Elucidating the chlamydial growth characteristics, infection factors, and host responses to persistent chlamydial infection in women

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Certificate of original authorship

I, Mark Thomas, declare that this thesis is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Life Sciences at the University of Technology Sydney. This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis. This document has not been submitted for qualifications at any other academic institution. This research is supported by the Australian Government Research Training Program.

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List of abbreviations

% v v^{-1}	Percentage volume of total volume
% w v^{-1}	Percentage weight of total volume
16S rRNA	16S ribosomal ribonucleic acid
AB	Aberrant body
ACTS	Australian Chlamydia Treatment Study
bp	Base pair
Bpdl	Bipyridal
BSA	Bovine serum albumin
BSC	Biosafety cabinet
cDNA	Complementary deoxyribonucleic acid
CST	Community state type
Ct	Chlamydia trachomatis
CtD	Chlamydia trachomatis D serovar
CXCL9	Chemokine (CXC motif) ligand 9
DAPI	4‟,6-diamidino-2-phenylindole
DC	Dendritic cell
DMEM	Dulbecco‟s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DPBS	Dulbecco‟s phosphate-buffered saline

dsDNA	Double-stranded deoxyribonucleic acid
EB	Elementary body
euo	Early upstream open reading frame
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FRT	Female reproductive tract
FTH1	Ferritin heavy chain 1
g	Gravity of Earth
GAG	Glycosaminoglycans
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gDNA	Genomic deoxyribonucleic acid
GFP	Green fluorescent protein
GLUT1	Glucose transporter-1
h	Hours
h PI	Hours post infection
H+L	Heavy and light chains
HctA	Histone Hc1-like *Chlamydia trachomatis* protein
HctB	Histone Hc2-like *Chlamydia trachomatis* protein
HIV	Human immunodeficiency virus
HREC	Human Research Ethical Committees
Hsp	Heat shock protein
Hsp60	60-kilodalton heat shock protein
Hsp70	70-kilodalton heat shock protein
HSV	Herpes simplex virus

htrA	High temperature requirement A
HIF-1α	Hypoxia inducible factor-1α
IB	Intermediate body
IDO1	Indoleamine 2,3-dioxygenase
IF	Immunofluorescence (microscopy)
IFNG	Interferon gamma
IFU	Inclusion forming unit
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL1A	Interleukin-1-α
IL6	Interleukin 6
IL8	Interleukin 8
IL10	Interleukin 10
inc	Inclusion (protein)
IRF1	Interferon regulatory factor 1
kDa	Kilodalton
kU	Kilo unit
LPS	Lipopolysaccharide
maU ml^{-1}	Milli-Anson Units per milliliter
MCC	Minimum chlamydicidal concentration
MEC	2-*C*-methylerythritol 2,4-cyclodiphosphate
MEP	Melavonate methylerythritol 4-phosphate
MIC	Minimum inhibitory concentration

MOI	Multiplicity of infection
MOMP	Major outer membrane protein
NAAT	Nucleic acid amplification testing
NK	Natural killer (cell)
NTC	No-template control
OM	Outer membrane
omcB	Outer membrane protein B
omp	Outer membrane protein
ompA	Outer membrane protein A
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PID	Pelvic inflammatory disease
Pmp	Polymorphic membrane protein
Pmp21	Polymorphic membrane protein 21
qPCR	Quantitative polymerase chain reaction
QUT	Queensland University of Technology
RB	Reticulate body
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
s	Seconds

SNP	Single nucleotide polymorphism
SPG	Sucrose phosphate glutamate
SRA	Sequence read archive
STI	Sexually transmitted infection
SYBR	Synergy Brands
T3SS	Type three secretion system
TARP	Translocated actin-recruiting protein
TBE	2-amino-2-hydroxymethyl-propane-1,3-diol, boric acid ethylenediaminetetraacetic acid
TFI	Tubal factor infertility
TLR	Toll-like receptor
TNC	Tenascin-C
TNFA	Tumour necrosis factor alpha
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol, boric acid ethylenediaminetetraacetic acid
TRITC	Tetramethylrhodamine
trpBA	Tryptophan synthase B/A
U	Unit
USC	University of the Sunshine Coast
UTS	University of Technology Sydney

Abstract

Chlamydia trachomatis is an obligate intracellular parasite and the leading cause of sexually transmitted bacterial infections in the human urogenital tract. Clinical manifestations of chlamydial infection include urethritis, cervicitis, pelvic inflammatory disease and tubal factor infertility. These pathological conditions are caused by the immune response to both acute and chronic chlamydial infections. Evidence suggests a high proportion of infections remain subclinical until spontaneous resolution or the commencement of symptoms leads to a diagnosis. Therefore, a substantial proportion of the morbidity and burden associated with chlamydia can likely be attributed to unresolved and untreated infections. While treatment with azithromycin is highly effective, treatment failure does occur. The mechanisms of treatment failure and its effects on fertility are poorly understood. Unlike other bacterial pathogens, *C. trachomatis* lacks stable genotypic resistance to macrolides. Another key difference between *Chlamydia* and many other bacteria is the constant interaction with its host cell. Thus, it was hypothesised that the unique intracellular niche and developmental cycle of *C. trachomatis* are important microbial factors which could affect treatment efficacy. To test this, several host and chlamydial factors were investigated. 16S rRNA gene amplicon sequencing of vaginal and cervical swabs and endometrial biopsies from participants of a case-control fertility study revealed that similarities in the microbial populations of the vagina and cervix were not predictive of those in the endometrium. While there was no association

between microbial community compositions and fertility status identified, *Ureaplasma* spp. were overrepresented amongst infertile women. The endometrial expression of several genes involved in immunity and reproductive function showed no association with microbial community composition, however, the gene which encodes tenascin-C was over-expressed in women who had a self-reported history of miscarriage. Comparisons of clinical isolates from women treated for chlamydial infections showed no significant differences in developmental or stress phenotypes but suggested that the subtle differences observed using *in vitro* models may not truly reflect the complexity of *in vivo* infectious processes. Finally, analysis of host and chlamydial gene expression before and after antibiotic treatment showed no association with outcome but yielded valuable information about the host and pathogen during the period following treatment. In particular, chlamydial gene expression was upregulated after they had survived treatment with azithromycin. This project has contributed towards current knowledge and increases the field"s understanding of the host and chlamydial factors involved in treatment failure and infertility. Additionally, it provides insight for future investigations of these important and complex interactions between humans and the bacteria which have evolved alongside us.

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Thesis statement

I am submitting my work titled “Elucidating the chlamydial growth characteristics, infection factors, and host responses to persistent chlamydial infection in women” as a conventional thesis, in accordance with the University of Technology Sydney (UTS) Graduate Research School (GRS) Research Candidature Management, Thesis Preparation and Submission Procedures.

Author contribution statements

Chapter 3

Wee, B. A.*, **Thomas, M**.*, Sweeney, E. L., Frentiu, F. D., Samios, M., Ravel, J., . . . Huston, W. M. (2018). A retrospective pilot study to determine whether the reproductive tract microbiota differs between women with a history of infertility and fertile women. *Aust N Z J Obstet Gynaecol, 58*(3), 341-348. doi:10.1111/ajo.12754

*these authors contributed equally to the study.

BAW conducted all bioinformatics analysis and contributed to the drafting of the manuscript. MT conducted laboratory components, RT-qPCR and related statistical analysis and contributed to the drafting of the manuscript. ELS contributed to the design and interpretation of RT-qPCR and contributed to the drafting of the manuscript. FDF contributed to the design and interpretation of RT-qPCR and contributed to the drafting of the manuscript. MS contributed to participant recruitment, questionnaire design and data collection, and contributed to the drafting of the manuscript. JR contributed to bioinformatics design and analysis of the microbiota data and contributed to the drafting of the manuscript. PG contributed R scripts, bioinformatics design and analysis of the microbiota data and contributed to the drafting of the manuscript. GM contributed to bioinformatics design and analysis of the microbiota data and contributed to the drafting of the manuscript. PT contributed to design and interpretation of RT-qPCR and contributed to the drafting of the manuscript. JAA conceived the concept of the study, conducted clinical

recruitment, epidemiological and questionnaire analysis and contributed to the drafting of the manuscript. WMH developed the case-control study design, contributed to statistical analysis and interpretation and contributed to the drafting of the manuscript.

Chapter 4

Amba Lawrence (QUT) conducted the initial isolation of clinical isolates from ACTS swabs and the growth and infectivity assays. Sam Kroon (UTS) performed the iron deprivation chlamydial persistence assays and analysed the results. Mark Thomas (UTS) was responsible for assisting with aspects of experimental design, expansion of the clinical isolates for use in persistence models, penicillin persistence growth assays, the penicillin-azithromycin recovery assays, all confocal imaging shown, interpretation and analysis of data, construction and standardization of figures, and drafting of the paper with the intent of future publication. Wilhelmina Huston (UTS) developed major experimental design components, drafting of the paper, analysis and interpretation of findings. Peter Timms assisted with drafting and aspects of the experimental design. Jane Hocking conceived ACTS and provided access to the clinical isolates of *C. trachomatis* used in this study.

Chapter 5

Mark Thomas (MT) was involved in experimental design, analysis and interpretation of the results, drafting of the manuscript for future publication, and all of the technical components and procedures described. Wilhelmina Huston was responsible for the study conception, analysis and interpretation of the results, and drafting. Peter

Timms assisted with drafting of the manuscript and some components of the experimental design. Jane Hocking conceived ACTS and provided feedback on drafted versions of the paper.

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CHAPTER 1:
LITERATURE REVIEW

Foreword

Chlamydia trachomatis is a Gram-negative, obligate intracellular, bacterial pathogen of the human urogenital tract. Globally, it is not only the most prevalent bacterial sexually transmitted infection (STI), but also the leading cause of preventable blindness. While each year approximately 83,000 Australians test positive for *C. trachomatis* infections of the urogenital tract, it has been estimated that up to 500,000 people may unknowingly harbor this frequently asymptomatic infection [1, 2]. When the host‟s immune system fails to resolve a newly acquired infection, *C. trachomatis* can employ a variety of virulence mechanisms to evade, suppress and modulate the host‟s immune response. If the pathogen remains untreated and establishes a chronic infection, its ongoing presence can trigger a sustained pro-inflammatory host immune response. While acute infection has been shown to produce mild to moderate disease states, prolonged infection and the resulting inflammatory cycle are believed to cause the majority of pathology and sequelae. In female patients, disease states range from mild to moderate morbidities such as cervicitis, endometritis and salpingitis; through to more serious cases of pelvic inflammatory disease (PID), tubal infertility and even life-threatening ectopic pregnancy. Even though early antibiotic intervention can prevent the progression of infection and pathology, a proportion of patients remain infected after treatment. Not only are these unsuccessfully treated patients at an inherently greater risk of disease progression and pathological sequelae, they also generate an epidemiologic risk within the greater population. This is largely due to their role as inadvertent vectors, who unknowingly continue to harbor and transmit

the pathogen. While it is not entirely clear how *C. trachomatis* is able to survive in such cases, this unique pathogen is gradually becoming better understood.

Part 1: *Chlamydia trachomatis*

Overview

Species of the genus *Chlamydia* are Gram-negative bacterial pathogens of many vertebrate species, including humans, pigs, mice, snakes, guinea pigs, snakes and birds. While their preferred host species differ, all *Chlamydia* are obligate intracellular pathogens with striking similarities in their developmental biology and genetic identity. *Chlamydia trachomatis* is the only species of the genus known to infect and cause pathology in the reproductive tracts of humans. Additionally, certain strains of *C. trachomatis* can cause trachoma – which is an ocular form of chlamydial infection the world‟s leading cause of preventable blindness.

Chlamydial developmental biology

Overview of chlamydial development

The developmental cycle of *Chlamydia* spp. is highly conserved and must occur within a protective membrane-bound vesicle commonly referred to as the chlamydial inclusion. As **Figure 1.1** illustrates below, the two distinct developmental forms which characterise this cycle are the infectious elementary body (EB), and the replicative reticulate body (RB). Infection begins with the adhesion of EBs to the host-cell surface, after which they penetrate the cell and begin to develop the inclusion. Once this protective ultrastructure has been formed, the EB differentiates into the replicative RB form and undergoes multiple

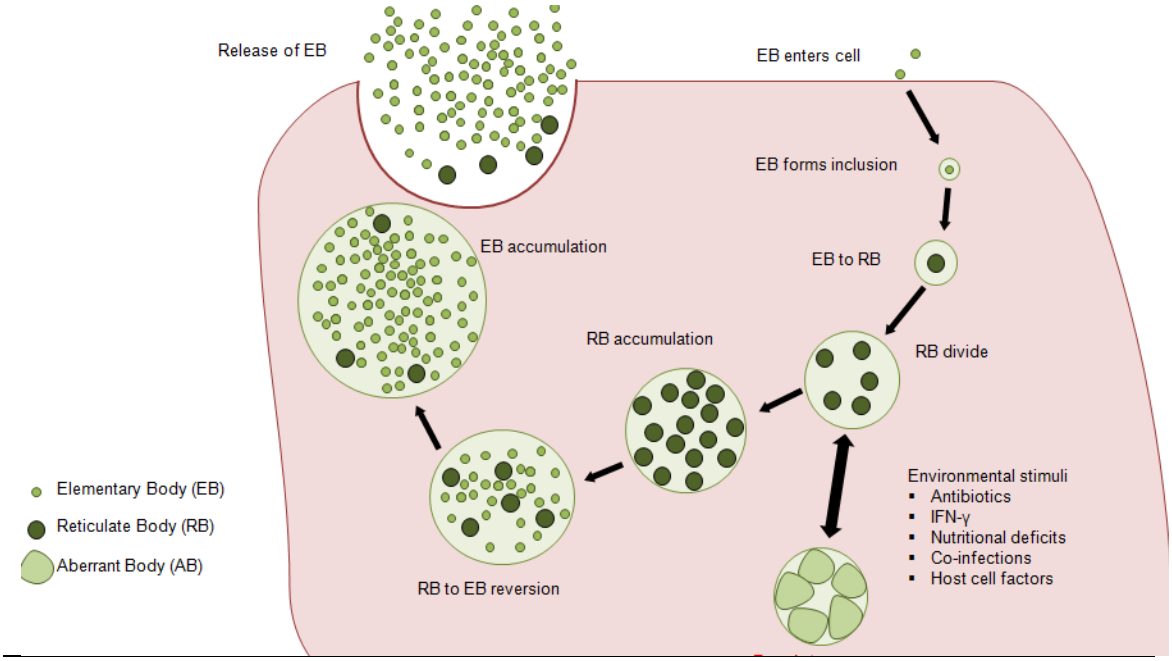

Figure 1.1. Overview of the chlamydial developmental cycle. A brief description of each key stage of the cycle is shown. The process begins with the attachment of an EB to a ligand on the surface of its host. From here, the EB enters its host by traversing the cell membrane (red outline) and in doing so, forms a cytoplasmic inclusion. Development continues within this protective inclusion, as the EB differentiates and becomes an RB. Undergoing consecutive rounds of replication, these rapidly dividing cells accumulate within the expanding inclusion. Replication among the RBs eventually stalls, as they cease replication and begin to revert into EBs; which soon-after exit their host and go on to infect other suitable cells. Also shown in the figure is a list of several environmental stimuli which are known to cause stress and induce chlamydial persistence during development organism. Figure is my own original work.

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rounds of binary fission. RBs continue to accumulate within the inclusion until the point at which they gradually begin to undergo asynchronous reversion to become EBs, exit the host cell, and propagate further infection amount the neighbouring cells [3]. Throughout each stage of its developmental cycle, the parasitic pathogen is known to extensively modulate, hijack and exploit its host-cell using a variety of secreted proteins [4].

Adhesion and invasion of the host cell

In order to reach the intracellular niche required for its developmental cycle, *C. trachomatis* must first attach itself to the outer membrane of a suitable host cell. This initial step of adhesion appears to involve the sulfate-dependent ligation of the EB envelope membrane and the target host-cell receptor [5, 6]. Previous studies into the chlamydial membrane proteins and surface molecules involved in this ligation have identified glycosaminoglycans (GAGs), chlamydia major outer-membrane protein (MOMP), 70-kDa heat-shock protein (Hsp70), outer-membrane complex (protein) B (OmcB) and polymorphic membrane protein-21 (Pmp21) as ligands of importance [5, 7-11]. Once attached, EBs induce their internalisation into the host-cell cytoplasm, form where they are translocated to a perinuclear location, before differentiating to become the replicative and non-infectious RB form. This is understood to require the activation and utilisation of the chlamydial type-III secretion system (T3SS) to insert effector proteins into the cytoplasm of the host cell [12]. The entry and internalisation of chlamydial EBs is believed to require the hijacking of several host cell processes, which facilitate the remodeling of cytoskeletal actin [13], macromolecules and microfilaments [14] to incorporate the membrane-associated EB into the cytoplasm of its host cell. Recent evidence suggests *Chlamydia* may also exploit a host cell pathway which resembles that of macropinoycytosis [15].

EB to RB differentiation in C. trachomatis

The genomic DNA (gDNA) of EBs exists for much of the time as a highly condensed form of chromatin, which greatly inhibits gene expression and prevents DNA replication. Evidence suggests that both the conformation and condensation

of this chromatin is mediated by two histone-like proteins, HctA and HctB [16, 17]. In order for the EBs to decondense their genome, recommence active gene expression, and begin their differentiation into RBs, they must first disrupt the strong binding interactions within the chromatin. How such a process is coordinated, while the vast majority of the genome is unable to be actively transcribed, is only partly understood. Early research into the mechanisms of chromatin dissociation suggests that *euo* is expressed early in the developmental cycle and produces a HctA C-terminal-specific protease that is capable of dissociating the gDNA-HctA complex [18]. This early theory of *euo*-mediated gDNA decondensation withstanding, more recent evidence appears to show HctA is not a substrate of proteolytic activity during this initial stage of development [19]. The same study also reported that 2-*C*-methylerythritol 2,4-cyclodiphosphate (MEC), which is a small metabolite produced by the non-melavonate methylerythritol 4-phosphate (MEP) pathway, appears to be responsible for the dissociation of chromatin from both histone-like proteins [19]. Although this suggests that the MEP pathway may play an important role in the early development of *C. trachomatis*, the extent of its involvement and the regulatory mechanisms of this biochemical pathway, in chlamydial persistence, are still unclear. Another recently proposed model of differentiation suggests that the size of the RB itself may be instrumental in the process [20]. The authors observed that prior to undergoing reversion to the infectious EB form, the mean size of RBs was decreased despite their continued replication. As an aside, I would like to quickly mention that examining chlamydial persistence in the context of this model could provide insight into the processes by which the aberrant forms revert to the typical developmental cycle and morphology.

RB accumulation and terminal differentiation

During the chlamydial developmental cycle, RBs undergo a series of changes to their metabolism, morphology and gene expression. The order in which these changes occur is highly conserved throughout the genus; yet the timing and duration of each stage differs among strains and indeed between species [21-23]. Traditionally, the transcriptional profiles present during the cycle have been used to temporally-classify the stages of development as either early, mid-cycle or late [24]. Throughout development, access to the genes required for each of these stages has been demonstrated to involve both IhtA – a small RNA (sRNA) translational inhibitor of HctA production and σ^{28}, which is a factor required for HctB production [25, 26]. The binary fission of RBs occurs predominantly during the mid-cycle stage, and requires coordinated expression of a number of genes, which encode the machinery and metabolites necessary for replication. This is reflected by the transcriptome during this stage, which is characterised by increased expression of *recA* (encodes 16S rRNA), *dnaA* (initiator of chromosomal replication), *polA* and *polE* (encode DNA polymerase subunits), *parB* and *minD* (involved in DNA partitioning and segregation), *mutS* (involved in DNA mismatch repair), and *ftsW* and *ftsK* (involved in cytokinesis) [24, 27]. While replication and RB accumulation continues, a gradually increasing proportion of RBs terminally differentiate back to the infectious EB form. This asynchronous reversion involves the recondensation of chromatin, which unsurprisingly appears to be mediated by increased expression of both *hctA* and *hctB* [24, 28]. It also involves structural modifications in the outer membrane (OM). This process has been attributed to the increased levels of proteins that form complexes within the OM (OmcA and

OmcB), and those which alter and exchange the structurally important disulphide bonds of other OM proteins [28].

Chlamydial persistence

Persistent chlamydial infection

C. trachomatis infections of the urogenital tract are notorious for their reemergence and detection in patients who have previously been infected and treated. The underlying cause of these persistent infections has long been a contentious issue amongst clinicians and researchers alike, with anecdotal and scientific evidence supporting several possibilities. Suggested causes include insufficient antibiotic accumulation, gastrointestinal colonisation, re-infection by sexual partners, new infections, and antibiotic resistance [29].

Developmental stress response

Outside the previously mentioned clinical context, persistence is a developmental stress response believed to be a positive and beneficial adaptation unique to the genus *Chlamydia* [30, 31]. The persistence phenotype is characterised by several reversible morphological, transcriptional and metabolic changes to both RBs and the surrounding protective inclusion [32-36]. The collective profiles of these changes vary depending on the inducer of persistence; however, the morphologically aberrant RBs, altered inclusion sizes and the reversible loss of both cultivability and replicative capacity are considered to be the universal hallmarks of *in vitro* persistence. These morphological variations have been compiled by Hogan *et al* [37]; and these most likely arise due to the different

effects each inducer has on both *Chlamydia* and the infected host cell at transcriptional, translational and epigenetic levels [38].

Inducers of persistence

A well-defined list of environmental conditions and exogenous stimuli are understood to induce chlamydial persistence *in vitro*. Early studies into the effects of penicillin on *Chlamydia* focused primarily on the morphological changes it produced in the inclusion and RBs but were unable to fully explain the antibiotic‟s impact on development [35]. Ongoing research into penicillin-induced persistence has furthered our understanding of how it affects chlamydial development, gene expression, membrane protein presentation, sensitivity to other antibiotics as well as other novel compounds [39-43]. Although it is not entirely clear how penicillin induces persistence in *Chlamydia*, recent findings strongly suggest it inhibits the cytokinesis stage of binary fission, and subsequently prevents the continuation of replication as well as terminal differentiation by the RBs [39, 40]. Recent findings also indicate the antibiotic may affect or inhibit chaperone activities between the pathogen‟s penicillin-binding proteins (PBP) and chlamydial *N*-acetylmuramyl-L-alanine amidases involved in cell (RB) separation [44]. While the interferon gamma (IFN-γ; reviewed in [45]) persistence model is the most extensively investigated nutrient-deprivation model, *C. trachomatis* is sensitive to reductions in several other metabolic precursors. Use of the iron-chelating agent deferoxamine mesylate in a number of studies has shown that iron restriction not only induces persistence in *C. trachomatis*, but also alters the pathogen‟s signaling pathways in an attempt to inhibit host-cell apoptosis [46, 47]. *In vitro* glucose deprivation has also been demonstrated to induce persistence in *C. trachomatis*,

and it appears as though the pathogen relies on glucose as its sole carbon source and possesses no alternative biosynthetic pathways [33, 48].

