and the organism's serovar classification.

Negative results are reported when the fixed stained cultures contain no applegreen fluorescing inclusion bodies (27).

Control Slides

The positive controls should show characteristic apple-green fluorescent staining. The negative controls should show no apple-green fluorescing inclusion bodies. The appearance of the positive and negative controls should be used as a reference in evaluating patient specimens.

8. LIMITATIONS

- Performance of the MicroTrak® *Chlamydia trachomatis* Culture Confirmation Test has been established for use in the detection and identification of *C. trachomatis* in tissue culture only.
- Optimal performance of this test depends on proper collection and transport of an adequate patient specimen and proper culture and staining techniques.
- Specimens may be inoculated into McCoy or HeLa 229 tissue cultures. Other systems have not been validated.

9. EXPECTED VALUES

The MicroTrak® *Chlamydia trachomatis* Culture Confirmation Test was studied on fresh specimens from 3786 patients (1353 male; 2433 female) at low and high risk for chlamydial infection. Of the 3786 specimens tested, 396 (10%) were positive for *C. trachomatis*. Of the positive specimens, 161 (41%) were from male patients, 234 (59%) were from female patients, and 1 specimen, sex unknown.

Expected values may vary depending upon the patient population tested.

10. PERFORMANCE

The MicroTrak® *Chlamydia trachomatis* Culture Confirmation Test was compared with reference iodine staining techniques in three independent laboratories. McCoy cell monolayers were inoculated with specimens, incubated at 35–37°C in the presence of carbon dioxide, and then fixed and stained. Two of the monolayers were blind passaged, incubated, and then stained and read by both methods.

Analysis of Known-Positive Samples

Site 1 analyzed patient samples previously identified as *C. trachomatis*-positive by iodine staining.

100 known-positive specimens (50 males and 50 females) were cultured using a glass vial and coverslip system and then analyzed by the MicroTrak® test and by iodine staining. Table 1 shows the number of samples detected by each method after primary culture (40 hours incubation for the MicroTrak® test, 48–72 hours for iodine staining) and after passage. Inclusion bodies were detected in a total of 96 specimens. Iodine detected 92 of these specimens, and the MicroTrak® test detected 95.

Table 1 – Detection of Known-Positive Samples (N=100)

	Number Detected After Primary Culture	Total Detected After Passage
MicroTrak	93	95
Iodine Staining	92	92

Analysis of Undiagnosed Clinical Specimens

Sites 2 and 3 analyzed a total of 3786 fresh specimens from both low- and high-risk populations. Two different culture techniques were used. The study design is summarized in Table 2.

Table 2 – Study Design

Culture Technique	Number of Specimens Male	Number of Specimens Female	Hours of Culture Incubation MicroTrak	Hours of Culture Incubation Iodine
Coverslip/Vial	113	628	48	48-72
Microtiter Plate	1240	1607	48*	48-72

*** 48–72 hours after passage.**

Table 3 shows results obtained after passage. A sample was scored positive if a positive result was obtained in primary culture or after passage. Based on this data, the sensitivity of the vial/coverslip technique was 100% and the specificity was 99%. The sensitivity of the microtiter plate technique was 93% and the specificity was 98%.

Table 3 – Comparison of Results After Passage (N=3786)

MicroTrak	+	+	-	-
Iodine	+	-	+	-
Coverslip/Vial Culture Technique	85	12	0	842
Microtiter Plate Culture Technique	311	81	22	2453

Table 4 compares positive results obtained using the MicroTrak® test and iodine staining both at primary culture and after passage. With the coverslip technique, the MicroTrak® test identified 73 specimens as positive after primary culture, 24% more than those detected by iodine staining after primary culture. With the microtiter plate technique, the MicroTrak® test identified a total of 336 positive specimens after primary culture, which is 28% more than iodine staining at primary culture and comparable to the total of 333 positives detected by iodine staining after passage.

Table 4 – Comparison of Results After Primary Culture and After Passage

	Detected After Primary Culture	Total Detected After Passage
Coverslip/Vial Culture Technique		
MicroTrak	73	97
Iodine	59	85
Microtiter Plate Culture Technique		
MicroTrak	336	372
Iodine	263	333

Evaluation of Data Obtained Using Duplicate Analysis

A subset of patient samples was cultured in duplicate and stained by the MicroTrak® test after a 48-hour incubation period. Of the 128 samples identified as positive, 117 were detected by reading

only the first sample, an additional 11 were detected by also reading the second sample. Therefore, duplicate analysis increased the positive detection rate by 9%.