Interferon-gamma and chlamydial infection

The effects of IFN-γ on chlamydial development and infection are well established (reviewed in [45]). Previous studies investigating the relationship between this proinflammatory cytokine and *Chlamydia* have focused on tryptophan depletion by the enzyme product of indoleamine 2,3-dioxygenase-1 (*IDO1*) [49]. The increased expression of *IDO1* and synthesis of this catabolic enzyme by the host cell in response to IFN-γ rapidly reduces the intracellular stores of tryptophan, an amino acid which *C. trachomatis* must acquire for its metabolic processes [50, 51]. As previously mentioned, there is a large volume of *in vitro* evidence that this decreased availability of tryptophan induces chlamydial persistence in the urogenital tract (reviewed in [52]). Interestingly, IFN-γ also decreases cellular levels of iron in infected cells by downregulating their transferrin receptor expression [53]. Conditions of intracellular iron deprivation are also known to induce chlamydial persistence [46, 54, 55]. A brief summary of this process is illustrated in **Figure 1.2**. Although it has long been understood that IFN-γ has many effects outside the context of tryptophan depletion, recent evidence has

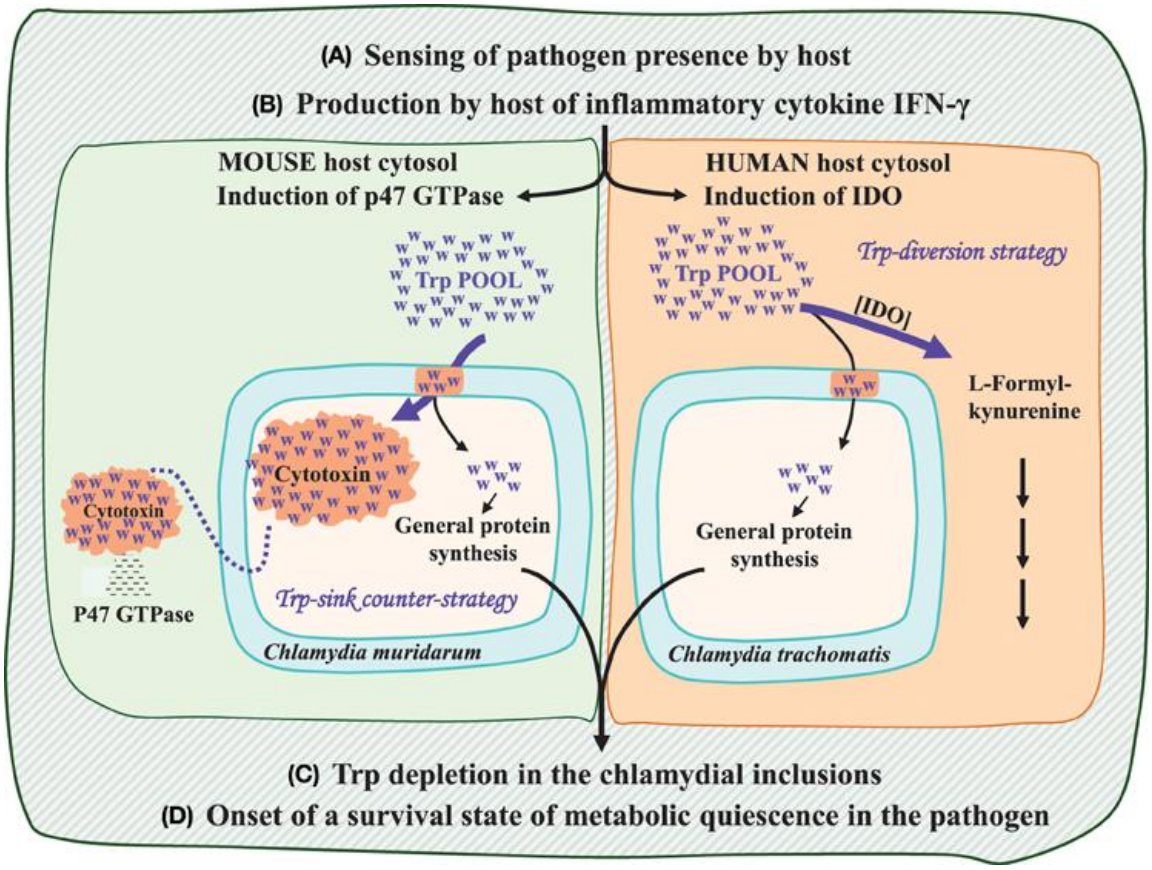


Figure 1.2. The production of IFN-γ by human and murine host cells is a key defence against intracellular pathogens such as *C. trachomatis*. The detection of *C. trachomatis* triggers increased IFN-γ production in both the host and localised immune cells. The similarities in the commencement (A and B) and outcome (C and D) of this process between human and murine cells have been established. The effects of homologous IFN-γ in both species however have been observed to be different. In human cells, a key role of the IFN-γ is to reduce the intracellular levels of tryptophan by enzymatic degradation. *In vitro* experiments suggest this is exploited by *Chlamydia* spp., who enter persistence in response to the decrease in the essential amino acid. Figure has been directly reproduced from [30].

suggested that IFN-γ causes a dramatic reduction in host cell glycolysis [56]. Importantly, the authors demonstrated that this effect was accompanied by reduced expression of two hypoxia-dependent genes, glucose transporter-1 (GLUT1) and hypoxia inducible factor-1α (HIF-1α). The effects of host-cell hypoxia on the chlamydial developmental cycle are well established and hypoxic conditions have been suggested to reduce the chlamydicidal effects of doxycycline [57]. Additionally, the complex relationship between IFN-γ, tryptophan depletion and chlamydial persistence has been shown to be influenced by the vaginal microbiota [52]. Novel *in vitro* co-culture experiments by Ziklo and colleagues reportedly demonstrated that the supplementation of indole, a heterocyclic organic metabolite of some species of lactobacilli, can be used in chlamydial biosynthesis of tryptophan [58]. Biosynthesis of tryptophan from indole by the chlamydial proteins encoded within the *trpBA* operon of *C. trachomatis* is believed to facilitate the pathogen‟s reversion from its persistent state back to its typical developmental cycle [59]. Thus, it is likely to be a crucial factor in the outcome of infection and the development of immunopathological sequelae [60]. These complex interactions are summarised below (**Figure 1.3**).

RB morphologies and molecular markers of persistence

Persistence is a reversible non-replicative state in which the RBs continue to undergo DNA replication and modulated gene expression. It is believed that the cessation of replication is caused by the RBs‟ inability to successfully divide while in this state. While the exact mechanism of such inhibition is not entirely clear, the most plausible explanation is that the altered expression levels of two genes involved in cytokinesis (*ftsK* and *ftsW*) [27, 34, 61]. This inability of RBs to

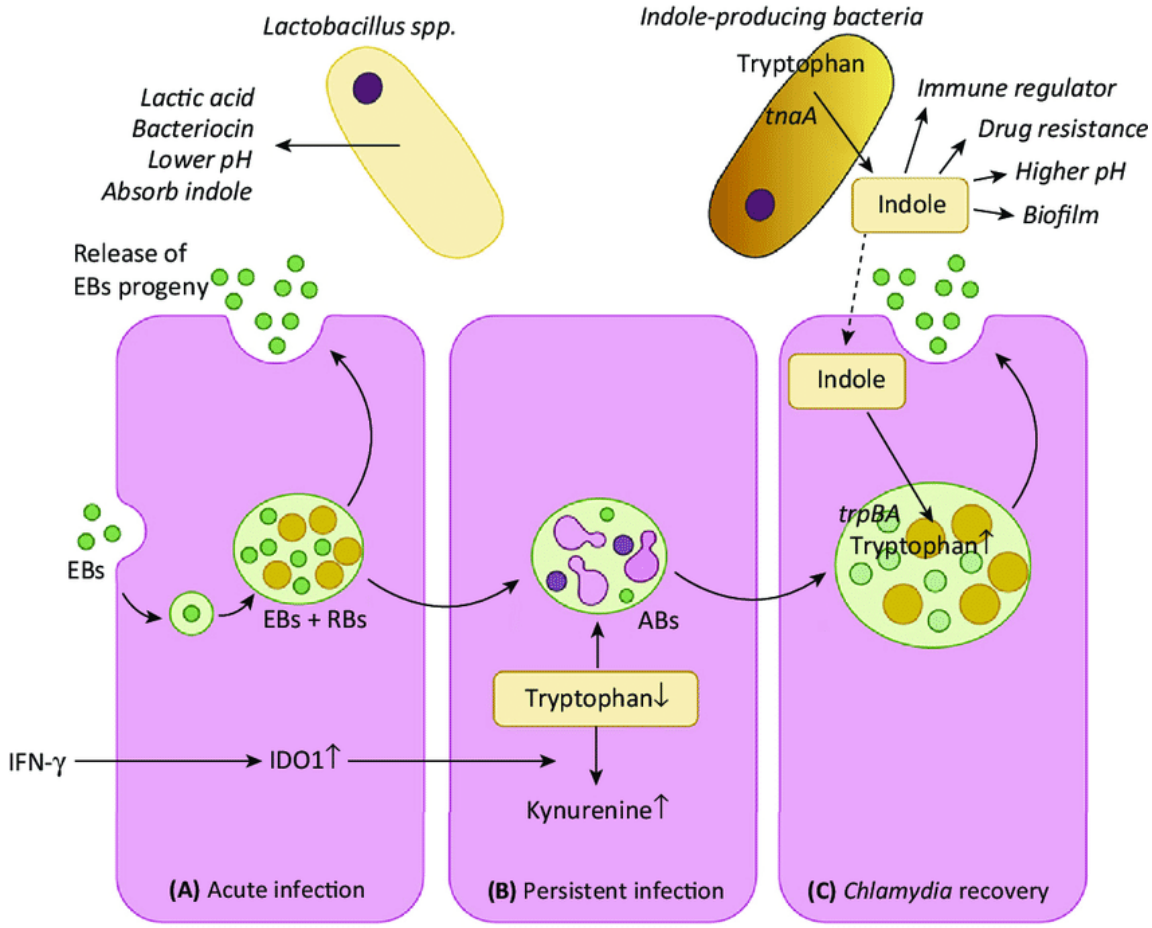


Figure 1.3. The relationship between *C. trachomatis*, IFN-γ and indole-producing bacteria. Tryptophan depletion within the host cell can create environmental conditions which are stressful enough to cause *C. trachomatis* to enter persistence. It has been proposed that in order to survive the attempts made by the host's immune system to clear the infection, *C. trachomatis* uses the metabolites of other microbes to facilitate its survival and recovery from persistence. Specifically, it is believed to use indole, which generated by the vaginal microbiota including some lactobacilli, to biosynthesise its own tryptophan. Thus, it is able to re-enter and continue with its typical developmental cycle. The image used in this figure is directly reproduced from [60].

undergo cytokinesis is largely responsible for their morphological transition into the enlarged pleomorphic aberrant body (AB) form. While it is understood that *Chlamydia* increase their overall expression of *groEL* during persistence, previous studies have identified certain chlamydial 60-kDa heat-shock protein (cHsp60)-encoding genes that are over-expressed in some models of persistence, making them attractive molecular markers of the stress response. Real-time PCR comparison of the expression levels of *ct755*, *ct604* and *ct110* have been suggested to be able to distinguish between active infection and chlamydial persistence. Specifically, ct755 expression levels predominate during active infection, while chlamydial persistence is characterised by higher levels of ct604 expression [62]. It should also be noted that these morphologies and molecular markers (and others) vary among the stimuli used to induce in vitro chlamydial persistence, which likely reflects their individual effects upon both the pathogen and its host cell.

The host immune system and *C. trachomatis*

Immunopathology and sequelae associated with* C. trachomatis *infection

While *C. trachomatis* urogenital tract infections are frequently asymptomatic, they have long been implicated in numerous disease states of the urinary and reproductive tracts of humans [63, 64]. The most commonly presented forms of symptomatic infection are cervicitis in females, and urethritis in males; which Stamm reminds us likely reflects the pathogen's portals of entry into the reproductive tract [65]. In females, unresolved and/or untreated infections may ascend past the endocervix, and into the upper reproductive tract. This may in turn result in endometritis or salpingitis, and also places the patient at a higher risk of

developing pelvic inflammatory disease (PID) and tubal factor infertility (TFI) [64]. Most of these pathologies, and their sequelae, have been attributed to the immune response triggered by infection [66-69]. Although the complexities of both chlamydial biology and the human immune system have limited our understanding into their intrinsic relationship, several models of symptomatic infection and disease progression to pathology have been proposed. These are perhaps best summarised by a comprehensive review by Menon and colleagues which examined published studies of the factors involved [70]. **Figure 1.4** below illustrates the four models by which the pathogenesis associated with chlamydial infections is believed to occur. Ascension of chlamydial infections beyond the endocervix and as far as the ovaries is one such proposed model. While there exists substantial evidence that the bacterial pathogen can reach these distal regions of the female reproductive tract [71, 72], the ascension model does not explain how the immune response to *C. trachomatis* at these anatomical sites results in pathology. This process is perhaps better explained by the cellular paradigm model, which posits that a cascade of proinflammatory signaling originating from the infected epithelium leads to mononuclear infiltration and damage of the reproductive tract [73]. Similarly, the hypersensitivity model suggests that pathology may occur when the host immune system produces a delayed and excessive "hyper" response to chlamydial antigens. Specifically, the 60-kDa *Chlamydia trachomatis* heat shock protein (cHsp60) has been suggested to cause hypersensitivity; though its role in this process has been the topic of much discussion [70]. Many believe that cHsp60 may elicit the production of auto-antibodies which recognise the epitopes of homologous heat shock proteins produced by humans and other bacteria [74, 75]. While this is supported by epidemiological data and clinical studies implicating

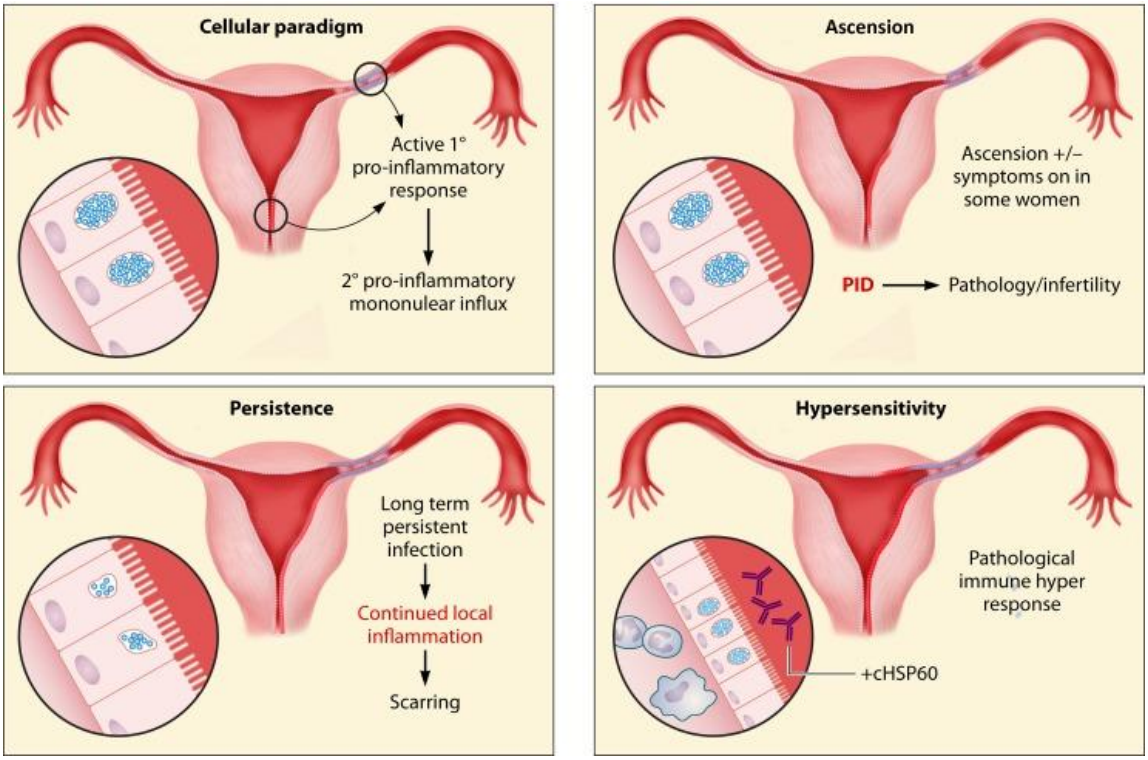


Figure 1.4. The four major models proposed by Menon and colleagues as mechanisms of chlamydial infertility in women. This figure summarises the models proposed which posit possible mechanisms of the immunopathology associated with urogenital *C. trachomatis* infections by: (top-left panel) dysregulated immune cascades (cellular paradigm); (top-right) ascension of the infection to and beyond the endometrium; (bottom-left) immune reactions to persistent infections; and (bottom-right) autoimmune antibody-mediated hypersensitivity. The image in this figure was directly reproduced from [70].

cHsp60 in such pathological processes, similar studies have questioned the degree to which exposure to cHsp60 is itself responsible for such a complex pathological process (reviewed in [76]). With similarities to the hypersensitivity model of disease progression, it has been also been suggested that chlamydial persistence may contribute towards reproductive tract pathology [37]. This model addresses the human and microbial factors driving pathology, by suggesting that chlamydial persistence can cause a prolonged and pathological interaction between the immune system and *C. trachomatis*. Arguably the most important evidence supporting this model comes from *in vitro* studies of the pathogen that revealed cHsp60 is overexpressed during persistence [77]. While a major limitation of the chlamydial persistence model of disease is that it relies heavily on findings from *in vitro* studies, it seems both possible and likely that chlamydial persistence does occur *in vivo*, as a strategy to evade the hosts‟ attempts to eliminate it, within its intracellular niche [52, 78, 79]. If this is indeed the case, it is likely that genotypic variation causing overexpression of cHsp60 or other immunogenic molecules are

part of the short- and long-term intracellular survival strategies employed by *C. trachomatis*.

Innate immunity to Chlamydia

Innate immunity in the female reproductive tract relies on several physical, chemical and cellular strategies to, among other things, prevent chlamydial infection [80]. These include mucous secretion [81], increased numbers of tissue-resident T lymphocytes and macrophages [82], natural killer (NK) cell activity [83], dendritic cells (DC) and an array of secreted antimicrobial compounds [84]. Immune surveillance of the mucosal epithelium for microbial pathogens such as *C. trachomatis* relies largely on toll-like receptors (TLRs), which recognise pathogen-associated molecular patterns (PAMPs) present on the surface of chlamydial cells. TLRs are expressed by epithelial cells lining the urogenital and reproductive tracts, as well as by local DCs, macrophages, neutrophils and natural killer (NK) cells [82, 85]. Once activated, these immune cells begin to directly destroy the pathogen and increase their secretion of predominantly Th-1 associated cytokines such as interferon-gamma (IFN-γ) and interleukin (IL)-12, as well as IL-8, IL-10, IL-1β, and IL-6. Studies have shown that this localised proinflammatory response is elicited by IFN-γ and IL-12-stimulated $CD4^+$ T cells, which are believed to be necessary for both the resolution of the initial infection, as well as the development of a lasting resistance to re-infection [86, 87]. Parallel to this, a sufficient Th-2-associated response by IL-4 and IL-2-stimulated $CD4^+$ T cells is also necessary in order to prevent a cycle of destructive inflammation [88]. Furthermore, a balanced Th-1/Th-2 response is essential for the development of an enduring protective immune response. Evidence from murine infection models has

strengthened suggestions that the innate immune response alone is able to eliminate the pathogen in some models of infection [89], even in the absence TNF-α [90], but not others. The degree to which murine TNF-α is involved in the immune clearance of *Chlamydia* appears largely dependent upon both which type of mucosal tissue is infected, and by which species [citation]. This fact, as well as the key differences Th-1 and Th-2 immune pathways of mice and humans perhaps best highlights the incongruence between the genital infection models of both species [91]. Although it is apparent that the translational value of animal models may be somewhat diminished by the fundamental limitations of cross-species comparisons, they provide an invaluable and ongoing contribution to our understanding of the important factors involved in basic chlamydial biology, infection and the host response to the pathogen, is invaluable [92].

Adaptive immunity to chlamydia

The role of adaptive immune response in chlamydial infection is to develop, retain and elicit a targeted response which protects against reinfection. This process has been demonstrated to involve Th-1-associated cells like M2 macrophages and dendritic cells, which secrete IL-8, IL-10, IL-1β and IL-6 in order to recruit and activate naïve T cells and B cells [93, 94]. Following their migration along the chemotactic gradient to the site of infection, these Th-2-associated cells are presented with chlamydial peptide fragments by antigen-presenting cells (APC). This group of cells includes dendritic cells, macrophages and epithelial cells [95], all of which present the antigenic fragments on major-histocompatibility complex (MHC) class I & II receptors. Characterisation of this process has revealed the presentation of various chlamydial peptide fragments, many of which originate

from proteins found in the chlamydial outer membrane [96, 97]. Physical contact with the peptide-MHC complex of APCs by cells of the adaptive immune system triggers the activation of their capacity for direct cytotoxicity, antigen-memory, antibody production or further cytokine secretion. This enables the immune system to not only initiate and sustain a more targeted immune response against the current infection, but also initiate a more rapid response to future infections. It has been previously shown that in order to interrupt this process and increase its chances of survival, *C. trachomatis* is able to suppress the transcription factors essential for the production of MHC I & II molecules in its host cell [98, 99]. This in turn prevents antigenic-peptide presentation to $CD4^+$ and $CD8^+$ naïve T cells, thus the process is believed to be another chlamydial survival strategy. A summary of these adaptive immune components and its relationship with the innate defences against infection are shown in **Figure 1.5**.

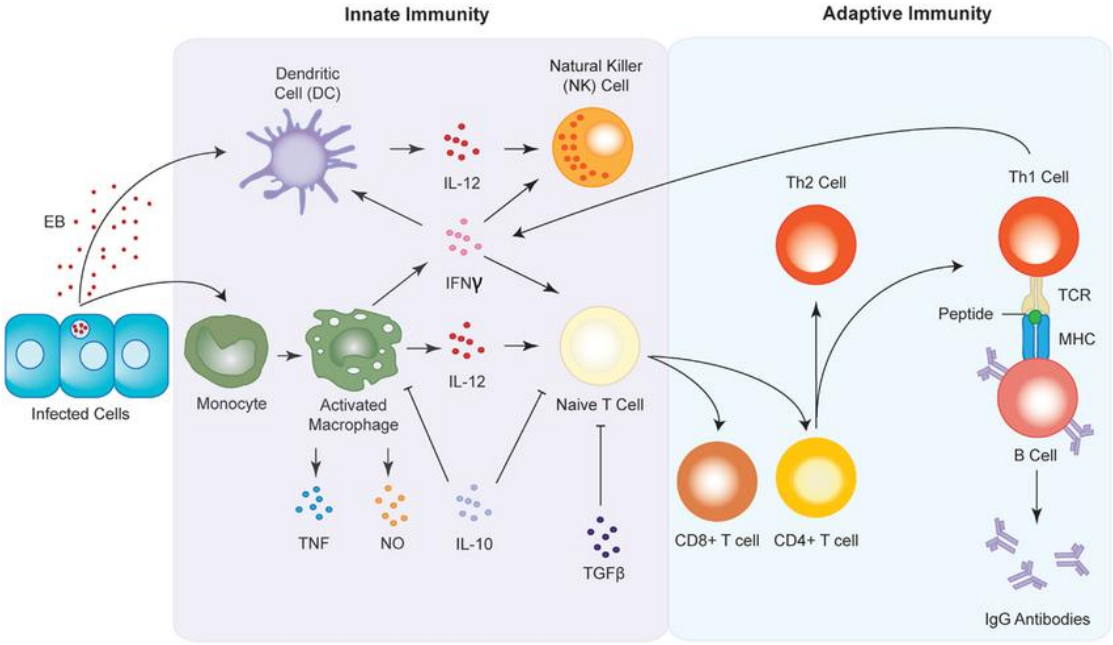


Figure 1.5. The innate and adaptive components of the host immune defence against chlamydial infection. EBs exit the infected host cells (in blue), marking

the end of the chlamydial developmental cycle. Of the EBs released into the surrounding microenvironment, some go on to infect a new, suitable nearby-host cell, while others are phagocytosed by professional APCs; which triggers them to release pro-inflammatory cytokines to recruit cells of the adaptive immune system. Proteolytic degradation of the EBs by APCs produces antigenic peptide fragments which are presented to the Th-2-associated cells which help to facilitate clearance of the infection and provide a lasting immunity to reinfection. Figure reproduced directly from [100].

Treatment of *C. trachomatis* infections of the urogenital tract

First-line treatment strategies

The established antibiotic treatment options for urogenital tract *C. trachomatis* infections either a single oral dose of azithromycin 1 g, or a seven-day course of doxycycline 100 mg taken orally, twice a day [101]. Azithromycin is prescribed in the majority of cases largely due to its convenience and increased likelihood of patient compliance [102]. Clinical studies and meta-analyses have estimated that azithromycin has a combined efficacy of 80–97% when used to treat either urogenital tract or rectal *C. trachomatis* infections [102-104]. Variations in its efficacy have been attributed to differences in the host cells at each anatomical site [105], hypoxic conditions [57], the host immune system [106, 107] and infectious load [108]. It has also been proposed that the microbiota, chlamydial persistence and heterotypic resistance may affect the outcome of treatment [109]. Collectively, the literature supports the suggestion that doxycycline, not azithromycin, should be the gold standard of antibiotic treatment for cases of *C. trachomatis* infections in Australia [110].

Chlamydial infections and doxycycline

Doxycycline is a tetracycline antibiotic and is indicated for treatment of urogenital and anorectal chlamydial infections [111]. Its antibiotic effects against *C. trachomatis* occur through its binding of the bacterial 30S ribosomal subunit, which turn inhibits protein synthesis by the organism [112]. Meta-analyses evaluating the efficacy of doxycycline to treat *C. trachomatis* infections suggest

that 100% of urogenital tract infections [113] and 99% of anorectal infections are resolved by treatment [114]. Thus, its outperformance of azithromycin in the treatment of anorectal infections has made it the preferred treatment of these cases.

Mechanism of action of azithromycin and resistance by Chlamydia

Azithromycin is a macrolide antibiotic with a broad spectrum of antibacterial activity. Its high degree of efficacy in treating *C. trachomatis* infections of the urogenital tract infections is a results of the antibiotic‟s relatively long (40 - 68 h) half-life, its high lipid solubility, and its distribution in serum; the latter of which confers a high degree of accumulation within macrophages, which facilitates the antibiotic‟s transport to the site of infection [115, 116]. The antibiotic exerts its bacteriostatic effects by reversibly binding the to the V domain of 23S ribosomal RNA (rRNA), thus preventing bacterial protein synthesis by the 50S ribosomal subunit [117]. Previously encountered mechanisms of bacterial resistance to macrolides have been shown to arise through either the acquisition of genes encoding active reflux of the drug, modifications to L4 ribosomal protein targets, point mutations in 23S rRNA-encoding genes, or methylation of a single adenine residue in the 23S rRNA V domain [118]. In Chlamydiae, however, mutations in both 23S rRNA-encoding gene copies, as well as L4 ribosomal protein-encoding genes, have been demonstrated to confer a drastic reduction in both the biological fitness and subsequent viability of laboratory-generated, macrolide-resistant strains [119-121]. Furthermore, studies consistently report the absence of genotypic or phenotypic macrolide resistance in clinical isolates after treatment with azithromycin [122-125]. The combined findings of *in vitro* and clinical studies which look at the effects of azithromycin on *C. trachomatis* suggest that its

survival in cases of treatment failure does not result from a classic microbial resistance strategy. Instead, it appears as though the pathogen withstands the effects of azithromycin by a unique process [124], one which is related to its constant interaction with its host cell [108, 126].

Part 2: The reproductive tract microbiome of women

The role of the female reproductive tract microbiome

Collectively, the vulva, vagina, cervix, endometrium, fallopian tubes and ovaries form the female reproductive tract (FRT). This unique anatomical region is populated by communities of predominantly non-pathogenic bacteria, some of which are found exclusively within this niche environment. Traditionally, research has focused on the relationship between microbial pathogens of the FRT and gynecological health and disease. In recent times, more attention has been given to the commensal bacteria which colonise the vagina and how they are able to prevent infection by opportunistic and professional pathogens [127]. This process and some of the important contributing factors are described by **Figure 1.6**. Recent improvements in sequencing technologies and data analysis have allowed faster and more efficient identification of the organisms present at anatomical sites throughout the human body [128]. PCR amplification and deep sequencing of bacterial 16S ribosomal RNA (rRNA) genes has highlighted the broad diversity of the bacterial communities within the FRT [129, 130]. Although many genera and species of bacteria have been found to inhabit the vagina, these dynamic communities are most commonly classified based on the most prevalent bacterial species identified [131]. The five community state types (CSTI-V) revealed using 16S rRNA sequencing are all characterised by a predominance of *Lactobacillus*

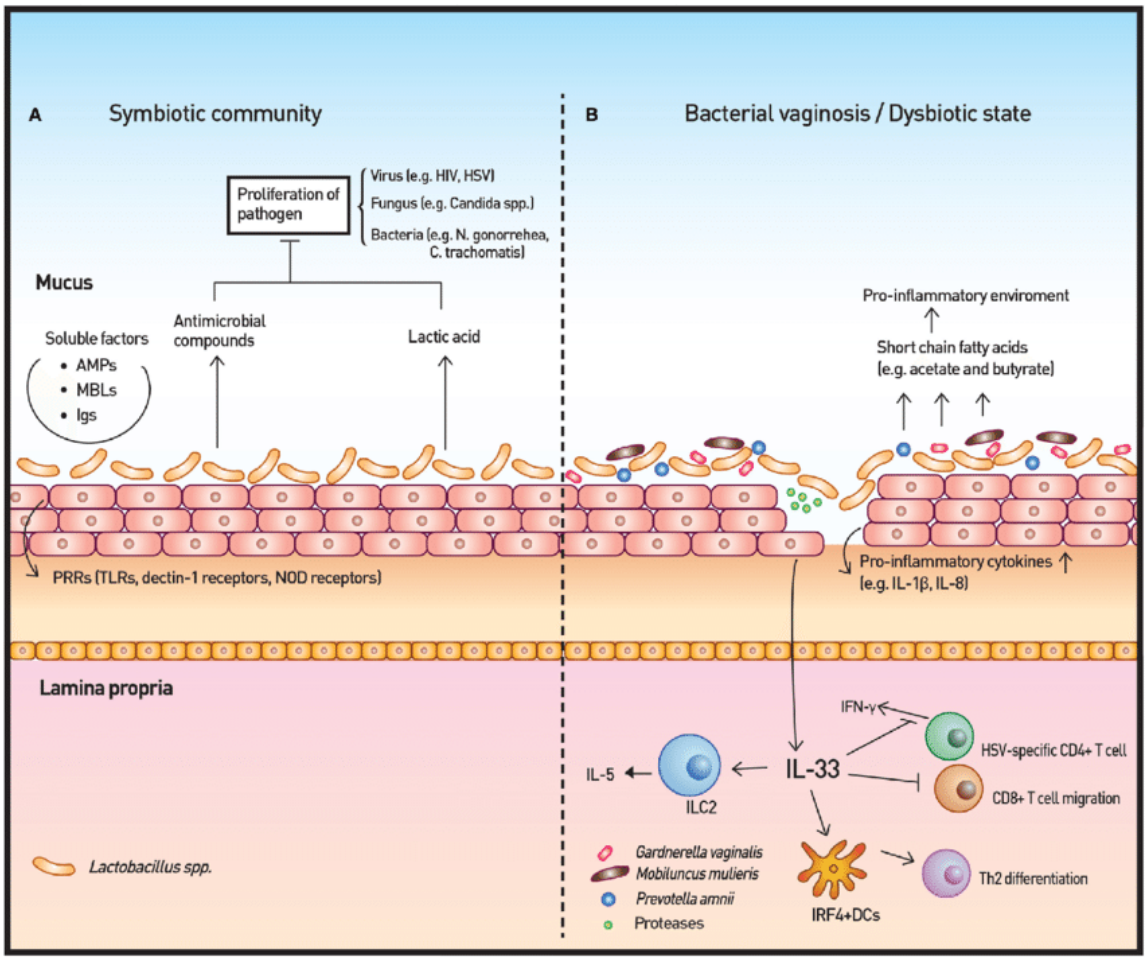


Figure 1.6. The role of the vaginal microbiota in gynecological health and dysbiosis. Symbiotic bacteria, microbial pathogens and the mucosal surface of the vagina are each important determinants of gynecological health. (A) The relationship between the bacterial and human cells of a healthy vaginal mucosa. Several different species of beneficial lactobacilli have been shown to promote health in the epithelial layer of the vagina. Through symbiosis, the growth of these beneficial bacteria helps to create and maintain a stable environment. This not only favours their continued growth, but also helps to suppress or eliminate some of the pathogenic microbes associated with vaginal dysbiosis. (B) Shifts and changes in the abundance of these protective bacteria, damage to the mucosal epithelium and changes in both localised and general immunity are all believed to be factors which can contribute to bacterial vaginosis, as well as other gynecological dysbioses. Figure directly reproduced from [132].

spp., except for CSTIV, which has been described as a heterogeneous mixture of species [133].

Pathologies attributed to shifts in microbial populations

All host-microbiome relationships are complex; which makes it unclear whether shifts in a community cause – or are a result of – gynecological pathologies. Bacterial vaginosis (BV) is a relatively common disease of the FRT characterised by vaginal odour and discharge [134]. While the causative agent of BV is unclear, it has been shown that patients with CST I, II, III and V during good health almost always have heterogeneous communities with a decreased relative abundance of the predominant *Lactobacillus* species during BV [135-137]. This apparent decrease in the relative abundance of lactobacilli has been largely attributed to a significant increase in the diversity and relative abundance of other bacterial species in the vagina [138-140]. In conjunction with this, patients with BV have been shown to be at an increased risk of sexually transmitted pathogens such as *C. trachomatis*, *Neisseria gonorrhoeae*, Herpes simplex virus 2 (HSV-2), and Human immunodeficiency virus 1 (HIV) [141-145]. Increases in the presence of these pathogens in the vagina have been attributed to a spectrum of BV-associated sequelae including cervicitis, endometritis, salpingitis, tubal factor infertility (TFI), and pelvic inflammatory disease (PID) [144, 146, 147]. The vaginal microbiome is especially important during pregnancy and appears to influence gestational outcomes. Evidence has shown that the rate of preterm birth is significantly increased in patients with BV; as well as those with abnormal microbial diversity, composition, and stability [148-151]. Once again, lactobacilli appear to play an important role, with a study by Relman *et al.* (2015) [152] noting

a significantly higher rate of preterm birth in subjects with CSTIV – the only CST in which lactobacilli are not the predominant bacteria.

The human vaginal microbiome and *C. trachomatis*

Commensal bacteria are those which can typically be found on the epithelial surfaces of various tissues throughout the human body. Importantly, they exist at these sites despite the absence of pathology or an overt immune response to their presence at that particular location [153, 154]. The duration and frequency of asymptomatic *C. trachomatis* urogenital tract infections are well established (reviewed in [155]). This has caused some discussion about whether *C. trachomatis* is in fact a commensal, opportunistically pathogenic bacterium, rather than a professional pathogen of the urogenital system [156]. While it should be stressed these ideas do not reflect the current consensus in the field, it provides several interesting avenues for discussing the pathogen in the context of treatment failure, the relationship between the host immune system and reproductive tract microbiome, and even vaccination [157, 158]. While the link between experimentally defined microbial populations and chlamydial infection outcomes is not fully understood, recent studies have identified an association between the presence of the pathogen and some community state types (CSTs) of the vagina [159, 160]. Specifically, women with vaginal CSTs III and IV and *Lactobacillus iners* were found be at a higher risk of chlamydial infection. A recent review by Molenaar and colleagues examining the evidence from studies involving *C. trachomatis* in the context of the vaginal microbiome emphasised the importance of lactobacilli, hormonal homeostasis and oral contraceptives in the pathogenesis of chlamydial infections [161]. As each of these has in turn been associated with

the immune system and inflammatory processes, it again highlights the interplay between these important host and microbial factors. The mechanisms proposed by this review are summarised below in **Figure 1.7**.

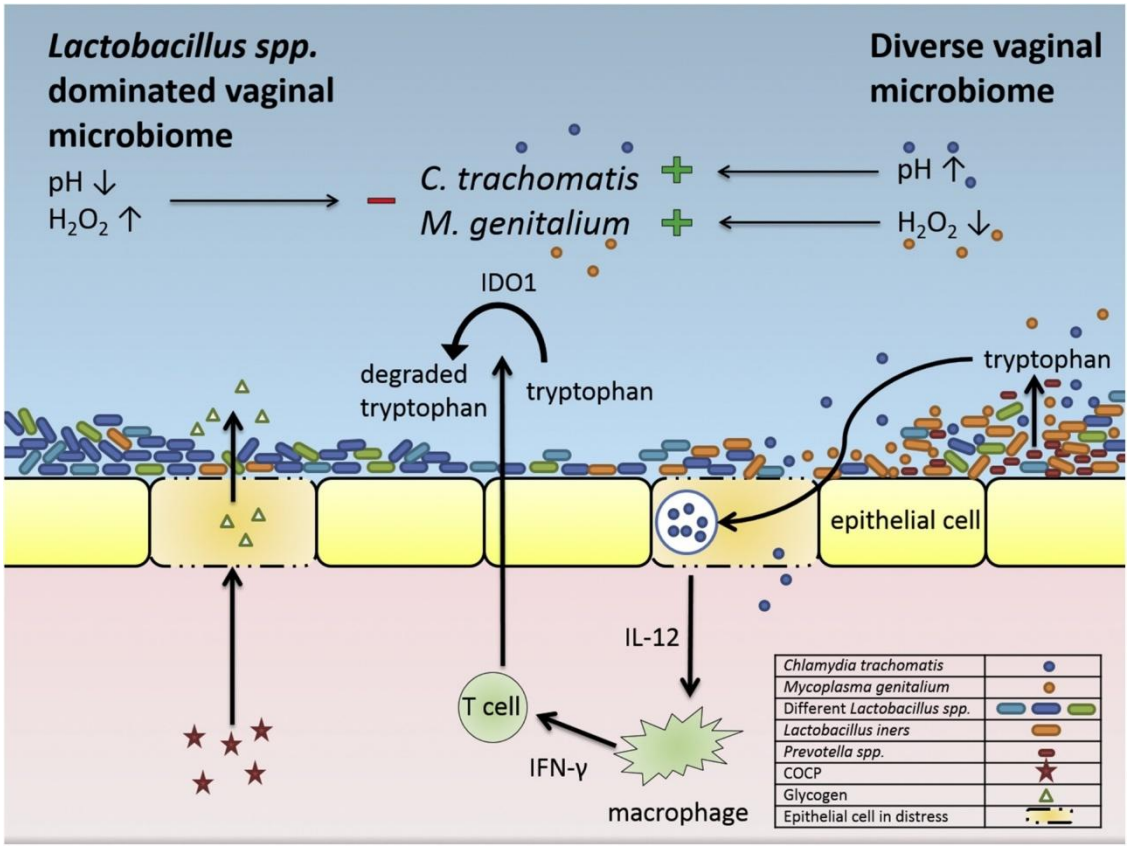


Figure 1.7. An overview of the relationship between diverse microbial populations and the immunopathology associated with intracellular pathogens such as *C. trachomatis* and *Mycoplasma genitalium*. These bacteria are frequently identified in and isolated from patients who remain asymptomatic. It is believed that this arises from homeostasis of the microenvironment. Changes in some of these contributing factors, such as shifts in the population or dominant species have been shown to shift this balance, which in turn may result in the pathology associated with the presence of *C. trachomatis* and other similar pathogens of the urogenital tract. Figure directly reproduced from [161].

Part 3: Scope and purpose of the project

Research Problem

While *C. trachomatis* infections of the urogenital tract are readily treatable with azithromycin, a small number of patients experience treatment failure – which has been associated with an increased risk of infertility. While the mechanisms of antimicrobial resistance in other bacteria are well established, findings from previous studies collectively suggest that *C. trachomatis* does not have the capacity for developing or acquiring the genetic components required for resistance to macrolide antibiotics such as azithromycin. Instead, it appears that in the cases of treatment failure which are known to occur, the pathogen survives the effects of the antibiotic through a complex combination of host and pathogen factors – which remain poorly understood. Reflecting this, the research problem outlined in this project was addressed by several different questions and experimental studies. Ultimately, this study will provide a better understanding of the roles that the host immune system, commensal micro-organisms, and *C. trachomatis* have in treatment failure and its sequelae.

Overview of the research project

This project included three stages of investigation to better understand the components of chlamydial infertility and treatment failure. Specifically, I

investigated the microbiome and host immune response of the female reproductive tract, clinical isolates of *C. trachomatis* associated with treatment failure, and gene expression of both the host and *C. trachomatis* during treatment. The initial focus was to examine how the microbial community composition of the vagina, cervix and endometrium was associated with both the host immune response and fertility status of women. To better understand the chlamydial factors involved in treatment failure, I examine several clinical isolates of the pathogen obtained from women treated for chlamydial infections, some of whom experienced treatment failure. Finally, I looked at the relationship between host and chlamydial gene expression in a separate subselection of women from the same cohort study, in order to assess their association with the participant's treatment outcomes. Combined, the findings from each component of this research project advance knowledge in how these host and microbial factors contribute towards chlamydial treatment failure and infertility in women.

CHAPTER 2: GENERAL METHODS AND MATERIALS

Part 1. Methods

General research methodology

Ethical approval

All research was conducted in accordance with the Australian Code for the Responsible Conduct of Research and the National Statement on Ethical Research in Human Research. Applications were submitted and approved by the respective Human Research Ethical Committees (HREC) prior to any of the applicable research being conducted. The committees to which the applications were submitted as well as the ethics approval numbers provided to facilitate the research are stated in the methods section of each data chapter.

Statistical analysis of research data

A variety of statistical tests and comparisons were conducting of the data generated in this research project. These were performed using either GraphPad or IBM SPSS software packages depending on the type of data and analysis required. The exact software packages, as well as descriptions of the tests and parameters used, are stated in the methods section of each data chapter.

Australian Chlamydia Treatment Study

Several of the studies described in this research project were conducted using clinical isolates, biological samples and participant data, accessed through collaboration with investigators of the Australian Chlamydia Treatment Study

(ACTS). A complete description of the ACTS design has been published by the investigators [162]. This longitudinal cohort study investigated the primary treatment outcomes of women attending one of two publicly funded sexual health clinics in Sydney and Melbourne. Strict eligibility criteria were used to recruit women who had tested positive for chlamydia by either nucleic acid amplification testing (NAAT) or a strand displacement assay at either of the sexual health clinics. Following their recruitment to the study by a nurse-administered questionnaire, a cervical swab specimen (day 0, pre-treatment) was collected by the attending health care professional. Participants received a 1 g dose of azithromycin to treat infection and returned after seven days so that a second specimen (day 7, post-treatment) could be collected. Weekly self-collected high-vaginal swabs and a diary of sexual activity were provided by each participant for the remainder of the 56 days. A summary of the specimens collected, and their intended uses has been adapted from the ACTS publication and is shown in **Table 2.1**. These included PCR, mass spectrometry testing of the azithromycin concentration in the cervical epithelium, and *ompA*-typing of any chlamydial strains detected after treatment. The primary treatment outcomes of ACTS participants were determined by the authors using an algorithm which incorporated the test of cure results, genotyping, azithromycin absorption, and sexual compliance data of each participant. Participant outcomes were defined as treatment success (clearance), new infection (different genotype), reinfection (sexual non-compliance) or treatment failure (PCR positive; sufficient absorption of azithromycin confirmed; same genotype; sexual compliance).

Mammalian cell culture

Cell culture conditions

All cell culture experiments were conducted in a PC2 cell culture facility. All users were trained to inspect and observe both the morphology and media of cultures, to ensure cells were exhibiting typical growth, free from microbial contamination. Cultures were routinely tested every three months for contamination by commonly encountered species of *Mycoplasma* (see "Testing of cell lines for *Mycoplasma*" section in this chapter for full description). Class-II biosafety cabinets (BSC) were fumigated biannually in accordance with the manufacturer's guidelines in addition to routine cleaning and between-use sterilisation by Virkon, 70% ethanol, and UV light. Equipment and reagents used for cell culture were confined to the room and maintained by all users. The regularity with which cell culture experiments were conducted in the lab often necessitated continual passage of several mammalian cell lines, for up to 3 months at any given time. Different cell lines were maintained in isolation, to avoid cross-contamination of cultures. Steps taken included the cleaning and sterilisation of biosafety cabinet equipment between cell lines, as well as the routine, sterile pre-measurement of cell-culture reagents, such as growth media and DPBS. Established cultures were isolated from *Chlamydia*-infected cultures in a separate incubator, and where possible, individual cell lines were stored in specific incubators, especially HeLa cells.

Table 2.1. Details and testing of swab specimens collected from ACTS participants. Adapted from [162].

Day	0	7	14	21	28	35	42	49	56	If positive at day 28, 42 or 56
Number of swabs collected	4	2	2	2	2	2	2	2	2	4-5
Culture	X									X
PCR	X	X	X	X		X		X		X
Chlamydia organism load	X	X	X	X	X	X	X	X	X	X
Genotype	X	X	X	X	X	X	X	X	X	X
Sequencing	X	X	X	X	X	X	X	X	X	X
β-globin	X	X	X	X	X	X	X	X	X	X
Test of cure					X		X		X	
Azithromycin absorption		X								
Blood	X									X
Rectal swab										X
Location (Clinic/Home)	C	C	H	H	H	H	H	H	H	C

Testing of cell lines for Mycoplasma contamination

In accordance with facility regulations and GLP best practices for cell culture, cells in our lab were routinely subjected to mandatory in-house testing for contamination with *Mycoplasma* species. Testing was performed using the Lonza MycoAlert™ Mycoplasma Detection Kit, in accordance with the manufacturer's instructions. This required each cell line being used since the last round of testing to be concomitantly cultured in a separate 75 cm^2 culture flask, with antibiotic-free

growth media, for two weeks. No more than 24 h prior to testing, each cleanroom user performed a final passage of their respective cultures, with the addition of steps that allowed for the collection of two different samples for submission. One of these samples was a cell-free fraction of used growth media collected immediately prior to passage, while the other contained both media and cells from the culture. Samples were stored for no longer than 24 h at 4˚C and cultures were only discarded after a clear result was indicated. All of the samples provided by our group were negative for contamination using this testing method.

Independent testing of cell lines for **Mycoplasma** *contamination*

In addition to the routine testing performed by the facility, members of the research group also used an independent, ad hoc testing method to rule out mycoplasmal contamination. This was conducted using a conventional PCR method, originally designed and published by Young and colleagues in 2010 [163], to amplify a target sequence unique to the 16S rRNA gene of *Mycoplasma* spp. Most of the samples tested using this method originated from adherent cell lines, cultured in 75cm^2 flasks under typical incubation conditions (37˚C, 5% CO_2 and 95% humidified air), which exhibited atypical growth or cell morphologies during examination by light microscopy. Collection of the cultured cells for testing began with the removal of expired growth media from the flask, followed by several gentle washes with DPBS 1X. Immediately after the final wash, 1.5ml of 2X trypsin was added to each monolayer. Each flask was then incubated under standard conditions for around five minutes, or until detachment and separation of the trypsinised cells was observed. The entire volume of each sample was then transferred onto fresh, sterile 15 ml Falcon tubes. Total DNA was extracted and

purified from each sample using the DNeasy Blood and Tissue Kit (QIAGEN), as specified in the kit instructions. Conventional PCR was then performed, using the details in **Table 2.2**, to amplify any mycoplasmal DNA present in the samples. The products of PCR were separated using agarose gel electrophoresis (1.5% agarose in TBE; 100V for 60 minutes), then examined under UV light. Contamination with *Mycoplasma* was indicated by the presence of a 270 bp amplicon amplicon alongside the appropriate experimental controls.

Table 2.2. Details of the PCR used to perform ad hoc testing of cell lines for contamination with *Mycoplasma* spp.