11. CRITICAL PARAMETERS

- Proper collection and transport of the patient specimen is critical to the performance of this test.
- Proper culture and staining technique is critical to the performance of this test.
- Specimens may be inoculated into McCoy or HeLa 229 tissue cultures. Other systems have not been validated.
- Appropriate positive and negative controls should be tested concurrently with patient specimens to verify the performance of the culture system, reagent, staining procedure, and microscope.
- Cell monolayers must remain moist during all stages of the procedure.
- The working reagent (ie, reagent reconstituted with exactly 3.0 ml reconstitution diluent) is optimized to detect all 15 known serovars of *C. trachomatis*. Dilution or adulteration of the working reagent or pooling of different reagent lots may result in loss of sensitivity.
- In the given test procedures, studies have shown that 30 µl of reagent is sufficient to cover the cell monolayer. Modifications to the procedures may lead to difficulty in covering the entire monolayer and to loss of sensitivity.
- Vial/Coverslip Technique: Use only clean slides that have been checked for nonfluorescence. Foreign matter may cause irregular fluorescent patterns. Take care to avoid contamination during specimen handling.
- The fluorescence microscope must be equipped with a filter system for observing fluorescein isothiocyanate (FITC) and with a correctly aligned bulb of adequate intensity.

12. PROBLEM SOLVING

Controls

If positive controls cannot be distinguished from negative controls, the following steps should be taken to determine the source of the error:

1. **Check the reagent expiration date.** If the reagent has expired, discard it and prepare and stain new controls using unexpired reagent.
2. **Check the microscope alignment** and reread the control slide.
3. **Verify the temperature of the incubator.**
4. **Stain a second set of controls**, observing the staining procedure carefully.
5. **Review the storage history of the reagent** for conformity with the instructions in this package insert.
6. **Investigate the viability of the C. trachomatis strain** used for the positive controls.

If a positive control does not show countainstained cells or bright apple-green fluorescence through the fluorescence microscope, clean the microscope objective and eyepieces. If this does not help, replace or realign the bulb and check the filters.

If the negative control appears positive, check culture stocks. The original negative control culture may have been contaminated.

Staining

If whole cells are stained apple-green, the cells may have dried out before fixing, or the reagent may have dried on the cells before the excess reagent was removed. Keep cells moist at all stages of the test.

Bacterial contamination may obscure cell monolayers, and, in some cases, bacteria may be nonspecifically stained by the antibody reagent. However, chlamydial staining is distinguishable from nonspecific bacterial staining by the characteristic morphology of the chlamydial inclusion bodies.

Dense staining around the perimeter of a coverslip or microtiter well may indicate that the reagent dried before rinsing or during the incubation. If a coverslip or well has this staining pattern, repeat the test by inoculating a new monolayer, and ensure that the incubation chamber has sufficient humidity to prevent drying.

Yellow staining indicates autofluorescence and should be disregarded.

Reading

If a culture is questionable (ie, is unreadable, has a poor cell monolayer, or contains atypical inclusions), inoculate a fresh cell monolayer with the original specimen and repeat the test.

Dense, irregular fluorescent patterns that mimic chlamydial inclusions may be caused by foreign matter in the specimen, or on the slide if the vial/coverslip technique is used. Take care to avoid contamination during specimen handling and use clean slides.

Artifacts may be caused by residual medium. If artifacts are a problem, try removing the residual medium by carefully rinsing the coverslips or microwells with a small amount of PBS after aspirating the medium. (See Section 6, Fixing of Monolayers, step 1.)

If too few cells are present on a vial coverslip or in a microwell, the problem may be due to damaged cells, to overly vigorous handling of the cells during rinsing, or to toxicity of the specimen. Repeat the culture and staining procedure, being sure to:

- check the appearance of the monolayers before inoculating the specimen
- rinse the coverslip or microtiter well gently, taking care not to disturb the cell monolayer.

The following conditions may cause nonspecific background haze. Note the recommendations for eliminating these problems:

- Mounting medium may not have been placed under the coverslip. Add it if necessary.

Microtiter Plate Technique:

- Removal of rinse water may have been inadequate. Ensure that all water has been aspirated from the wells before adding mounting medium.

Vial/Coverslip Technique:

- The slide may have been chemically treated by the manufacturer. Rinsing the slide with acetone for 5–10 minutes before use may alleviate this problem.
- Rinsing may have been inadequate. Be sure to rinse the coverslip in a beaker of deionized water for 10 seconds.
- The coverslip may have been mounted on the slide with the cell side up rather than down. Check the placement of the coverslip.

13. RISK AND SAFETY

Sodium Azide

R22	Harmful if Swallowed.
R32	Contact with acids liberates toxic gas.
S36	Wear suitable protective clothing.

Prepared in accordance with requirements for EEC label.
EINECS 247-852-1

Notice: Adulteration of reagents, use of instruments without appropriate capabilities, or other failure to follow instructions exactly as set forth in this labeling can adversely affect performance characteristics and stated or implied label claims.

The price of reagents includes a royalty for a license under U.S. Patent Nos. 4,582,791 and 4,218,845 for use of this product only.

Monoclonal antibodies developed for Trinity Biotech plc, by Genetic Systems Corporation, Woodinville, Washington, USA.

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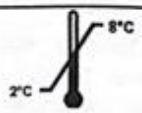
GUIDE TO SYMBOLS

Consult Instructions for Use



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