Reaction Mixture

Component	Volume (µl)	Reagent
PCR Mastermix	25	Amplitaq Gold 360 (2X)
Forward primer (10 µM)	1.5	GGGAGCAAACAGGATTAGATACCCT[1]
Reverse primer (10 µM)	1.5	TGCACCATCTGTCACTCTGTTAACCTC[1]
Water	17	dH_2O
Template	5	DNA purified from cultured cells
Total	50 µl	

Cycling Conditions

Temperature	Time	
95 ˚C	5 min	Taq activation
94 ˚C	30 sec	
55 ˚C	30 sec	40 cycles
72 ˚C	30 sec	
72 ˚C	10 min	Final extension
4 ˚C	∞	Final hold

[1]Oligonucleotide sequences from the 2010 publication by Young and colleagues [163].

Establishing growth of immortalised mammalian cell lines

Immortalised mammalian cell lines were resuscitated from storage in liquid nitrogen and maintained for routine use. Working in a class II biosafety cabinet, cells in 2 ml cryovials were thawed and slowly transferred to a 50 ml sterile centrifuge tube containing 10 ml of pre-warmed (37˚C) growth media. The cell and growth media solutions were gently mixed before centrifugation at 500 × *g* and 28˚C for 5 min. Following the removal and disposal, of the supernatant, pelleted cells were *gently* resuspended in 15 ml of pre-warmed growth media and transferred to 75 cm^2 tissue culture flasks. These were transferred to clean incubators and grown at 37˚C with 5% CO_2 and 95% humidified air. The morphology and growth media of newly resuscitated cell lines were inspected every 24 h until they achieved confluency.

Maintenance of established mammalian cell lines

The general appearance and health of cultured cells were monitored regularly by visual inspection. Additionally, routine assessments of cell morphology and culture confluence were conducted using phase-contrast light microscopy. As cultures approached or achieved 100% confluency, they underwent passage into one or more fresh, sterile 75 cm^2 tissue culture flasks. Passage began with the disposal of expired growth media into a discard bottle containing a bleach solution (12.5% NaOCl). The monolayer of each flask was gently washed twice with 10 – 15 ml of warm, sterile DPBS 1X, to remove any remaining growth media. Immediately after the second wash, 2 ml of pre-warmed 2X trypsin was evenly distributed across each monolayer. Flasks were re-sealed and incubated at 37˚C with 5% CO_2 and 95% humidified air for 5 min, to facilitate detachment of the monolayer and begin

separation of the cells. Once detachment was confirmed by phase-contrast light microscopy, 10 ml of fresh growth media was added to each flask to neutralise the trypsin and prevent excessive proteolysis of the cell surface receptors. Repeated, gentle aspiration with a serological pipettor was used to further separate and mix the separated cells in growth media. Passage of cells for next-day use was consistently achieved by combining up to half of the resuspended cells with fresh, warmed growth media, to a total volume of 12 ml, and transferring the diluted cells into a fresh, sterile tissue culture flask prior to incubation for 24 hours at 37˚C with 5% CO_2 and 95% humidified air.

Cell counting and plate culture

Cell culture experiments were routinely conducted using multi-well tissue culture plates. This required seeding each well with a specified number of cells for an evenly distributed monolayer. To achieve this, cells cultured in 75 cm^2 tissue culture flasks underwent passage by the regular method, to the step of resuspension in fresh media. The cell suspension was transferred to a fresh, sterile 50 ml centrifuge tube and aspirated to ensure an even distribution of suspended cells. Following this, 10 µl was removed and placed in a sterile 1.5 ml Eppendorf tube. An equal volume of trypan blue dye solution (0.4% in 1X DPBS) was added to the cell sample, which was gently mixed by pipette aspiration as little as possible – to preserve the viability of cells in the dye mix. A 10 µl aliquot was transferred to a Bright-Line™ haemocytometer with a coverslip, and the numbers of cells in each of the four counting grids was determined. This was used in calculating the concentration of cells in the 50 ml centrifuge tube, as well as the volume required to achieve the desired seeding concentration. The suspension was

gently agitated, and the required volume was transferred to another fresh, sterile centrifuge tube. Fresh growth media was added to the new centrifuge tube to achieve the desired seeding volume, and the diluted cells were mixed by inversion. The appropriate volumes for seeding were added to the wells of each plate, which were then gently rocked once in each direction for even distribution of the cells across the surface of the wells. Experiments requiring cells to be grown on coverslips necessitated the addition of sterile No. 1.5, 13 mm glass coverslips to the well prior to seeding the cells. Tissue culture plates seeded with cells were rested for 30 min on a completely flat surface, before being transferred to an incubator to develop at 37˚C with 5% CO_2 and 95% humidified air.

Bacterial culture

Isolation, expansion and semi-purification of Chlamydia

Clinical isolates of *C. trachomatis* were initially propagated from the cervical swab samples of ACTS participants by Amba Lawrence. To obtain a sufficient amount of infectious EBs, each of the clinical isolates were isolated, then expanded by successive rounds of cultivation. The isolation process began by thawing cervical swabs preserved in SPG from -80˚C to room temperature, on ice. Sterile 1mm silica beads were then added to each sample and each tube was vortexed (40 sec) to resuspend the swab material. 150µl of SPG from each sample was used to inoculate 80%-confluent monolayers of McCoy B cells cultured in six-well plates. Infected cultures were centrifuged at 500 × *g* and 28˚C for 30 min, and incubated at 37˚C with 5% CO_2 and 95% humidified air. The media of each infected well was replaced at 4 h PI with standard growth media supplemented with 1 µg ml^{-1}

cycloheximide. Cultures were routinely monitored to assess their general appearance, overgrowth of microbial contaminants, and both the presence and development of each clinical isolate. Successfully infected cultures were further propagated using a similar process, in which they were lysed and homogenised in fresh SPG and used in further propagation. When clinical isolates propagated this way were able to successfully infect most of the monolayer using six-well plates, their cultivation was scaled up to allow expansion. To achieve this, for each of the clinical isolates being expanded, 12 ml of pre-warmed growth media was spiked with enough crude chlamydial stock to produce a significant number of infected cells in a 75 cm^2 monolayer. Fully confluent monolayers of McCoy B cells cultured in 75 cm^2 tissue culture flasks were then inoculated with the infectious media, centrifuged at 500 × *g* and 28˚C for 30 min, and incubated at 37˚C with 5% CO_2 and 95% humidified air. At 4 h PI, the growth media of each infected flask was replaced with 12 ml of fresh, pre-warmed growth media, supplemented with 1 µg ml^{-1} cycloheximide. The *C. trachomatis*-infected cultures were incubated under the same conditions as before until approximately 44 h PI. With UV sterilisation of the BSC and culture equipment performed between each clinical isolate, each of the cultures were then harvested and semi-purified in order to produce multiple low-volume, high-concentration chlamydial stocks. This was achieved by removing the expired media from each flask and adding up to 5 ml of ice-cold SPG storage buffer directly to the monolayer. A cell scraper was used to detach the monolayer into the buffer, which was then transferred directly to the next flask (after removing its expired growth media). This was repeated until all cultures of that particular isolate had been harvested into the buffer. The SPG-cell solution was then transferred to a fresh, sterile 50 ml centrifuge tube containing 15 ml of sterile,

1 mm silica beads. This was then vortexed for two minutes on the highest setting, to sufficiently lyse the host cells and release the chlamydial particles. A sterile transfer pipette was used to aspirate the lysate from the silica beads into a fresh, sterile 50 ml centrifuge tube. Cellular debris was collected by centrifugation at 500 × *g* and 1˚C for 5 min, after which the semi-purified supernatant was collected in a fresh tube and aliquoted into sterile cryovials. Aliquots of semi-purified infectious EBs were stored at¯80˚C until required.

Infection of mammalian cell lines with C. trachomatis

Cells were routinely grown to near-confluency, in tissue culture flasks and plates, for the purpose of *in vitro* infection experiments. Because several clinical and type strains of *C. trachomatis* were routinely used, handling procedures to avoid cross-contamination of stocks were followed as required. UV and chemical sterilisation of the BSC and cell culture equipment were performed between experiments using single strains of *C. trachomatis*, especially when expansion of strain stocks was undertaken. Where multiple strains were necessary for experiments, any incompletely-used stocks were discarded after use. Propagation of *C. trachomatis* was performed in several mammalian cell lines described in this thesis. This process began by retrieving chlamydial stocks with pre-determined concentrations of infectious particles from storage at -80˚C. Following thawing, chlamydial stocks were diluted in measured amounts of fresh, pre-warmed growth media, to the concentration of infectious EBs determined during the experimental design stage. After removing the expired media from each culture, they were overlaid with the infectious growth media. The necks and lids of tissue culture flasks and plates were sealed with parafilm in the BSC before centrifugation at 500 × *g* for 30 min

at 28 ˚C. After allowing any potential aerosols to settle, infected cultures were transferred from the centrifuge to an incubator set to 37˚C with 5% CO_2 and 95% humidified air. At 4 hours post infection (h PI), the infectious media of each culture was replaced with fresh, sterile, pre-warmed growth media supplemented with 1 μg ml^{-1} cycloheximide. Infectious cultures were further incubated under the same conditions for as long as was necessary for each experiment.

Microscopy

Immunofluorescence microscopy

Cultured cells infected with *C. trachomatis* were fixed for staining by first removing the infectious media from each well. Wells were then washed with 1X DPBS, filled with 96% methanol for 10 min, and washed with 1X DPBS twice more after removal of the fixative. Methanol-fixed monolayers were permeabilised with triton X-100 (0.5%) in 1X DPBS, and non-specific binding sites were blocked using bovine serum albumin (BSA; 1%) dissolved in 1X DPBS overnight. Once blocked, cultures were incubated for 1 h at room temperature with a primary antibody solution made by adding both 4‟,6-diamino-2-phenyindole (DAPI; 1/40,000) and rabbit sera (1/500) to 1X DPBS. The rabbit sera used contained antibodies raised against purified *C. trachomatis* high-temperature requirement A (HtrA) protein. The primary antibody solution was removed, and cultures were filled and emptied four times with a wash buffer (0.2% tween 20 in 1X DPBS). A secondary antibody solution (Alexa Fluor 488-conjugated goat anti-rabbit antibody (1/600; Thermo Fisher Scientific) in 1X DPBS) was added to each well and the plates were incubated for a further 45 minutes at room temperature. Non-specific

and unbound antibody was removed by washing each well another five times with wash buffer, and enough 1X DPBS was added to the wells to form a shallow, protective layer of hydration over the fixed, stained and labeled cells. Plates were either viewed the same day by fluorescence microscopy or were stored away from light at 4˚C and imaged within several days.

Quantifying infected cells

Fixed cultures treated with fluorescent antibodies and stains were visualised using a GE IN Cell 2200 high-throughput fluorescent microscope and its accompanying software. After a manual inspection of the wells, an automated protocol was used to capture ten computer-randomised fields of view from each well, in both the FITC and DAPI channels of the microscope‟s 10x objective profile. Where possible, the software was also used to count the number of "cells" (chlamydial inclusions) in real-time from the FITC channel of captured images. The inclusion counts of each well were then averaged across the nine technical replicates and used to calculate the number of inclusion-forming units per ml (IFU ml^{-1}) obtained in each of the experimental culture conditions.

Confocal imaging of Chlamydia-infected cultures

Cultures on 13 mm (No. 1.5) coverslips were stained and labelled using the same process for methanol-fixed cultures, with the addition of a mouse anti-α-tubulin (1/500; Thermo Fisher Scientific) in the primary antibody solution, and an Alexa Fluor 546-conjugated goat anti-mouse antibody (1/600; Thermo Fisher Scientific) in the secondary antibody solution. Stained coverslips were mounted to clear glass slides using *n*-propyl gallate (NPG) and sealed using clear varnish around the edge of the coverslip. Slides were stored away from light and visualised using the Nikon

Eclipse Ti-S Confocal Microscope and the Nikon Elements imaging software. Using the 60x oil-immersion objective, cultures were manually brought into focus and visually inspected for host and chlamydial cell morphologies and abundance – typically in the GFP (chlamydial inclusions) and TRITC (host-cell α-tubulin) channels. Once the correct focal plane (middle of the host cells), a representative image was obtained by capturing a z-stack of ten images in the GFP/DAPI/TRITC channels to form a composite image at the z-position which provided the most detail in focus.

Nucleic acid analysis

DNA and RNA extractions

All nucleic acids were extracted and purified using commercial assay kits, in accordance with the manufacturer‟s instructions. DNA was extracted in a clean area of the general lab after cleaning all surfaces and instruments with 70% ethanol and wiping down with paper towel. RNA extractions were performed in a class II BSC cleaned with 70% ethanol and RNase-Zap™ to mitigate contamination and degradation of the samples by RNases. Elution of all nucleic acids was always performed using ultrapure dH_2O unless a second elution sample was required as a backup. Nucleic acids were all stored long-term at -80˚C, short-term at -20˚C, or used as aliquots at 4˚C.

Polymerase chain reactions (PCR)

Standard and some quantitative PCR reactions were conducted using 20-50 µl sample volumes in sterile 0.2 ml flat/dome cap clear 8-tube strips. The majority of qPCR reactions were performed using the QIAGEN 0.1 ml tubes designed to fit the

72-well rotor of the RotorGene Q quantitative thermocycler. All reactions used 1X AmpliTaq Gold® 360 Master Mix, but the final concentrations of dH_2O, sense and antisense nucleotides, SYBR Green I dye, and target sequence varied between applications. Assays intended for qPCR were first tested on a conventional platform using identical reaction mixes, as SYBR green dye has been previously shown to have very low-level inhibition of polymerase activity at higher concentrations [164]. The basic thermocycling protocol included steps for denaturation of the Taq-antibody complex (95˚C, 10 min) then 25-35 cycles of amplification (95˚C, 30 s; 55˚C, 30 s; 72˚C, 30 s) and a final elongation (72˚C, 7 min) to ensure separation of the polymerase and the amplicons. qPCR included a capture of fluorescence in the green (FITC) channel during the extension steps of amplification cycles. The products of conventional PCR assays were visualised by agarose gel electrophoresis.

Agarose gel electrophoresis

Agarose gels were prepared for electrophoresis by fully dissolving molecular-grade agarose powder (1-2% w/v) in tris-borate-EDTA buffer until the solution appeared clear. Ethidium bromide dye was added to a final concentration of 1:50,000. Gels were allowed to set completely at room temperature using the Owl Horizontal EasyCast™ system (Thermo Fisher Scientific) before being placed in an empty and clean electrophoresis tank. TBE buffer was gently poured into the tank until the gel was completely submerged, ensuring no air bubbles were under the gel tray. A volume (usually 10 µl unless otherwise specified) of each PCR product was transferred into a fresh tube or onto a parafilm-coated surface before being was mixed with a 6X DNA loading buffer. This contained bromophenol blue

and xylene cyanole dyes for visualization during electrophoresis, and glycerol to weigh-down DNA in the sample. After gentle mixing, the entire volume of each sample was carefully loaded onto a lane of the gel. Electrophoresis at 100 V for 1 h was typically used to resolve the products, which were then visualised by exposing the gel to ultraviolet light in a GelDoc camera cabinet, using the autoexposure feature.

Part 2. Materials

Buffers, solutions and chemicals

Name	**Composition**
2X Trypsin	2X working stock solution of trypsin-EDTA in sterile DPBS.
6X DNA loading buffer	0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 30% v/v glycerol in dH_2O.
Azithromycin	100 mg ml^{-1} aliquots.
Bleach	12.5% v/v sodium hypochlorite in water.
Blocking buffer	1% w/v bovine serum albumin in DPBS.
β-mercaptoethanol	10X stock.
DPBS	2.67 mM KCl, 1.47 mM KH_2PO_4, 136.9 mM NaCl, 8.1 mM Na_2HPO_4; pH 7.1-7.4.
n-propyl gallate	80% v/v glycerol, 4% w/v *n*-propyl-gallate, 0.02 M Tris; pH 7.4.
Penicillin	100 KU ml^{-1} aliquots.
Permeabilisation buffer	0.5% Triton-X100 in DPBS.
Sodium dodecyl sulfate (SDS)	10X stock solution.
SPG buffer	5 mM glutamic acid, 10 mM sodium phosphate, 250 mM sucrose; pH 7.4.
Tris-borate-EDTA (TBE) buffer	89 mM Tris base, 89 mM boric acid, 2mM ethylenediaminetetraacetic acid; pH 8.0.
Wash buffer	0.2% v/v Tween-20 in DPBS.

Mammalian cell lines and *C. trachomatis* isolates

Cell line	Description
McCoy B	Murine fibroblasts (ATCC® CRL-1696™) were routinely used in the propagation, expansion, quantification and growth and infectivity comparisons of C. *trachomatis* isolates.
HEp-2	Human epithelial cell line (ATCC® CCL-23™) originating from a historical HeLa cell contamination. Used in growth and infectivity comparisons of *C. trachomatis* isolates.
HeLa	Widely-distributed human cervical epithelial cell line (ATCC® CCL-2™) originally sourced from Henrietta Lacks in 1951 – without informed consent. HeLa cells were used to compare the growth and infectivity of *C. trachomatis* isolates.
MCF-7	Human mammary epithelial cell (ATCC® HTB-22™) used in chlamydial development and stress response experiments. While the suitability of this cell line for chlamydial culture is well documented [98, 165-168], it was originally and has always been used by our lab under the belief it was the ECC-1 endocervical carcinoma cell line (ATCC® CRL-2923™) previously obtained from the ATCC. External testing during the final stages of this project determined this was not the case. As our lab has never purchased or handled this cell line, the reasons for this discovery remain unclear.
Caco-2	Human colorectal carcinoma cell (ATCC® HTB-37™) used to compare the growth and infectivity of *C. trachomatis* isolates.
ARPE-19	Adult human retinal epithelial cell (ATCC® CRL-2302™) used to compare the growth and infectivity of *C. trachomatis* isolates.
SiHa	Human cervical epithelial cell (ATCC® HTB-37™) used to compare the growth and infectivity of *C. trachomatis* isolates.

D/UW-3/Cx	Widely studied D serovar lymphogranuloma venereum (LGV) strain of *C. trachomatis* (ATCC® VR-885D™). D/UW-3/Cx was used as a positive control for chlamydial growth and development in several experiments.
1-017-1	Serovar K clinical isolate of *C. trachomatis* originating from a participant in the Australian Chlamydia Treatment Study (ACTS) who experience treatment failure. 1-017-1 was isolated from an endocervical swab provided prior to treatment.
1-017-13	1-017-13 is a clinical isolate of *C. trachomatis* originating from the same ACTS participants as 1-017-1 but was isolated from the swab provided after treatment failure was determined to have occurred.
1-020-1	A serovar D clinical isolate of *C. trachomatis* isolated from the endocervical swab of an ACTS participant whose infection was resolved by antibiotics.
1-028-1	A serovar E clinical isolate of *C. trachomatis* cultivated from the endocervical swab of an ACTS participants whose infection was resolved by antibiotics.
1-049-1	A serovar K clinical isolate of *C. trachomatis* from the swab of an ACTS participant whose infection was resolved by antibiotics.

Antibodies

Description	**Composition and origin**
Primary chlamydial antibody	Whole fractionated rabbit serum containing antibodies raised against the HtrA protein of *C. trachomatis* (South Australian Health and Medical Research Institute (SAHMRI); Adelaide, Australia).

Secondary antibody	Invitrogen Goat-anti-Rabbit IgG (H&L), conjugated with Alexa Fluor® 488 dye (Thermo Fisher Scientific; MA, USA).
Tubulin antibody	Mouse anti-α-tubulin antibody produced in mouse (Sigma-Aldrich Co. LLC; MO, USA).
Anti-tubulin antibody	Invitrogen Goat-anti-Mouse IgG (H&L) conjugated with Alexa Fluor® 546 dye (Thermo Fisher Scientific; MA, USA).

Proprietary kits

Use	**Description and supplier**
DNA isolation	DNeasy Blood and Tissue Kit (QIAGEN; Venlo, Netherlands; Cat. No. 69504).
RNA isolation	Purelink™ RNA Mini Kit (Thermo Fisher Scientific; MA, USA; Cat. No. 12183018A).
cDNA synthesis	Superscript™ III First-Strand Synthesis System (Thermo Fisher Scientific; MA, USA; Cat. No. 18080051).

Software

Purpose	**Description**
Data and statistical Analysis	GraphPad Prism 7 (GraphPad Software, CA, USA)
	SPSS (IBM, NY, USA).
	Microsoft Excel 2010 (Microsoft, WA, USA).
Microscopic image analysis	ImageJ 1.51-1.52e (National Institute of Health, USA)
	IN Cell Investigator Software (GE Healthcare,

	IL, USA).
	Nikon NI-S Elements Software (Nikon, Tokyo, Japan).
RT-qPCR interface	Rotor-Gene Q-series Software v2.3.1.49 (QIAGEN, Venlo, Netherlands)
Word processing	Microsoft Word 2010 (Microsoft, WA, USA).

Equipment and machines

Use/name	**Description and supplier**
Autoclave	Systec D-200 Horizontal Benchtop Laboratory Autoclave (Systec GmbH, Linden, Germany).
Benchtop centrifuge	Beckman Coulter X-15R Series Benchtop Centrifuge (Beckman Coulter, CA, USA).
Confocal microscope	Nikon Ti-S Confocal Microscope (Nikon, Tokyo, Japan)
Conventional thermocycler	Eppendorf™ Mastercycler™ Pro Nexus Eco and Gradient (Eppendorf, Hamburg, Germany).
Fluorescent microscope	Nikon Eclipse Ti-E Inverted Microscope (Nikon, Tokyo, Japan)
	IN Cell Analyzer 2200 (GE Healthcare, IL, USA).
Gel Doc	Gel Doc System (Edutek, NY, USA).
Haemocytometer	Brand® Neubauer Improved Cell Chamber (Brand GmbH, Wertheim, Germany).
Incubator	Heracell™ 150i CO_2 Incubator (Thermo Fisher Scientific, MA, USA).
Microcentrifuge	Heraeus™ Pico™ 21 microcentrifuge (Thermo Fisher Scientific, MA, USA).

NanoDrop spectrophotometer	NanoDrop™ One/OneC Microvolume UV Spectrophotometer (Thermo Fisher Scientific, MA, USA).
Phase-contrast light microscope	Nikon TS100 Inverted Microscope (Nikon, Tokyo, Japan).
Quantitative thermocycler	Rotor-Gene Q (QIAGEN, Venlo, Netherlands).
Vortex	Vortex-Genie 2 (Scientific Industries, Inc, NY, USA).
Waterbath	Thermo Scientific Precision 28 l water bath (Thermo Scientific, MA, USA).

CHAPTER 3: A CASE-CONTROL STUDY OF THE VAGINAL, CERVICAL, AND ENDOMETRIAL MICROBIOTA IN WOMEN WHO HAVE A HISTORY OF INFERTILITY USING 16S rRNA GENE AMPLICON SEQUENCING

Chapter notes

This chapter is a more detailed version of a journal article published by our group and several collaborators. It contains a significant amount of the data generated by the first study of my PhD research project. In this paper, we examined the relationship between the vaginal microbiome, endometrial gene expression, fertility, and other gynecological factors. As part of my thesis, these findings contributed towards improving our understanding of these points, especially how the microbial diversity in the female reproductive tract relates to gynecological health. The details of the publication are as follow:

Wee, B. A.*, Thomas, M.*, Sweeney, E. L., Frentiu, F. D., Samios, M., Ravel, J., Gajer, P., Myers, G., Timms, P., Allan, J.A. and Huston, W. M. (2018). A retrospective pilot study to determine whether the reproductive tract microbiota differs between women with a history of infertility and fertile women. *Aust N Z J Obstet Gynaecol, 58*(3), 341-348. doi:10.1111/ajo.12754

*these authors contributed equally to the study.

Abstract

The human vaginal microbiome is critical for local homeostasis and defence against pathogens. However, far less is known about the microbiota inhabiting the upper female reproductive tract and its impact on fertility. Here we have examined the association of the vaginal, cervical, and endometrial microbiota for women with a history of infertility compared to women with a history of fertility using a case-control study design. A total of 65 specimens from the reproductive tracts of 31 women (16 controls and 15 cases) were analysed using 16s RNA gene amplicon sequencing. The dominant microbial community members were consistent in the vaginal and cervical specimens of 23 women from which sequences were obtained from both sites. However, for the 11 women where endometrial sequences were obtained, there were significantly different proportions of these most abundant taxa relative to that in the individual‟s vaginal specimens (p=0.0264). When the microbial communities were analysed using the previously described reproductive tract community state types, three endometrial specimens formed a separate cluster within community state type IV. In addition, *Ureaplasma* spp. were found to be over-represented in the vaginal and cervical microbiota of women with a history of infertility (p=0.011-0.42 (unadjusted)). This was further confirmed using a separate PCR that also validated that the species was *Ureaplasma parvum*, additionally women with *Ureaplasma* were more likely to be colonised by *Lactobacillus iners*. When we examined the expression of immune genes within the endometrium, there was no correlation with case-control status or microbial community composition. However, expression of the human tenascin-C gene

showed a significant correlation with a self-reported history of miscarriage. The presence and structure of the microbial communities in the upper female reproductive tract is not always the same as the lower reproductive tract, and it may have important implications for fertility and outcomes of assisted reproductive technologies.

Introduction

The human microbiome has been widely documented to impact the body's physiology, immunity and nutrition (reviewed, [169-171]). Prior to the application of advanced sequencing technologies to characterize the microbiota, the importance of the vaginal microbiota for the female reproductive tract physiology was already understood [172]. The microbiota of the vagina functions in part as a defence against pathogens [173] and is speculated to have implications for reproductive function [174]. In 2011, using 16S rRNA gene amplicon sequencing, the human vaginal microbiota in reproductive-age women was comprehensively characterised [131]. The microbial communities present within this study were categorised into five community state types (CST), based on community composition [131]. The five CSTs were defined by the presence or absence of a dominant *Lactobacillus* sp., including CSTI: *Lactobacillus crispatus*, II: *L. gasseri*, III: *L iners*, IV: a diverse array of strict and facultative anaerobes lacking a dominant *Lactobacillus* sp., and V: *L. jensenii* [131]. CSTIV was subsequently further described to consist of three separate clusters where distinct groups of taxa were observed to consistently cluster together and suggested to possibly have distinct ecological functions [133]. A longitudinal study has demonstrated that the vaginal microbiota is highly dynamic, with changes in the communities observed after sexual contact, menses, douching, and lubricant use; although some CSTs were more stable and rapidly returned to the profile present prior to the event [175]. Women who experienced symptomatic bacterial vaginosis were found to have vaginal microbiota dominated by anaerobes and generally an absence of

Lactobacillus, although some carried *Lactobacillus iners* prior to the symptomatic episode [135]. Characterisation of the vaginal microbiota during pregnancy demonstrated that the diversity and richness of the vaginal microbiota was significantly reduced during pregnancy, with *Lactobacillus* spp. dominating the vaginal microbiota in most pregnant women [176]. A study where vaginal, cervical, and endometrial specimens were collected from 11 women over a 12-week period surrounding the time of implantation of an intrauterine levonorgestrel device (LNG IUS) found that after insertion of the device, the microbiota was dominated by *L. crispatus* [177]. This study represented the first published microbiota profile that included the vagina and the upper reproductive tract (endometrium). A separate study on hysterectomy samples using a species-specific PCR approach identified that the microorganisms that colonised the upper reproductive tract did not correlate with the presence or absence of selected host inflammatory markers [178]. The role of the vaginal microbiota in terms of risk for infection with pathogens should be considered in the context of a likely complex interplay with the host response to any insults, pathogens, and microbiota. One example of this complexity was that women with a *L. crispatus*-dominated vaginal microbiota produced cervicovaginal mucous that was more resistant to HIV infection [179].

In addition, recent evidence indicates that the reproductive tract microbiota could contribute to adverse pregnancy outcomes [152], although pathogens have long been implicated in prematurity, preterm rupture of membranes [174] or stillbirth (reviewed [180, 181]). There is also speculation [174] and emerging reports that implicate the reproductive tract microbiota in outcomes from assisted reproductive technology treatments [182]. Recently, the endometrial microbiota was tested by

examining the tip of the transfer catheter used for single embryo transfer for women receiving IVF treatment [183]. This study identified lower relative abundance of *Pseudomonas* spp. and *Flavobacterium* spp., as well as a higher community diversity index for women who proceeded to pregnancy compared to those where embryo transfer did not result in pregnancy [183]. However, this study did not identify the community state types or *Lactobacillus* species, and so whilst it indicates a possible role for the microorganisms in the endometrium for outcomes of assisted reproductive treatments there is a need for further insight into the specific microbial taxa or communities. A separate study examined the vaginal microbiota in women undergoing *in vitro* fertilization embryo transfer (IVF-ET) and demonstrated that the microbiota diversity index was higher for women who had a live birth from the IVF cycle, when compared to women who did not have a live birth [184]. However, this study did not profile the taxa present, but rather compared the diversity index, providing a relatively limited insight into the role of the vaginal microbiota during pregnancy. The presence of a non-*Lactobacillus*-dominant microbiota in the endometrium correlated with IVF embryo transfer failure in a recent study [185], however, there was no extraction control reported in the study and many reagent-contaminating bacteria were described in the results. Hence, whilst these reports do support that the reproductive tract microbiota in women is relevant to fertility and pregnancy outcomes, much remains to be tested, such as examining infertility or pregnancy outcomes in the context of specific taxa, species level analysis, or the community profiles. In this present study, we further examine the possible association of the reproductive tract microbiota with female fertility. We investigated the differences in reproductive tract microbiota (vaginal, cervical, and endometrial) in women with a history of

infertility (cases) who had required assisted reproductive treatments, compared to women with no history of infertility (controls). We aimed to profile the community state types throughout the reproductive tract, and to test for over-represented taxa in women with a history of infertility in order to provide a more in-depth insight into the reproductive tract microbiota composition that may be a contributing component to infertility.

Methods

Study design

This retrospective case-control study was designed to compare the reproductive tract microbiota of women with a prior or current history of infertility (cases) to that of women with no history of infertility (controls). Participants categorised as cases were attending gynecological or fertility care with a current or a recent previous history of infertility that required assisted reproductive technologies in order to conceive. Only five women in the infertile group were still undergoing treatment for infertility (current infertility); the remaining ten were having treatment for other gynecological reasons having previously required fertility treatment to achieve pregnancy. Participants designated as controls were women attending gynecological care for a hysteroscopy procedure for benign reasons (hysteroscopy for an IUD (IUS) change, or hysterectomy) with no history of infertility. Gynecological and obstetric history was collected using a self-completed questionnaire.

Ethics approval and consent to participate

Ethical approval to conduct this study was granted by the Uniting Care Health Human Research Ethics committee (Approval number 1227), Queensland University of Technology Human Research Ethics Committee (Approval number 1300000004), and

University of Technology Sydney Human Research Ethics Committee (Approval number 2015000700). Participants were required to provide informed written consent prior to participation in the study.

Specimen collection

At the time of hysteroscopy in the operating theatre, a sterile speculum was inserted into the vagina, and swabs collected from the posterior fornix and the endocervical canal. The vagina was prepared for surgery with betadine antiseptic surgical solution. An endometrial biopsy was collected using an endometrial curette and stored in 0.5 ml of RNAlater®. Study samples were immediately taken to the Tissue Bank on the hospital campus and frozen at -80°C, except for the endometrial sample that was incubated overnight at 4°C and then frozen at -80°C the following day.

In addition to comparing the microbiota profiles of the participants on the basis of their assigned case and control categories, we also analysed other aspects of the participants‟ gynecological history. These other attributes included the type of contraceptive used, any history of miscarriages, any history of endometriosis, and age of the participants at the time of sample collection. The grouping the participants into three contraceptive categories (pill (oral contraceptives), LNG

IUS (intrauterine levonorgestrel device) or other) was conducted using the data collected prior to the specimen collection procedure. The „other" contraceptive category included methods such as rhythm, condoms, tubal ligation or no use of active contraception at all. Participants were divided into the following age groups for categorical analysis; 28-33, 34-39, 40-45, and 46-49 years old.

Sample processing

Swab samples

Swabs in TE buffer were thawed before undergoing enzymatic and mechanical lysis [131]. The first enzymatic lysis step involved the addition of 10 µl mutanolysin (7,000 U ml^{-1}; Sigma Aldrich), 2.5 µl lysozyme (100 mg ml^{-1}; Sigma Aldrich) and 1.5 µl lysostaphin (1 mg ml^{-1}; Sigma Aldrich), and incubation at 37°C for 1 h. Following this, a secondary lysis solution was added to each of the samples that contained 50 µL of proteinase K solution (600 mAU ml^{-1}; QIAGEN), 1.25 µL RNase A solution (100 mg ml^{-1}; QIAGEN) and 55 µL sodium dodecyl sulphate (SDS; 10% vol vol^{-1} in dH_2O). Samples were then incubated at 55°C for 1 h. Mechanical lysis was then performed on each sample. This involved the addition of three 3 mm sterile silica beads (Ajax Finechem Pty. Ltd.,) to each tube before disrupting the samples for 30 s at 3450 oscillations min^{-1} using the Biospec Mini-Beadbeater-16. Extraction and purification of total genomic DNA (gDNA) from the resulting lysates was conducted immediately using the DNeasy Blood and Tissue Kit (QIAGEN), in accordance with the manufacturer's recommendations. Total gDNA was eluted in ultrapure DNase/RNase-free distilled H_2O (dH_2O; Thermo

Fisher Scientific), aliquoted, and stored at -80°C (see below for description of extraction controls (negatives) and batch process).

Endometrial tissue samples

Samples were thawed on ice and a 30 mg section was isolated for processing. This was performed using a new sterile surgical scalpel blade for each individual sample. In cases where the provided tissue had a mass of less than 30 mg, the entire sample was processed. Preparation of a crude lysate involved a similar process to the one described for the swab samples. Briefly, this method differed slightly to account for the physical nature of the sample, and used a longer secondary enzymatic lysis incubation time of 90 min. Any tissue remaining after this step was collected by centrifugation (3 min; 10,000 × *g*), and only the supernatant was collected, before continuing with the mechanical lysis step of the DNA extraction process. gDNA was extracted and purified from the crude lysate and evaluated as described above. A trial participant sample set was extracted using each of these methods. DNA purity and quantity were assessed by spectrophotometry, and a test 16s rRNA gene PCR analysis was conducted to confirm that the protocol produced DNA that yielded a visible PCR product. DNA purity and quantity were assessed by spectrophotometry from each of the batch extractions (both swabs and endometrium). Samples that did not have sufficient yield or purity were not sent for sequencing (two vaginal, eleven cervical, and five endometrial). DNA extraction was batched with ~10 samples per batch (116 samples in 11 batches) and one negative control was prepared using the exact same protocol (i.e. lysis, extraction, PCR) for either the swab or tissue method. All extraction controls (negative controls) were sequenced and analysed.

16S rRNA gene amplification and sequencing

Primers amplifying the variable (V) V1 to V3 regions of the bacterial 16S rRNA gene were used (27F and 534R). The 27F primer was comprised of a mix of 7 primers that have been previously shown to accurately amplify correct ratios of *Lactobacillus* spp., as well as other species of bacteria [186]. These primer sequences included: 5‟AGAGTTTGAT**YM**TGGCTCAG3‟ (four oligo mix), 5‟AGGGTTCGATTCTGGCTCAG3‟ (*Bifidobacterium*-specific), 5‟AGAGTTTGATCCTGGCTTAG3‟ (*Borrelia* species-specific), 5‟AGAATTTGATCTTGGTTCAG3‟ (*Chlamydia* species-specific). 534R primer: 5‟TTACCGCGGCTGCTGGCA3‟.

16S rRNA gene amplicons were generated according to the Illumina 16S metagenomic library preparation protocol (Part no. 15044223 Rev. B) in a 384-well plate in an 8 µL reaction comprising 4 µL of Life Technologies AmpliTaq Gold 360 master mix, 2 µL primer mix and 2 µL of gDNA. Amplification of the 16S rRNA gene was performed under PCR cycling conditions adapted from Romero et al., 2014 [187]; 5 minutes denaturing at 95°C, 29 cycles of 30 seconds at 94°C, 30 seconds at 52°C and 60 seconds at 72 °C followed by a final step of 10 minutes at 72°C. All amplicon products, regardless of whether an amplicon was detected or not, underwent secondary PCR to attach barcodes and sequencing adapters using the Nextera XT v2 library preparation kit and sequenced on the Illumina MiSeq with 300bp paired end chemistry (v3) by the Australian Genome Research Facility (AGRF). Sequencing was carried out with the forward read (R1) at the V3 region and (R2) starting at the V1 region of 16S rRNA gene amplicon.

Sequence analysis

Paired end reads were assembled using PEAR v0.9.9 [188] with a minimum overlap of 10bp and minimum assembly length of 500 bp. Assembled sequences were then filtered using a quality score cutoff of Q20 (-q 20) using Qiime's split_libraries_fastq.py script. Sequences were then assigned to taxonomic clusters at 97% identity cutoff using the STIRRUPS pipeline based on the vagina-specific curated V1-V3 16S rRNA gene sequence database [189]. QIIME was also used to cluster sequences into operational taxonomic units (OTUs) at a 97% identity threshold using the pick_open_reference_otus.py script [190]. A representative sequence from each OTU was assigned a taxonomic classification based on the Greengenes database (v 13_8) [189, 191]. The dataset is available in the sequence read archive (SRA) database under accession number: PRJEB18626. Non-specific amplification and sequencing of human DNA was removed by using Kraken [192] to search against 3 human genome assemblies available on RefSeq, i) Genome Reference Consortium Human Build 38 patch release 8 (GCA_000001405.24), ii) HuRef (GCA_000002125.2) and iii) CHM1_1.1 (GCA_000306695.2). The number of reads remaining after each processing step are shown in **Appendix I**. Laboratory extraction controls were performed with each series of extractions and these were also sequenced (11 DNA extraction batch negative controls) (mean=288 raw reads, min=12, max=672) due to previously reported risks of laboratory reagent contamination in metagenome studies [193] (**Appendix I**).

Statistical analysis

Over-represented taxa were identified using the group_signifiance.py script implemented in QIIME v1.9.0 [194]. Taxonomic assignments from STIRRUPS and the OTU tables from QIIME were randomly subsampled (rarefied) in ten replicates down to 1000 sequences results per sample using multiple_rarefactions.py in QIIME. Each of ten rarefied replicates was then individually tested for significantly over-represented taxa and OTUs (STIRRUPS and QIIME, respectively). Differentially abundant taxa and OTUs between case and control samples were determined using the non-parametric t-test and Kruskal-Wallis test as implemented in the group_significance.py script [194]. R-plots were generated using the R Package Phyloseq v1.12.2 [195]. Analysis of consistency of taxa between the three specimen sites was conducted by comparing the mean log ratios of the cervix or endometrium relative to vaginal results for each taxa for each individual using a Mann-Whitney test (GraphPad Prism 6.0).

We compared the proportions or distributions of the demographic (age), obstetric (parity, gravidas, miscarriage), and gynecological factors (contraceptive, endometriosis) between the case and control groups using SPSS (specific test used was relevant to the data type and is indicated with each p value within the results). The dataset is available in SRA database under accession number: PRJEB18626.

Ureaplasma detection and quantification

The UMS-125 sense (5‘‘GTATTTGCAATCTTTATATGTTTTCG 3‘‘) and UMA226 antisense (5‘‘ CAGCTGATGTAAGTGCAGCATTAAATTC 3‘‘) primer pair were used for PCR detection of the human *Ureaplasma* species (*U. parvum* and *U.*

urealyticum) in patient samples as per previously published protocols [196]. Amplicons were analysed using both agarose gel electrophoresis of the PCR products and high-resolution melt curve analysis following qPCR.

Total RNA extraction from endometrial tissue

Tissue samples preserved in RNALater (Ambion) were extracted in batches (batches consisted of ~11 samples including one negative control). A 125 W Omni benchtop tissue homogenizer (Thomas Scientific) was used to lyse and homogenize the samples (on ice). The RNeasy mini kit (QIAGEN) was used to isolate total RNA, in accordance with the manufacturer's recommendations. Reverse transcription (RT) with random hexamers and 500 ng total RNA was performed using the SuperScript® III first-strand synthesis kit (Thermo Fisher Scientific). cDNA samples were stored at -80°C until required.

Endometrial gene expression analysis

Gene expression analysis was conducted to profile the localised immune response present in the endometrium. This involved the use of reverse transcription quantitative polymerase chain reaction (RT-qPCR) to determine the levels of six genes of interest (Interleukin-6 (*IL6*), Interleukin-8 (*IL8*), Syndecan-1 (*SDC1*), IL-1-alpha (*IL1A*), Tenascin-C (*TNC*), and Tumor necrosis factor alpha (*TNFA*)) relative to two housekeeping genes (Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and Peptidylprolyl isomerase A (*PPIA*)) in each of the cDNA samples. Reactions used AmpliTaq™ Gold 360 Mastermix and SYBR® I nucleic acid dye. The QIAGEN Rotor-Gene Q RT-qPCR cycler was used for thermocycling (see **Appendix II** for reaction and cycling conditions). This was followed by standard

(1°C/second) melt curve analysis. All qPCR experiments were performed in triplicate alongside no-template (NTC) and positive controls. The cycle threshold (Ct) value for each gene of interest represents the geometric mean value of three technical replicates from each cDNA sample. Ct values with a standard deviation (SD) greater than 1.0 were excluded. The ΔCt values presented were calculated by subtracting the Ct value of each gene of interest from the geometric mean of GAPDH and PPIA. The gene expression levels were analysed using a heat map and cluster dendrogram using the ward.D2 clustering algorithm based on Euclidean distances between samples as implemented in the vegdist and hclust R packages respectively. RT-qPCR gene expression data was statistically analysed and assembled into figures using GraphPad Prism 6. The Mann-Whitney test was used to compare the ΔCt values of two groups (e.g. Case-Control), and the Kruskal-Wallis test (with Dunn‟s post-hoc testing to correct for multiple comparisons) was used when three or more groups were compared (e.g. CSTs).

Results

Case-control groups were comparable for gynecological factors, although age was significantly different

This study was a retrospective pilot case-control examination of the reproductive tract microbiota of women, in the context of fertility. Participants sampled were women with a history of infertility, who had required assisted reproductive technologies to conceive (cases), compared to women with no history of infertility (controls) (**Table 3.1**). Participants were recruited when they attended for gynecological care for any reason that included a hysteroscopy, so we analysed the groups to see if this recruitment strategy resulted in any biases in gynecological features between the cases and controls. Many of the cases had completed their fertility treatment (only five of the 15 were in the process of trying to achieve a pregnancy at the time of the study and the remaining ten reported to have stopped or completed fertility treatment). All the participants in the control group reported that they were no longer trying to conceive. The statistical analysis of the gynecological history indicated that the two groups had similar proportions of the types of contraceptives in use, endometriosis, parity, gravidas, and miscarriage (**Table 3.1**). However, age was significantly different with a higher mean age ($p=0.05$) and distribution ($p=0.035$) in the controls compared to the cases.

Table 3.1. Comparison and analysis of fertility, epidemiological, and gynecological factors, grouping by case-control status.

		CONTROL (fertile; n=16)	CASE (infertile; n=15)	*P*
Age	Range (mean)	35–48 (42.75)	28–45 (37.6)	0.05[!]
	28-33	0	3	
	34-39	4	7	0.035[$]
	40-45	8	5	
	45-49	4	0	
Pregnancy ever		16 (100%)	12 (80%)	0.203[$]
Live birth ever		16 (100%)	11 (73.3%)	0.086[$]
Median live births (interquartile range)		2.5 (2–3)	2.0 (0–2)	0.049[^]
No longer seeking pregnancy		16 (100%)	10 (66%)	0.018[$]
Contraceptive Use*	Other	6 (37.5%)	9 (60%)	
	Pill	4 (25.0%)	5 (33.3%)	0.149[$]
	Mirena	6 (37.5%)	1 (6.6%)	
History of endometriosis	Y	6 (37.5%)	6 (40.0%)	0.589[$]
	N	10 (62.5%)	9 (60%)	
History of miscarriage	Y	8 (50%)	5 (33%)	0.283[$]
	N	8 (50%)	10 (66.7%)	

[!] t test; [$] Fishers exact chi-square; ^ANOVA

* other: included no current contraception method in place, tubal ligation, condoms, or rhythm method.

The microbiota profile was consistent in the cervical and vaginal samples from each individual, but the endometrium often had a distinct composition of microbiota

16S rRNA gene amplicon sequencing was conducted on DNA isolated from the vagina, cervix, and endometrium, where the specimen was successfully extracted (five vaginal, 11 cervical and five endometrial samples did not extract well and were excluded prior to sequencing). A total of 11,854,281 paired reads (mean=136,256 min=43, max=402,194 per sample) were obtained for 102 samples from 40 individuals. The quality and quantity of DNA extracted from each of these samples meant that the final total of individuals included for analysis in the study was 31 (16 controls and 15 cases). After removal of reads mapping to the human reference genomes, assembly of paired end reads and filtering of low-quality sequences a total of 5,358,265 assembled sequences remained (mean=63,513, min=15, max=246,712). Only samples with at least 1,000 sequences remaining (n=68) were analysed and are summarised in **Table 3.2** and **Appendix I**). The sequence depth per sample was variable, with the highest number of filtered sequences obtained from vaginal (mean=88,302) and cervical (mean=95,401) samples. Endometrial samples produced fewer reads with an average of 7,815 sequences per sample (**Appendix I**).

The genera associated with the most abundant sequences for samples with 1,000 reads and above are shown in **Figure 3.1** (proportional abundance). The genus *Lactobacillus* was the most dominant group identified and this was consistent

Table 3.2. Number and details of samples extracted and analysed.

	CASE	CONTROL	NEG[1]	TOTAL
Number of participants recruited	15	25		40
Participants excluded[#]	0	9		9
Participants included	15	16		31
Samples sequenced	41	46	11	98
Vagina[!]	15	16		31
Cervix[!]	10	13		23
Endometrium[!]	6	5		11
Total sites/samples analysed	31	34	11	76

[1] NEG refers to the negative extraction controls prepared alongside the sample extractions. A total of 11 were sequenced to control for laboratory/reagent contamination.

[#] participants who were recruited and consented to the study but were: 50 or over, did not pass the extraction quality assessment, or were unsuitable for either grouping.

[!]minimum of 1K filtered sequences

across all three reproductive specimen sites (**Figure 3.1**). **Appendix II** shows the relative proportions of all taxonomic assignments in the samples. The most abundant taxonomic units identified in this analysis were the same in both specimens for all 23 of the 31 women whose cervical and vaginal samples were successfully sequenced and analysed (**Figure 3.1**; 11 samples with no data). This was distinct from the 11 women with sequences from the endometrium and vagina, where five women (three cases, two controls) had different abundant taxa compared to the vagina. The most proportionally high abundant taxa in the endometrium (relative to the vagina) were significantly distinct from the vaginal sample compared to the relatively similar results for the cervix (as a ratio of

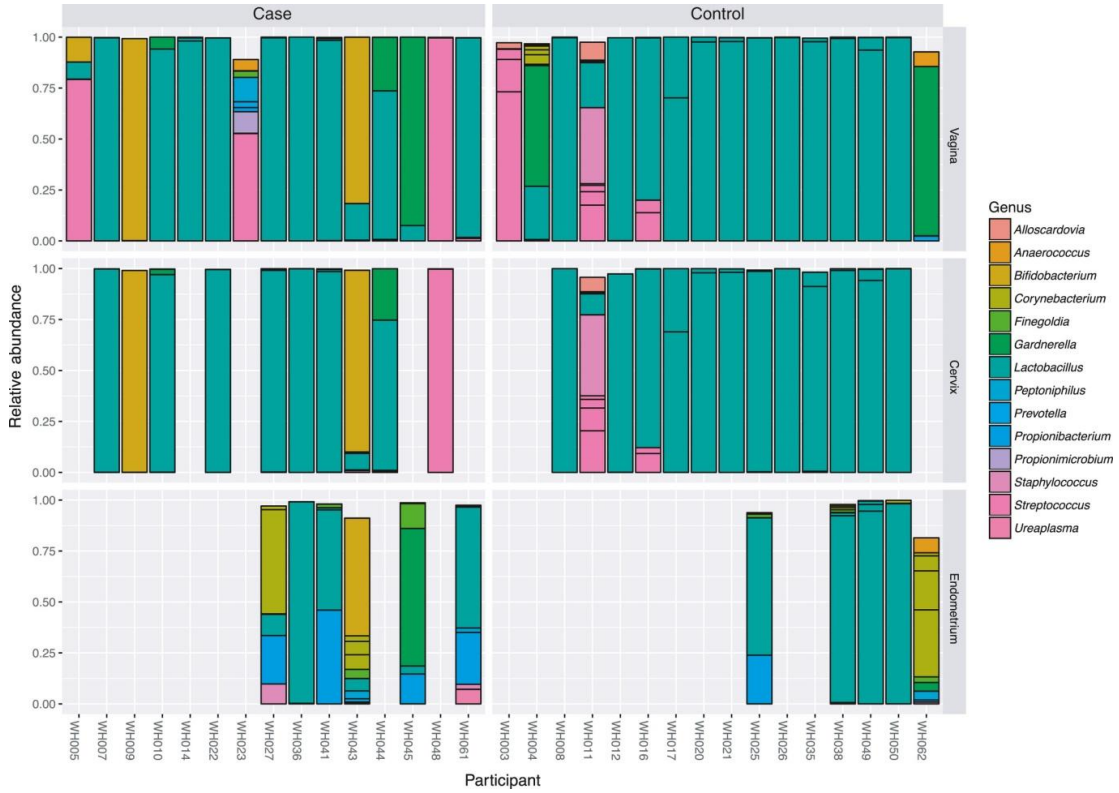


Figure 3.1. The relative proportion of the 25 most abundant taxonomic assignments. Bars are colored by genera and lines in the same color indicate different clusters. Participants (codes shown below the bars) were grouped according to their case-control status (indicated above the bars) for each of the three specimen sites (top to bottom, indicated to the right: vagina, cervix, and endometrium). Only samples with more than 1,000 assembled and assigned sequences are shown.

matched vaginal samples) (p=0.0264, Mann-Whitney test; cervix to vagina median ratios p=0.0009 n=53; endometrium to vagina median ratio p=0.1691 n=23).

The microbial community profile and the presence or absence of a dominant *Lactobacillus* species are known important factors in the ecology of the vaginal microbiome. The community clusters within the samples were analysed using a cluster dendrogram method (previously described by [131]). The gynecological

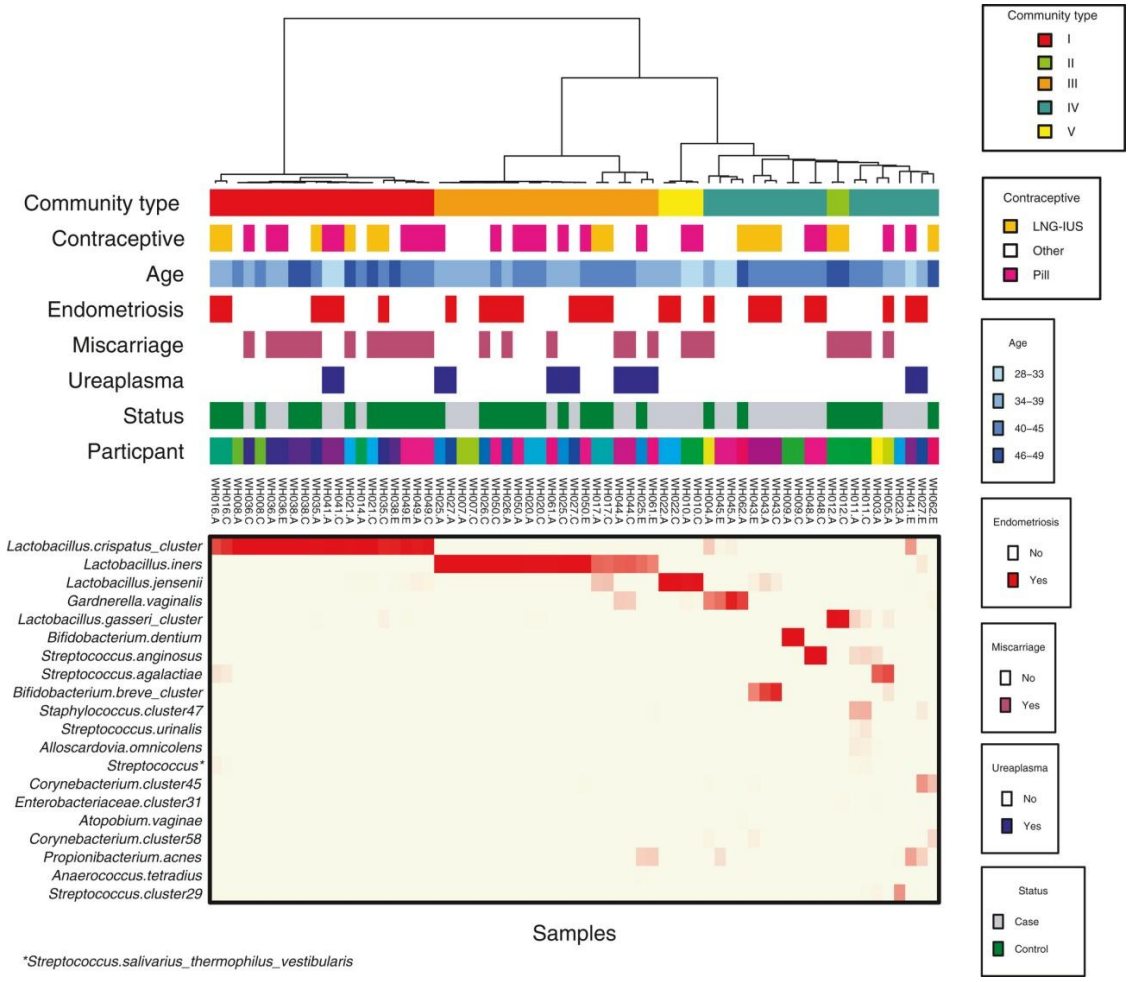


Figure 3.2. Community state types dominated by *Lactobacillus* were present in the majority of specimens. The heat map showing the proportions of the top 20 most abundant taxonomic groups assigned by STIRRUPS in the reproductive tract microbiota. Contraceptive in use, age, history of endometriosis, history of miscarriage, and case/control status are displayed above each sample (color keys are indicated to the right). Linkage clustering of the samples to the previously defined community state types (CST I, II, III and IV) [131] is shown at the top of the figure.

and demographic factors are annotated on the figure (**Figure 3.2**). The reproductive tract microbiota profiles in the participants were distributed across the previously described community state types. Of the 31 total participants and their vaginal microbiota profiles, nine corresponded with the CSTI (*L. crispatus*-dominant) profile; one corresponded with a CSTII profile (*L. gasseri*-dominant);

nine corresponded with a CSTIII profile (*L. iners*-dominant); ten corresponded with CSTIV; and two corresponded with a CSTV profile. Generally, each individual's specimens (vaginal, cervical, and endometrial) clustered together within the dendrogram, with the exception of two individuals whose endometrial samples that clustered together as a possible sub-cluster of CSTIV (discussed further below).

There were ten participants whose vaginal (and/or cervical) sample was consistent with CSTIV. However, there were three sub-clusters within the CSTIV. The participants in the first of these CSTIV clusters were found to have reproductive tract microbiota dominated by *Gardnerella vaginalis* (and co-clustering with *Prevotella* in some cases) (consistent with the previously described CSTIV [131], also described as CSTIV-B [175]). The second cluster consisted of a group where *Streptococcus agalactiae* or *Streptococcus anginosus* were dominant (somewhat consistent with the previously described CST IV- A [175]). This group also featured *Bifidobacterium* dominant profiles although this organism was not mentioned in the previously described sub-clusters of CSTIV and could prove to constitute another CSTIV sub-cluster. The final cluster consisted of three endometrial samples with a mix of *Cornyebacterium*,spp. and *Propionibacterium* spp. as the major features; each of these individuals had lower reproductive tract specimens that clustered separately on the dendrogram. These three may represent a previously undescribed endometrium-specific sub-cluster of CSTIV (we propose this community state type be termed CSTIV-E). This endometrial CSTIV-E community state type does not appear to relate to the community present in the lower genital tract, as the three individuals with CSTIV-E each had a different CST in the vagina and cervix (either CSTI, CSTIII, or CSTIV).

A risk to the interpretation of the study results is that the control group is significantly older than the case group. However, the five participants in the 45-49 age category that may introduce limitations to the interpretation of the study are spread across all of the community state types. There was no evidence of clustering based on age or case-control status on the dendrogram (grey-green bar, **Figure 3.2**).

To visualize the composition similarity between samples, a principle coordinates analysis of the Jensen-Shannon Divergence distance between samples was conducted. This enabled the assessment of whether any observed similarities were due to specimen site, age, case-control status, or contraceptive category. This analysis showed that there was no grouping of samples by any of these features (data not shown).

Testing for correlates of the participant gynecological or epidemiological data with the microbiota results was conducted. This was performed using non-parametric t-tests for features with two groups, and the Kruskal-Wallis test for features with more than two categories (e. g. age and contraceptive method combined). Associations of presence of microbial taxa with participant data were identified (**Table 3.3**). These are trends that appear to be influenced by high sequence proportions in a small number of participants (generally two to three participants) and should be interpreted with some caution, as none were significant after adjustment for multiple testing.

Gardnerella vaginalis was over-represented in both the vagina and cervix of women in the 28-33 age range (vagina: p= 0.005-0.015, cervix: p= 0.004-0.049). G*ardnerella* was over-represented within but not exclusive to this age category as

Gardnerella were detected in ≥1 individual in each age grouping. *Streptococcus* cluster 29 is defined by STIRRUPS to group *S. australis, S. cristatus, S. mitis, S. oralis, S. pneumoniae, S. pseudopneumoniae, S. infantis* or *S. sanguinis*, which cannot be resolved by the V1-V3 16S rRNA gene sequence alone. This cluster was found to be over-represented in women in the „*other*" contraceptive group (p= 0.021-0.049 range, 9/10 replicate tests). *Bifidobacterium breve* was over-represented in women with a history of endometriosis (p= 0.006-0.050; 5/10 replicates). *L. gasseri* was significantly correlated with a history of miscarriage (p= 0.017-0.037; 10/10 replicates). Although several trends in the endometrial samples were observed, the small number of samples included in this comparison did not provide sufficient confidence in these results.

Table 3.3. Associations between the presence of microbial taxa and the collected participant data. Statistical analysis of trending taxa that were present in a majority (greater than five out of ten) of rarified replicates were observed to return naïve p-values of <0.05. but were not significant after correction for multiple testing.

	Vagina	**Cervix**
Age: 28-33	*Gardnerella vaginalis* (10/10)	*Gardnerella vaginalis* (10/10)
Contraceptive method: Other	*Streptococcus* cluster29 (9/10)	none
Reported history of endometriosis	Bifidobacterium breve_cluster (5/10)	none
Reported history of miscarriage	*Lactobacillus gasseri*_cluster (10/10)	*Lactobacillus gasseri*_cluster (7/10)

Ureaplasma spp. were more frequently identified in the vaginal and cervical microbiota of women with a history of infertility (cases)

To identify significant compositional differences between case and control women, a non-parametric t-test using the group_significance.py script in Qiime on species level classifications that were assigned using STIRRUPS was performed [194]. Ten replicates of the assigned sequences were rarified down to 1000 sequences and as such only samples with more than 1000 sequences were included. Only a single genus, *Ureaplasma*, was found to be over-represented among cases in at least five out of the ten species-richness rarefaction replicates of the vaginal samples (p=0.011-0.042, unadjusted for multiple testing, **Figure 3.3**). A similar trend in the results of the QIIME OTU clustering was observed, where 7 out of 10 rarefaction replicates showed an over-representation of an OTU mapped to *Ureaplasma* spp. (p=0.019-0.05, unadjusted).

To further characterise *Ureaplasma* spp., a *Ureaplasma*-specific PCR targeting the multiple banded antigen (*mba*) gene was carried out on four case samples and a control sample that had *Ureaplasma* spp. sequences (**Figure 3.4**). A high-resolution melt (HRM) assay of the *mba* gene was performed to identify the *Ureaplasma* spp. (*U. parvum* or *U. urealyticum*) present within these samples. The results of HRM analysis indicated that the five samples all contained *U. parvum.* It is also notable that four of the five women with a history of infertility (cases) who were colonised with *Ureaplasma* also had the *L. iners*-dominant vaginal CSTIII (**Table 3.4**). The association between *Ureaplasma* and *L. iners* was found to be statistically

significant in the vaginal sequencing results using a nonparametric t test (p= 0.005-0.02, 10/10 rarefactions; unadjusted for multiple testing).

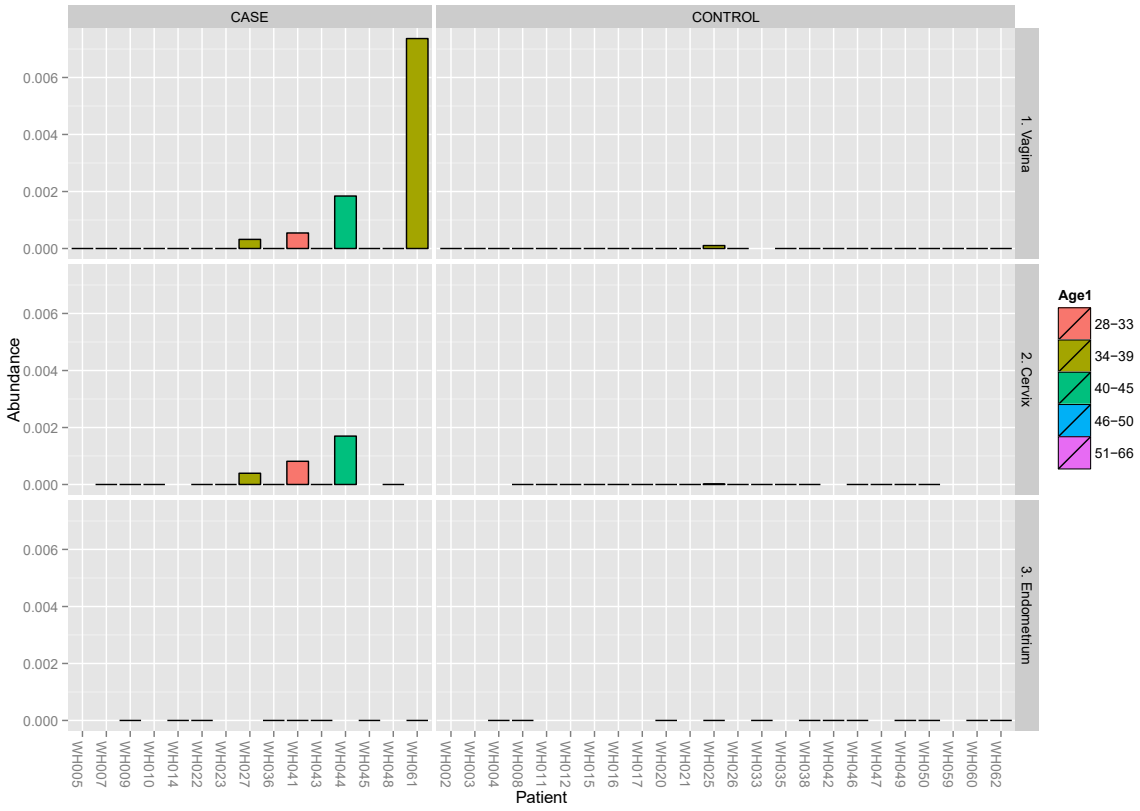


Figure 3.3. Relative abundance (%) of sequences per sample mapped to *Ureaplasma* species. Bars are colored according to the age category of the participant (color key to the right). The participants are grouped by case-control status (indicated at the top), and specimen sites on the left y axis. The proportion of *Ureaplasma* sequences from each swab site are shown (y axis).

Table 3.4. Number of filtered reads mapped to *Ureaplasma*.

Sample ID	Site	Status	Number of mapped reads
WH025.A	Vagina	Control	29
WH025.C	Cervix	Control	3
WH027.A	Vagina	Case	67
WH027.C	Cervix	Case	53
WH041.A	Vagina	Case	87
WH041.C	Cervix	Case	203
WH044.A	Vagina	Case	188
WH044.C	Cervix	Case	207
WH061.A[a]	Vagina	Case	1128

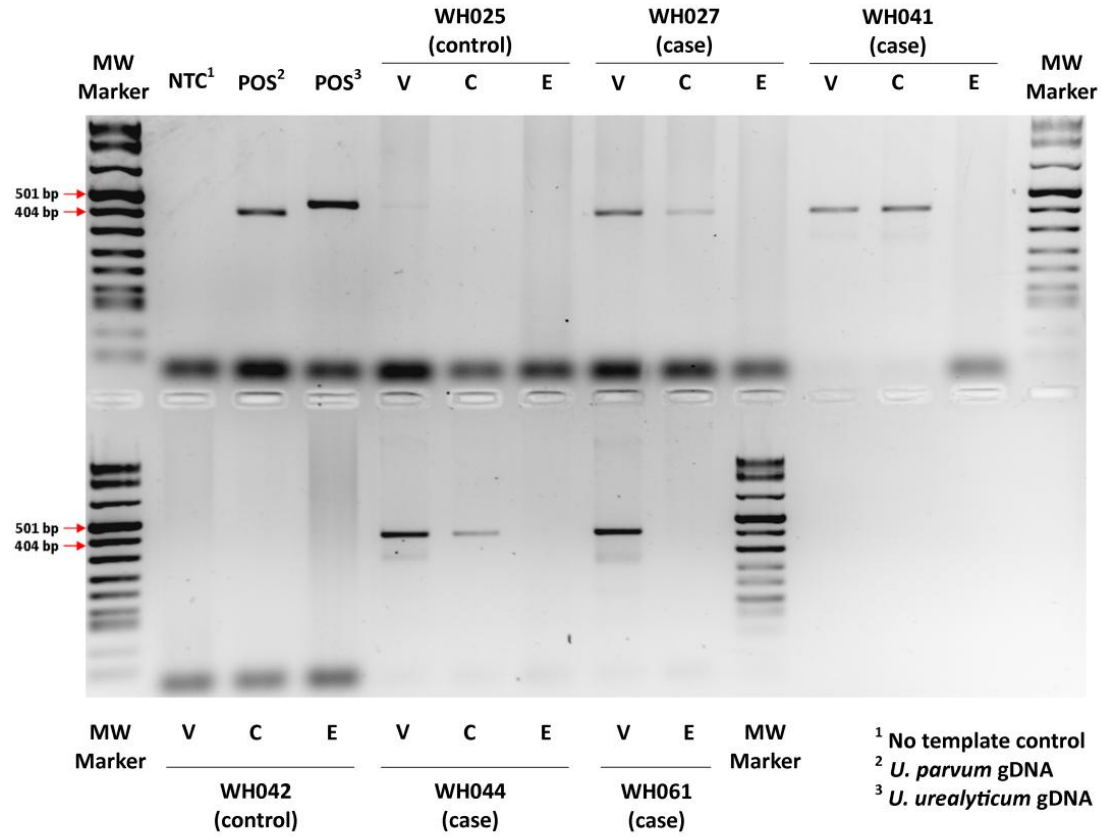


Figure 3.4. PCR amplification of the multiple banded antigen (*mba*) in samples with sequences corresponding to *Ureaplasma* spp. Primers were specific for *Ureaplasma* spp. and the size of the product was used to differentiate between *U. parvum* and *U. urealyticum.* The case-control status of the participants is indicated on the figure, as well as the specimen site (V: vagina, C: cervix, E: endometrium). WH061.C failed extraction quality control and was not analysed for any aspect of this study. A no template control (NTC[1]), and positive controls of gDNA from representative strains were included as indicated on the figure (POS[2] and POS[3]).

Alpha diversity was not significantly different in infertile women compared to fertile women, but was significantly higher in the endometrium of women using LNG-IUS

The ecological diversity or taxonomic richness of each sample was measured using Shannon Diversity index and compared to other samples [197]. The alpha-diversity indices were compared by grouping the participants based on their case-control status (**Figure 3.5**) and their self-reported contraceptive use (**Figure 3.6**). There was considerable variation in the alpha-diversity across both of these categories and the only significant difference detected was that the alpha-diversity index was higher in the endometrial specimens of LNG-IUS users compared to the endometrium of either uses of the oral contraceptive pill (pill), or users who did not use a pharmacological contraceptive treatment (other; $p=0.000899$).

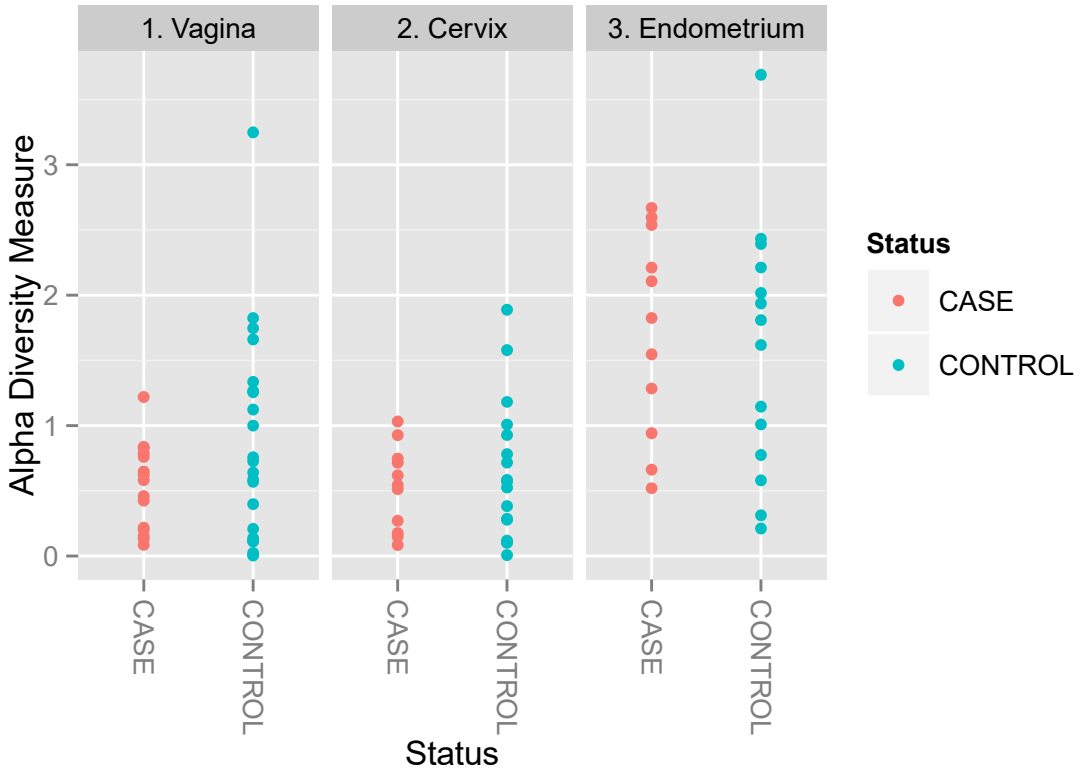


Figure 3.5. Alpha-diversity comparisons of the microbiota grouped by case-control status. This figure shows the alpha-diversity index scored using the Shannon diversity measure for each of the three anatomical sites samples. Participants were grouped on the basis of their medical and self-reported history of fertility (controls) or infertility (cases). Colour has been used to distinguish between the case-control categories of the participants, in the legend on the right of the figure.

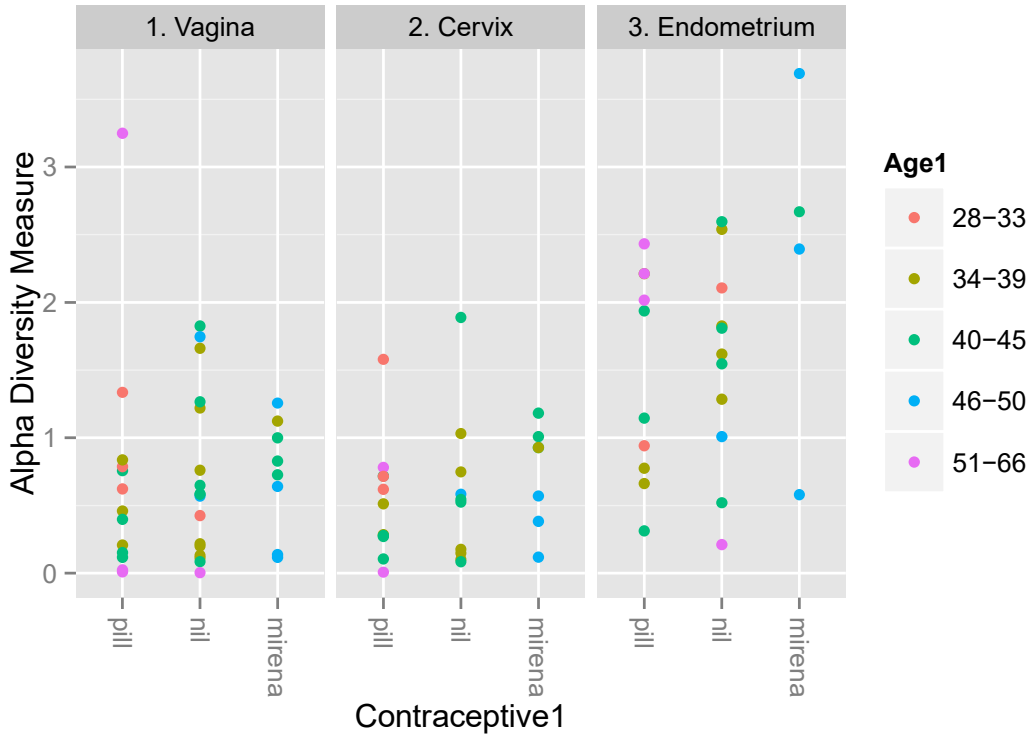


Figure 3.6. Alpha-diversity comparisons of the microbiota grouped by age. This figure shows the alpha-diversity index scored using the Shannon diversity measure for each of the three anatomical sites samples. Participants were grouped based on their self-reported age at the time of recruitment. Colour has been used to distinguish between the age categories of each participant, which is indicated the legend on the right of the figure.

Endometrial gene expression most significantly correlated with a history of miscarriage and contraceptive use, rather than a history of infertility or microbiota composition

RT-qPCR was used to assess if the composition of the microbiota was associated with the expression of select genes in endometrial tissue (selected based on previous reports of correlation with endometrial function, inflammation or disease). The expression levels of six genes, *IL1A*, *IL6*, *IL8*, *TNC*, *TNFA*, and *SDC1* were measured relative to the geometric mean of two reference genes (*GAPDH* and *PPIA*). The gene expression levels are displayed in **Figure 3.7** using a heat map and cluster dendrogram. There were two major clusters that were significantly correlated with the participant factors (**Figure 3.7**).

There were no significant differences in the expression of any genes tested in relation to case-control status (**Figure 3.8A**). High expression of *TNC* grouped in two clusters in the dendrogram and appears to be related to past history of miscarriage (**Figure 3.7**). Consistent with the dendrogram clustering, *TNC* expression was significantly higher in the endometrium of women with a history of miscarriage (p=0.0006, **Figure 3.8A**). The dendrogram also shows that higher *IL8* expression levels also grouped into two (smaller) clusters (**Figure 3.7**). These are likely associated with contraceptive usage as *IL6* and *IL8* expression levels were significantly higher in LNG IUS users (p=0.05 by Mann-Whitney test), whereas *SDC1* was significantly lower in LNG IUS users compared to OCP users (p=0.05 by Mann-Whitney test) (**Figure 3.8C**).

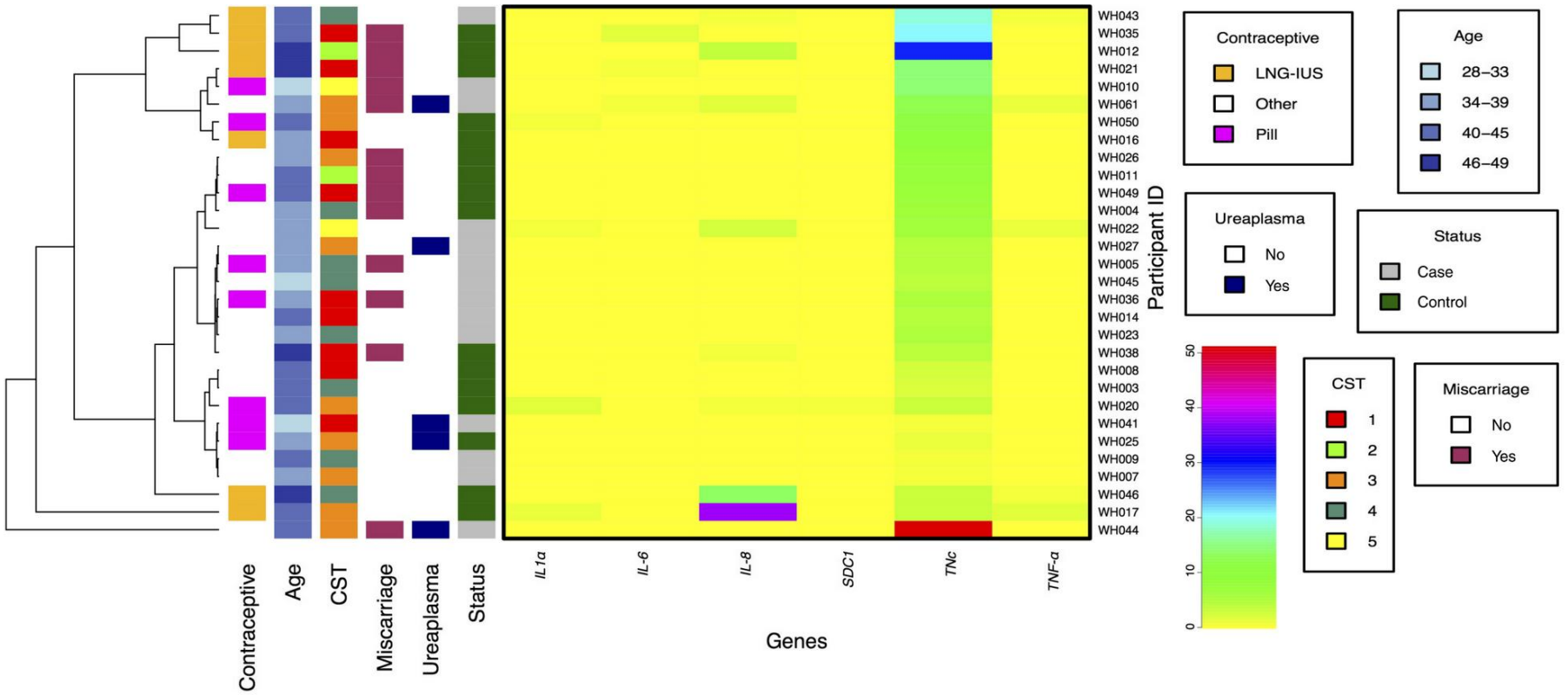


Figure 3.7. Endometrial expression of six selected genes with known implications for endometrial pathology or function. The dendrogram shows hierarchical linkage clustering of *IL1A*, *IL6*, *IL8*, *TNC*, *TNFA*, and *SDC1* expression. Participant data, including case-control status, presence of *Ureaplasma* spp., age, miscarriage, and contraceptive are indicated to the right of the heatmap. Colors represent the transformed mean ΔCt values for each specimen, calculated as $2^{-\Delta Ct}$.

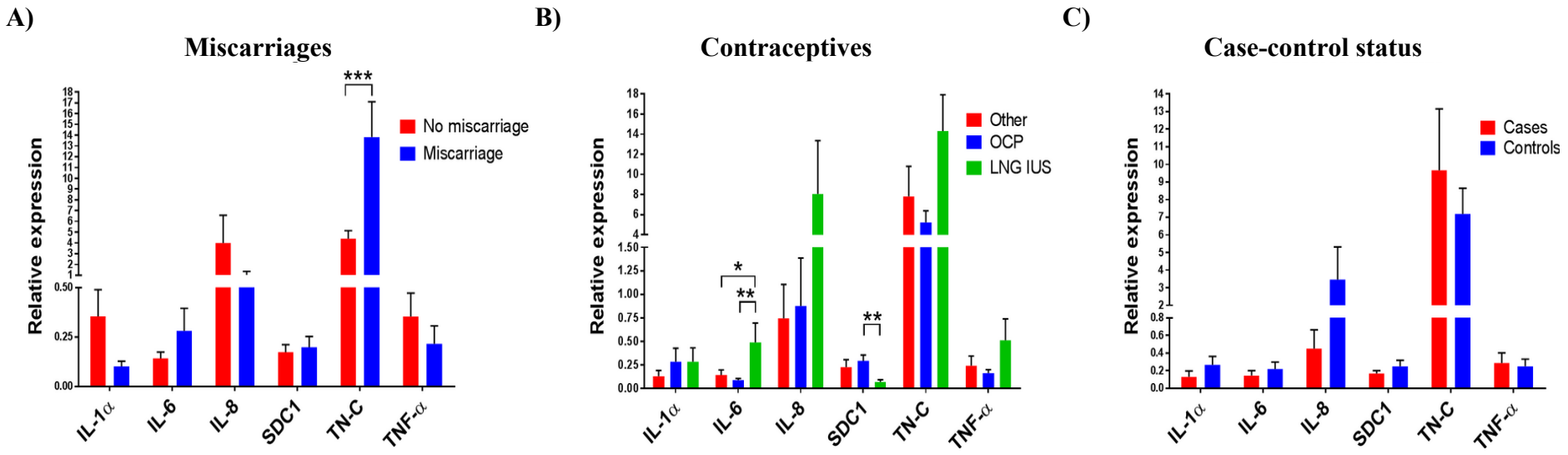


Figure 3.8. Expression of selected endometrial genes grouped by participant factors. The expression levels of six genes of interest relative to the geometric mean of GAPDH and PPIA, grouped according to participant data. Y-axes show transformed mean ΔCt values of each group, while the X-axes displays gene names. Bar color indicates participant grouping based on (**A**) miscarriage history, (**B**) contraceptives and (**C**) case-control status. Statistical tests performed were Kruskall-Wallis or Mann-Whitney tests (* $p<0.05$; *** $p=0.0006$).

Discussion

The objective of this study was to examine the reproductive tract microbiota (vagina, cervix, and endometrium) in women who had a history of needing fertility treatment to conceive, compared to fertile controls. Fertile women were those who had pregnancies with no need for assisted reproductive treatments or unknown fertility status as they reported actively and consistently avoiding pregnancies. Participants were recruited when they attended a clinic for either fertility or gynecological care that included a hysteroscopy. This opportunistic recruitment meant that cases were undergoing the procedure as part of their infertility treatment or clinical care or as part of gynecological care after a previous history of infertility. Therefore, these data do not necessarily reflect the microbiota at the time of infertility treatment (cases) or fertility (controls).

Many individuals within this study had a dominant *Lactobacillus* species within the reproductive tract microbiota, which is consistent with the previous literature [131, 175]. The highest represented taxa present were found to be the same in the cervical and vaginal swabs in all individuals where sequences were obtained from both specimens. However, the ratio of dominant taxa in the endometrium compared to the vagina were significantly different ($p=0.0264$). In addition, three of these formed a sub-cluster of CSTIV that may represent a newly described sub-cluster of CSTIV (CSTIV-E) found in the endometrium. It is acknowledged that this proposed CSTIV-E should be validated with a greater number of samples than analysed here. A cluster with a similar profile of *Propionibacterium* in the absence

of a dominant *Lactobacillus* was also observed in the endometrial fluid microbiota analysis in the recent study by Moreno and colleagues [185], supporting the validity of this possible endometrial community state type IV-E cluster. This previous study [185] also described a *Bifidobacterium* dominant cluster consistent with that described here within CSTIV.

Ureaplasma spp. were significantly over-represented in women with a history of infertility (cases) in the vagina and cervix samples. *Ureaplasma* spp. were also significantly associated with the presence of *L. iners* (CSTIII) in four of the five women. Using *Ureaplasma*-specific PCR, it was further confirmed *U. parvum* was present in all women with sequences from *Ureaplasma* spp. These findings provide a new contribution to previous knowledge regarding *Ureaplasma*. *Ureaplasma* have been previously identified within the reproductive tracts of both fertile and infertile women [198, 199] and were reported to be associated with preterm delivery (< 37 weeks of gestation) [200, 201] and other adverse pregnancy outcomes such as chorioamnionitis [201]. This association is supported by the findings of the present study, which has established a level of correlation between *Ureaplasma* spp. and both a history of infertility, and the presence of *L. iners* in the FRT. Surprisingly, *Ureaplasma* spp. were only detected in the endometrium of one woman within the study by 16S rRNA gene amplicon sequencing, but this specimen was not positive when the samples were further tested using *Ureaplasma*-specific PCR assays. Previous studies have reported the presence of *Ureaplasma* spp. within the upper reproductive tract of infertile women [198]; however, as this study involved only cross-sectional sampling of the women, perhaps these results suggest that *Ureaplasma* spp. only transiently colonise the upper reproductive tract. This possible transient association of *Ureaplasma* with

the upper reproductive tract is supported by the fact that several other taxa described in this study were found to be present in both between the lower and upper reproductive tracts, although in significantly distinct proportions.

A previous study that investigated women undergoing assisted reproductive technology embryo transfer found that reduced alpha diversity of the endometrial microbiota was associated with the embryo transfer not proceeding to a successful pregnancy [183]. In this study, the alpha-diversity index was not seen to be significantly different based on case-control status, however, this is possibly not a valid comparison as this study is retrospectively based on women with a history of needing fertility treatment (infertile cases) or not needing fertility treatment (fertile controls). There are other noteworthy trends that have been reported in other reproductive tract microbiota studies (reviewed in [202]). Specifically, in the present study, *B. breve* sequences were over-represented in women who reported a history of endometriosis, *L. gasseri* was over-represented in women with a history of miscarriage, *G. vaginalis* was more frequently found in the younger aged women (28-33 in this study), and *Streptococcus* cluster 29 sequences were over-represented in women not using hormonal contraceptives compared to women using hormonal contraceptives. Whilst not significant after adjustment for multiple testing and limited by small sample size, these associations have been noted for consideration in future studies of the reproductive tract microbiota.

There were no apparent differences in endometrial gene expression based on case-control (fertility) status. The major finding for endometrial gene expression was that *TNC* had significantly higher expression in participants with history of miscarriage. The product of *TNC,* TN-C, interacts with fibronectin and has well-established roles in modulating cell adhesion and migration [203]. It is also

involved in the regulation of matrix metalloproteases, and has been implicated in endometriosis, recurrent miscarriages, and numerous cancers throughout the human body [204-207].

In a previous study, it was shown that the vaginal microbiota was altered after IUD insertion [177, 208]; in this study the microbiota did not appear to be influenced by the type of contraceptive in use at the time of sampling, although this may relate to the study's smaller sample size. It was expected to observe differences in the endometrial gene expression in women using LNG IUS compared to other contraceptive, given that this is a local device that releases progesterone specifically to modulate the local microenvironment. *IL6* expression has long been shown to be modulated by IUS contraceptives [209, 210], as was also demonstrated within this study. It has also been suggested to be a predictive indicator of endometriosis [211], although within the current study there were no significant differences in the endometrial levels of *IL6* in relation to self-reported history of endometriosis by participants. *IL8* was also differentially expressed in the endometrium based on contraceptive use, with slightly higher levels detected in participants with an LNG IUS. *IL8* is of interest as it plays an important role in angiogenesis of the endometrium and increased localised expression of *IL8* is thought to contribute to the pathogenesis of endometriosis [212, 213]. *IL8* has also been suggested to act as an autocrine growth factor and stimulate tissue attachment in endometriosis [214-216]. *SDC1* upregulation or protein localization has been reported as a marker of endometritis [217, 218]. *SDC1* was significantly downregulated in LNG IUS users. *IL1A* is a proinflammatory cytokine with roles in facilitating embryo implantation and activating matrix metalloproteases involved in remodeling the extracellular matrix, yet no significant differences were

observed between the case-control groups during this study. No significant differences were observed in the expression of *TNFA*, despite the fact that *TNFA* has been shown to function in pathological conditions of the endometrium, including failed embryo implantation and endometriosis [219-221]. *IL6* expression has been shown to be modulated by IUS contraceptives [209, 210] and has also been suggested to be a predictive indicator of endometriosis [211]. Consistent with previous reports, *IL6* expression was identified as significantly higher in women using the LNS-IUS device compared to the expression levels of endometrial tissues from women using other forms of contraceptives.

This study has a number of limitations that should be considered in the interpretation of its findings. Participants were recruited opportunistically and therefore the microbiota were not sampled at the time of confirmed infertility treatment or fertility. It is apparent from the findings of a longitudinal study that some (but not all) individuals do have a propensity for a particular microbiota profile [175], and so some of the results presented here may reflect the pre-disposition of the reproductive tract microbiota in individuals that may well be relevant to their past history of infertility. Participant recruitment targeted individuals attending the gynecology clinic for procedures such as hysteroscopy and hysterectomy (often sometime after their fertility treatment or pregnancies were completed). The mean age of the control group was also significantly older than that of the case group. Three reproductive tract sites were sampled from each of the patients recruited. However, inconsistencies in sample extraction quality and/or bacterial DNA sequence results limited the number of individuals with sequenced microbiota from all three sites. The sampling protocol for specimen collection during the hysteroscopy specified the need to differentiate vaginal,

cervical and endometrial samples and surgeons were advised to avoid the surface of the vaginal canal when removing the cervical sample, however the risk of possible cross contamination is noted. Future studies should include a much larger sample size and prospective recruitment during fertility treatment to further measure the role of the microbiota and immune responses at the time of treatment for infertility.

Conclusion

These findings strongly support the hypothesis that the microbiota and local tissue immune responses are likely to be an important influence on infertility. This is also consistent with current commentary in the fertility treatment field [174] and a very recent longitudinal study that identified that the endometrial microbiota composition significantly correlated with IVF cycle success, despite the lack of controls in the study [185]. Hence, we suggest the requirement for more extensive investigations into the microbiota and endometrial tissue response during fertility treatment.

Combined, these data provide some suggestion that the presence of certain taxa and the local tissue response may interact to generate a less receptive endometrial environment for embryo transfer. There is already a common practice of endometrial scratching (reviewed in [222]) during the menstrual cycle prior to embryo transfer. Because of this, it may be possible in the future to harvest the scratching material to test the endometrial microbiota profile and expression of select endometrial genes to predict if the endometrial environment is optimal to proceed with embryo transfer. However, such a future innovation will require further larger sized and longitudinal studies of the reproductive tract microbiota on women undergoing fertility treatment during their cycles. It is possible that in the future, with further information, the analysis and possibly modulation of the microbiota and concomitant host response profile will form a component of infertility treatment programs.

CHAPTER 4: CLINICAL ISOLATES SHOW SUBTLE DIFFERENCES IN PERSISTENCE PHENOTYPES AND GROWTH *IN VITRO*

Chapter notes

A significant proportion of the experiments, analysis and the interpretation of data comprising this chapter were conducted as components of my PhD research project. These original works are part of a larger ACTS clinical isolate characterisation study. As such, they are accompanied by additional unpublished work performed by other members of the research group. Thus, Chapter 4 is presented as the manuscript of this nearly completed larger study. A modified version will likely be submitted for publication very shortly and will provide valuable knowledge to the field of chlamydial biology. Specifically, it will enhance knowledge of the implications and considerations of using *in vitro* models to compare the developmental phenotypes of clinical isolates of *C. trachomatis*.

Name	Contribution
Mark Thomas	Developed aspects of experimental design, conducted some of the major experiments, performed interpretation and analysis of raw data and drafted the paper with the intent of future publication.
Amba Lawrence	Conducted some of the major experiments.
Sam Kroon	Designed and performed some of the experiments.
Jane Hocking	Responsible for ACTS and the clinical isolates.
Peter Timms	Assisted with interpretation of the findings and drafting of the paper.
Wilhelmina Huston	Developed major experimental design components, provided critical review of drafted versions of the manuscript as well as the analysis and interpretation of experimental findings.

Abstract

Urogenital tract infection by *Chlamydia trachomatis* is the most common sexually transmitted bacterial infection globally. While progress has been made to better understand how type strains develop and respond to environmental stress *in vitro*, very few studies have examined how clinical isolates behave under similar conditions. Here, we examined and compared *in vitro* the development and stress-response phenotypes of several important clinical isolates alongside type strain D/UW-3/Cx. Chlamydial persistence was a focus of the study as it was hypothesised to be an important factor involved in the survival strategy of isolates associated with treatment failure. All of the *C. trachomatis* isolates produced the highest number of infectious EBs at 44 h PI in the McCoy B murine fibroblast cell line, as did the type strain. This reflects and supports previous findings that showed the cell line is exceptionally permissive to chlamydial growth [223]. Conversely, the clinical isolates showed higher levels of infectivity in the MCF-7 human epithelial cell line. Development of the type strain D/UW-3/Cx in each of the six cell lines tested both yielded higher numbers of infectious progeny and progressed at a rate which was orders of magnitude higher than that shown by each of the clinical isolates. The clinical isolates were shown to be more susceptible than D/UW-3/Cx to the effects of penicillin and iron deprivation persistence models in the MCF-7 cell line. While subtle differences between clinical isolates were observed in each of the experimental *in vitro* models used, there were no significant differences identified. This study reinforces the importance of using clinical isolates when trying to relate *in vitro* data to clinical outcomes, as well as

the importance of considering the adaptations many type strains have developed as a result of *in vitro* culture.

Introduction

Chlamydia trachomatis is an obligate intracellular pathogen and causes the most prevalent bacterial sexually transmitted infection (STI) worldwide. There are more than 83,000 chlamydia infections recorded in Australia each year [224]. The pathology and sequelae associated with chlamydial diseases are caused by the infected individual‟s inflammatory response and potentially influenced by a number of important host factors [70]. In women, the spectrum of chlamydial disease ranges from mild cases of cervicitis, endometritis and salpingitis, to more serious cases of pelvic inflammatory disease (PID), tubal infertility, and life-threatening ectopic pregnancy.

Presumptive cases of urogenital tract *C. trachomatis* infection are treated with either azithromycin or doxycycline. While evidence suggests doxycycline is slightly more effective at clearing infection, azithromycin is often prescribed for its simple one-off treatment dose [113, 225]. Azithromycin is a broad-spectrum macrolide antibiotic with a relatively long (40 - 68 h) half-life and high lipid solubility and accumulates within macrophages migrating to the site of infection [115, 116]. Despite its high efficacy, instances do occur in which monitored women remain infected with the initial strain after treatment. The reasons for such cases have not yet been determined. Suggested causes include insufficient antibiotic adsorption, chlamydial gastrointestinal colonisation and auto-inoculation, and reinfection by sexual partners [29, 162]. Direct macrolide resistance is generally not considered a probable cause due to the antibiotic‟s

mechanism of action, and the scarcity of clinical isolates with genotypic or phenotypic resistance to azithromycin [118, 122-125]. Furthermore, azithromycin-resistant mutants created *in vitro* with single nucleotide polymorphisms (SNPs) in their 23S rRNA and L4 protein-encoding genes show poor biological fitness and viability [119-121].

When combined, *in vitro* and clinical evidence suggests that *C. trachomatis* survives by other means, which likely reflect both the pathogen and its intracellular niche. One candidate mechanism is the persistence stress response, which is believed to be a positive and beneficial adaptation unique to the genus *Chlamydia* [30, 31]. The persistence phenotype is characterised by several reversible morphological, transcriptional and metabolic changes [32-36]. The collective profiles of these changes vary depending on the inducer of persistence; however, the morphologically-aberrant RBs, altered inclusion sizes and the reversible loss of both cultivability and replicative capacity are the universal hallmarks of persistence. These morphological variations have been reviewed by Hogan *et al* [37].

Several environmental conditions and exogenous stimuli have been demonstrated to induce chlamydial persistence *in vitro* [77]. Penicillin and IFN-γ are two of the most extensively studied of these stimuli and have both been used to characterise different aspects of the persistence phenotype (reviewed by [226]). Use of the iron-chelating agent deferoxamine mesylate in a number of studies has shown that iron restriction not only induces persistence in *C. trachomatis*, but also alters the pathogen‟s signaling pathways in an attempt to inhibit host-cell apoptosis [46, 47]. Interestingly, IFN-γ also decreases cellular levels of iron in infected cells by downregulating their transferrin receptor expression [53].

Previous investigations into chlamydial persistence have established that type strains have variations in their degree of responsiveness to certain stimuli, and have altered susceptibilities to antibiotics while in the persistent state [227-230] In the present study, we examined the *in vitro* phenotypes of several unique clinical isolates of *C. trachomatis*. Their relative abilities to infect and develop in different cell lines were measured, as were their responses to two widely used and physiologically-relevant models of *in vitro* chlamydial persistence. Their susceptibility to azithromycin during persistence is also investigated, to better understand whether persistence has implications for treatment with frontline antibiotics.

Materials and methods

Cell culture and cultivation of *C. trachomatis*

McCoy B, MCF-7, CACO-2, HeLa, SiHa and ARPE-19 cell lines (details in **Table 4.1**) were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10 % vol vol^{-1} fetal calf serum (FCS; Sigma), 4 mM analyl-glutamine (Sigma), 100 µg ml^{-1} streptomycin (Life Technologies) and 50 µg ml^{-1} gentamicin (Life Technologies). All cell lines were incubated at 37˚C in a humid environment containing 5% CO_2. Each cell line in use was regularly confirmed to be free of *Mycoplasma* contamination. All six clinical isolates used were obtained from the Australian Chlamydia Treatment Study (ACTS). This is a cohort study of women diagnosed with and treated for chlamydia, designed to identify repeat infections and potential cases of treatment failure [162]. Isolates were collected from infected women using swabs stored in a 2 ml cryovial tube containing a sucrose-phosphate glutamate (SPG) buffer, at -80˚C/dry ice. Tubes were thawed to room temperature on ice, and a small volume of each tube's contents was used to inoculate individual wells of McCoy B cells cultured in 96-well plates. Cultures were centrifuged and incubated under standard conditions. Once a multiplicity of infection greater than 0.8 had been achieved in 96-well plates, isolates were then further cultured in 6-well tissue culture plates, before finally being cultured in T75 tissue culture flasks. Semi-purified stocks of each culturable isolate were created by scraping the monolayer of each flask into SPG, mechanically lysing the collected cells with glass beads, and centrifugation of the crude lysate to remove

the host cell debris. The five clinical isolates used in this study are shown below in **Table 4.2**.

Table 4.1. Details of the mammalian cell lines used in this study.

Name	Cell Type	Origin	ATCC® Code™
McCoy B	Fibroblast	Fibroblast from *Mus musculus*	CRL-1696
MCF-7	Epithelial	Human mammary epithelium	HTB-22
CACO-2	Epithelial	Human colorectal adenocarcinoma	HTB-37
HeLa	Epithelial	Human cervical carcinoma	CRM-CCL-2
SiHa	Epithelial	Human cervical squamous cell carcinoma	HTB-35
ARPE-19	Epithelial	Human adult retinal pigmented epithelium	CRL-2302

Table 4.2. Identification and details of the *C. trachomatis* clinical isolates and strain used in this study.

ACTS Code	OmpA Serovar	Clinical Outcome
1-017 (1)	K	Repeat infection, first incident
1-017 (13)	K	Repeat infection, second incident
1-020 (1)	D	Successfully treated
1-028 (1)	E	Successfully treated
1-049 (1)	K	Successfully treated
Strain Code	OmpA Serovar	Information
D/UW-3/Cx	D	Widely used genital type strain

Infectivity and determination of viable progeny

Three identical sets of McCoy B, MCF-7, CACO-2, HeLa, SiHa and ARPE-19 cells were cultured in 96-well plates, in triplicates for each condition. Once confluent, each set was infected with each of the five clinical isolates and type strain D/UW-3/Cx, at a multiplicity of infection (MOI) of 0.5 calculated for the Mc Coy B cell line. Cultures were immediately centrifuged at 500 × *g* and 37°C for 30 minutes, then incubated under standard conditions (37°C, 5% CO_2, 95% air). At 4 hours post infection (h PI), the infectious media in each well was replaced with complete DMEM supplemented with 1 µg ml^{-1} cycloheximide before further incubation under standard conditions. To determine the infectivity of the selected isolates in each cell line, a culture set was fixed with 100% methanol at 30 h PI for evaluation by immunofluorescence (IF) microscopy. To evaluate the viable progeny counts of each isolate after 44 and 54-hour time points of infection, the remaining two culture sets had their expired media replaced with SPG buffer and were stored at -80°C. Cultures were thawed and thoroughly lysed and homogenised by repeated pipetting, before the resulting lysate was used to create a 10-fold dilution series in complete DMEM. These were then overlaid onto fresh cultures of McCoy B cells in 96-well plates, which were fixed at 30 h PI for evaluation by IF microscopy. Infected cells were counted in a minimum of ten fields of view per well, which was combined with the area-per-field-of-view value to calculate the number of infected cells per well. This was in turn used to calculate the infectious progeny numbers in the original viability plates.

Penicillin persistence model

Identical sets of cultures were used to compare, using two different approaches, the effects of benzylpenicillin on the viability and morphological appearance of *C. trachomatis*. Viability culture sets were established in 96-well plates, by seeding 2.5×10^4 MCF-7 cells and using a working volume of 200 µl per well. The MCF-7 cell line was selected for use in the chlamydial persistence experiments on the basis that it is a steroid hormone-responsive, human epithelial cell line and thus, a suitable cell line for such a model. Our previous use of this cell line also showed that it is quite permissive to chlamydia growth, making it ideal for use in experiments with clinical isolates. Microscopy culture sets were established in 24-well plates, by seeding 6×10^4 MCF-7 cells per well, on 13mm coverslips, in a working volume of 1 ml. After approximately 24 hours of growth under standard incubation conditions (37°C, 5% CO_2, 95% air), each of the plates were all infected with each of the clinical isolates and D/UW-3/Cx, at a multiplicity of infection (MOI) of 0.8. Infected plates were immediately centrifuged at 500 × *g* and 37°C for 30 minutes, then incubated under standard conditions. Each of the clinical isolates and D/UW-3/Cx in both the viability and microscopy culture sets had their media replaced at 4 h PI with fresh growth media ± benzylpenicillin. Untreated (-Pen) control wells received of only. Treated wells (+Pen) received complete DMEM supplemented with 1 µg ml^{-1} cycloheximide and either 0.02, 0.05 or 1.0 U ml^{-1} of benzylpenicillin. Each set of cultures was then incubated under standard conditions. At 44 h PI, half of the viability and microscopy sets (-recovery) were harvested and stored at -80°C, and fixed with methanol, respectively. The remaing matching halves of both sets (+recovery) had the media removed from their wells,

which were then given two five-minute washes with PBS 1X before being refilled with fresh, penicillin-free DMEM, supplemented with cycloheximide. These were then returned to incubation under standard conditions. At 110 h PI, the remaining halves of each set were harvested and fixed, respectively, for comparison. Confocal microscopy was performed on the cultures grown on coverslips, while the infectious yield of the viability culture set was determined using an infectious dilution series of pipette-lysed SPG. This was used to incolulate fresh, confluent monolayers of McCoy B cells grown in in 96-well plates. These infected plates were immediately centrifuged at 500 × *g* and 37°C for 30 minutes, then incubated under standard conditions. These cultures were fixed with methanol at approximately 30 h PI and then visualised via IF microscopy.

Assessing azithromycin susceptibility during persistence

The methodology of azithromycin-penicillin co-treatment detailed by the present work is similar to the penicillin persistence model described above. Briefly, identical sets of 96-well plates were seeded with 2.5 x 10^4 MCF-7 cells. Cell were infected with three of the clinical isolates and D/UW-3/Cx after around 24 hours of standard incubation. Although this is fewer than in previous experiments, 1-017 (1), 1-017 (13) and 1-049 (1) were selected in order to narrow down the scope and volume of work, and focus more closely on these three clinically important Serovar K isolate. Near-confluent monolayers were infected at MOI 0.8, using complete DMEM spiked with a volume of SPG buffer containing 2.0 x 10^4 EBs. Cultures were centrifuged at 500 × *g* and 37°C for 30 minutes, then incubated under standard conditions At 4 h PI, the media in each infectious culture was replaced with complete DMEM supplemented with 1 µg ml^{-1} cycloheximide ±

benzylpenicillin. Each of the three clincal isolates were exposed to both a no (-Pen) and a low dose (0.05 U ml^{-1}) of benzylpenicillin, while D/UW-3/Cx was exposed to both a no (-Pen) and a high dose (1.0 U ml^{-1}). Each of the cultures were incubated under standard conditions until 14 h PI, at which point azithromycin was added to half of each culture set. The dose of azithromycin used to treat (+Az) the cultures reflected each clinical isolate and the lab strain‟s previously-determined minimum inhibitory concentration (MIC) value for azithromycin. The other set of cultures (-Az) had an equivalent amount of DMSO diluted in DMEM added as a mock treatment. At 44 h PI, half of the culture sets (-recovery) were harvested into SPG and stored at-80°C. The remaing half of the cultures (+recover) underwent the same penicillin recovery treatment process described above, and were incubated under standard conditions before being harvested at 110 h PI and stored at -80°C. Viability was determined as previously described, using serial dilutions of the infectious lysed SPG enumerate the infectious progeny by IF microscopy.

Iron deprivation culture model

A modified version of the methods published by Thompson and Carabeo [231] was used to examine the effects of iron deprivation on clinical isolates of *C. trachomatis*. MCF-7 cells were cultured in 96- and 24-well plates and infected with each of the five clinical isolates and D/UW-3/Cx (**Table 4.2**: MOI of 1.0) as previously described. At 4 h PI, infections media was replaced in each of the cultures with complete DMEM supplemented with 1 µg ml^{-1} cycloheximide and 0-400 µM of the iron chelator 2,2‟-Bipyridyl (Bpdl). Untreated infected controls received equivalent volumes of 95% ethanol and no Bpdl. Cultures were incubated under standard conditions until 44 h PI, at which point they were either harvested

for viability, fixed for microscopy, or allowed to recover. This involved replacing the media in each of the recovery cultures with fresh DMEM supplemented with 1 μg ml^{-1} cycloheximide and 100 μM of iron chloride ($FeCl2_3$). These were then harvested for viability or fixed at 96 h PI, and compared to the pre-recovery (44 h PI) sets. Azithromycin and Bpdl co-treatment experiments had an additional 16 h PI media change, which included the addition of 0.064 μg ml^{-1} of azithromycin to the MIC-dose treated cultures.

Results

Differences between the infectivity and growth of clinical isolates are more profound than the differences caused by the cell line

Six cell lines were selected, to compare how susceptible they were to infection by four of the clinical isolates, and type strain D/UW-3/Cx (**Figure 4.1.**). The human mammary epithelial cell line (MCF-7) was found to be the most permissible for infection by each of the four clinical isolates, with 35-40% of all counted cells infected. Both the retinal cell line (ARPE-19) and cervical carcinoma cell line (SiHa) were observed to have the lowest percentage of infectivity (5-10%) for all four clinical isolates, while the type strain was observed to have produced inclusions in 25-30% of the counted cells in both these lines. While the type strain showed a similar level of infectivity across the McCoy B, MCF-7, HeLa and SiHa lines, it did show slightly lower levels in CACO-2 and ARPE-19. The same six cell lines used for the infectivity assay were infected and harvested at two timepoints, to determine the number of viable infectious progeny that had been produced during the developmental period. All four isolates produced the most infectious progeny (IFU ml^{-1}) in the McCoy B cell line (**Figure 4.2**) at both 44 (**Table 4.3**) and 54 h PI (**Table 4.4**). For example, clinical isolate 1-017(1) grown in McCoy B cells yielded $1.2x10^6$ and $1.4x10^6$ IFU ml^{-1} at each of the time points respectively; while at the same time points, growth in HeLa cells yielded only $3.7x10^4$ and

$4.2x10^4$ IFU ml^{-1}. Similarly, the type strain produced higher levels of progeny in the McCoy B cells ($9.8x10^6$), in CACO-2 ($9.4x10^6$) and HeLa cells ($7.1x10^6$), compared to the fewer in MCF-7 ($1.1x10^6$), SiHa ($1.9x10^6$) cells, and ~100-fold fewer in ARPE-19 cells ($5.9x10^4$). Growth in the ARPE-19 cell line was also shown to yield the lowest number of infectious progeny from the clinical isolates, with all four showing no detectable progeny. Less variation in the viability of clinical isolates was seen in the SiHa cell lines (**Figure 4.1**) than the other four which proved to be permissible for growth. 1-017(1) appeared to produce slightly more infectious progeny than 1-017(13) in the McCoy B cell line (44 h: $1.2x10^6$ vs $7.4x10^{4;}$ 54: $1.4x10^6$ vs $5.8x10^4$) than the other four cell lines permissible to its growth.

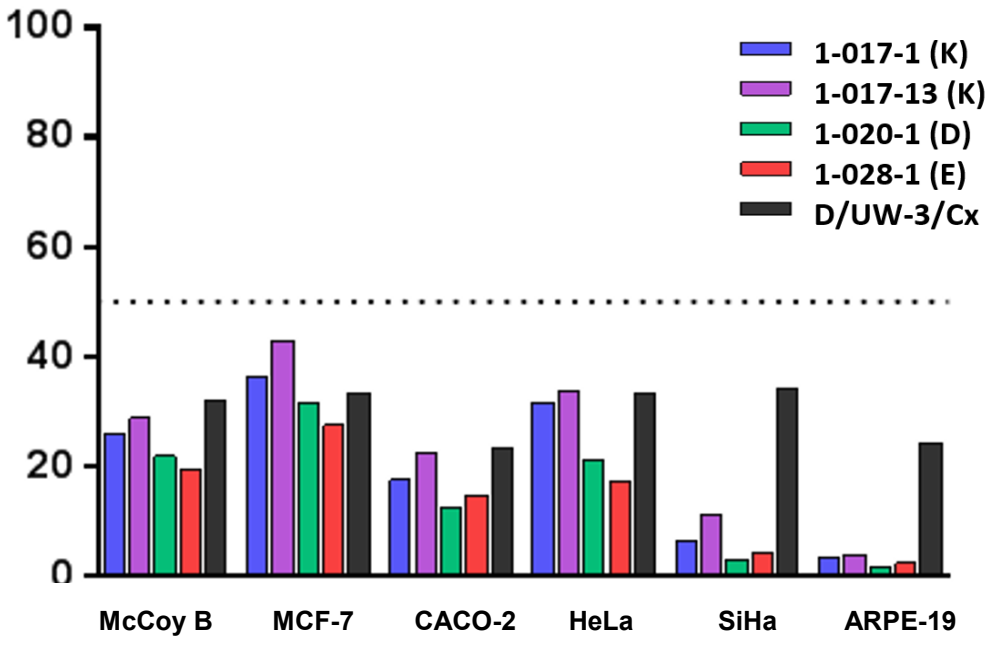


Figure 4.1. The percent infectivity of four clinical isolates and D/UW-3/Cx in various cell lines. Six routinely-used cell lines were cultured and infected with four clinical isolates and the laboratory strain shown in the figure legend. Infected cultures were fixed at 30 h PI and observed via IF microscopy. Percent infectivity was determined by calculating the total number of infected and uninfected cells in each monolayer.

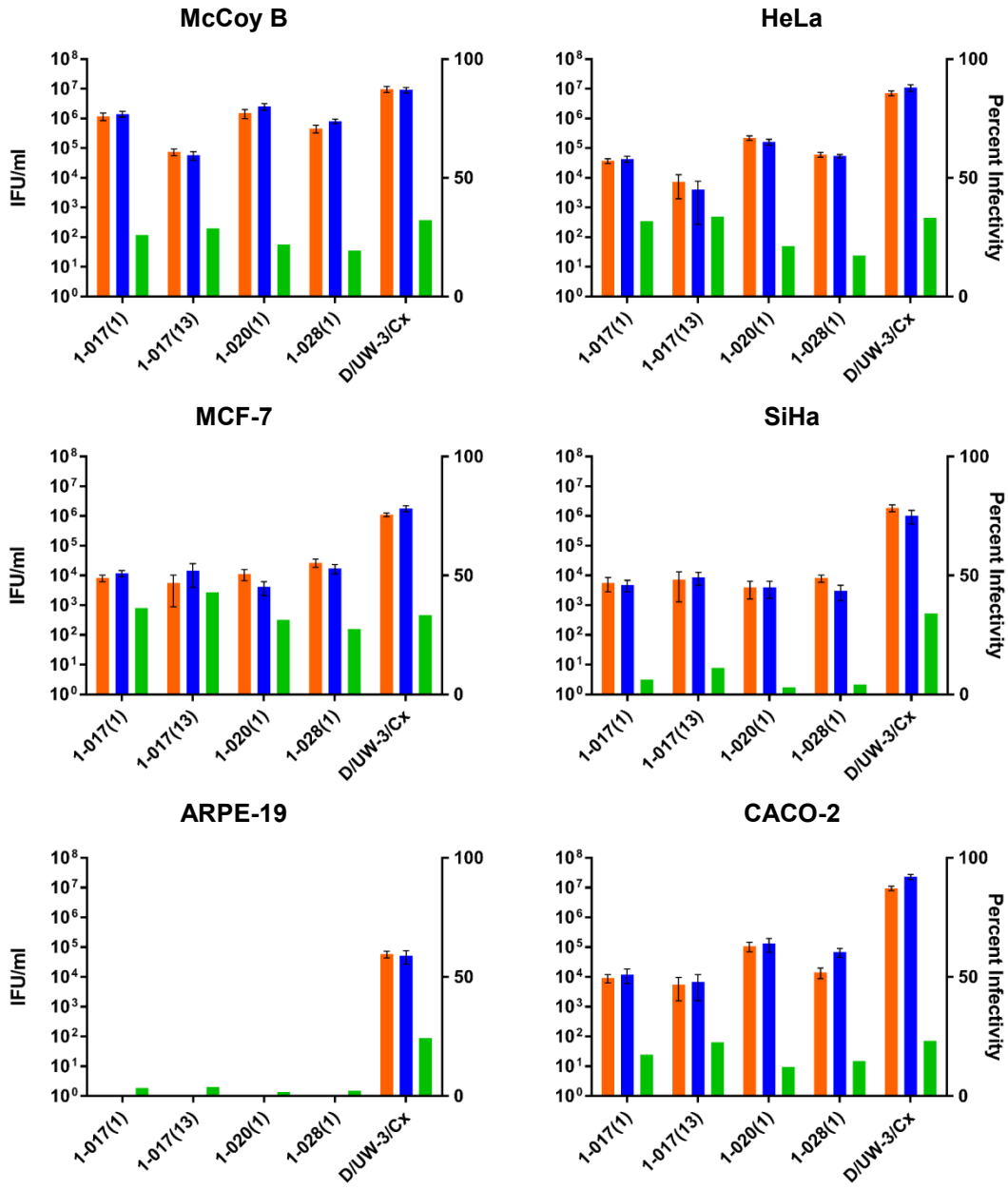


Figure 4.2. The growth and infectivity of *C. trachomatis* clinical isolates and a type strain in different mammalian cell lines. The same four clinical isolates and type strain D/UW-3/Cx were cultivated in six different mammalian cell lines. Infectivity is shown in green and the units (0-100%) are shown on the right Y-axis of each graph. The viability of each isolate/strain was measured by harvest, semi-purification and reinfection of triplicate cultures at two time points. The IFU/ml (left Y-axis) at both 44 and 54 h PI are shown as orange and green, respectively.

Table 4.3. Viability of the *C. trachomatis* clinical isolates and type strain at 44 h PI.

	HeLa	McCoy B	MCF-7	SiHa	ARPE-19	CACO-2
1-017(1)	3.72E+04	1.20E+06	8.21E+03	5.71E+03	0	9.29E+03
1-017(13)	7.35E+03	7.42E+04	5.63E+03	7.32E+03	0	5.65E+03
1-020(1)	2.19E+05	1.50E+06	1.14E+04	4.02E+03	0	1.09E+05
1-028(1)	6.10E+04	4.61E+05	2.71E+04	8.20E+03	0	1.43E+04
D/UW-3/Cx	7.08E+06	9.83E+06	1.11E+06	1.89E+06	5.88E+04	9.46E+06

Table 4.4. Viability of the *C. trachomatis* clinical isolates and type strain at 54 h PI.

	HeLa	McCoy B	MCF-7	SiHa	ARPE-19	CACO-2
1-017(1)	4.28E+04	1.41E+06	1.19E+04	4.83E+03	0	1.22E+04
1-017(13)	4.02E+03	5.80E+04	1.45E+04	8.69E+03	0	6.76E+03
1-020(1)	1.63E+05	2.54E+06	4.18E+03	4.02E+03	0	1.33E+05
1-028(1)	5.44E+04	8.09E+05	1.74E+04	3.06E+03	0	6.82E+04
D/UW-3/Cx	1.09E+07	9.13E+06	1.80E+06	1.04E+06	5.15E+04	2.33E+07

Clinical isolates show similar susceptibilities to conditions of iron deprivation at lower doses than D/UW-3/Cx

All five clinical isolates and the type strain were cultivated in MCF-7 cells and treated with 0, 100, 200 (D/UW-3/Cx only) and 400 μM bipyridal (Bpdl). Recovery was performed on one set at 44 h PI by way of adding 100 μM iron (as $FeCl_3$) to reverse the effects of Bpdl. Cultures were harvested at 44 and 96 h PI and viability was determined. As seen in **Figure 4.3A**, the type strain was able to recover from up to 100 μM of Bpdl ($7.1x10^4$ IFU ml^{-1} before recovery) following the supplementation of $FeCl_3$ into culture ($3.1x10^8$ IFU ml^{-1} after recovery). The five clinical isolates showed lower recovery, even from at the lowest dose of Bpdl, as seen in **Figure 4.3B-F**. For example (**Figure 4.2C**), at the 100 μM dose of Bpdl with and without recovery, 1-017(13) yielded $3.1x10^3$ and $7.0x10^2$ IFU ml^{-1} (very near the limit of detection for this assay) respectively. All five isolates produced viable infectious progeny in a manner that was dose-dependent, with slightly lower levels of infectious progeny present in cultures treated with 400 μM than those treated with 100 μM.

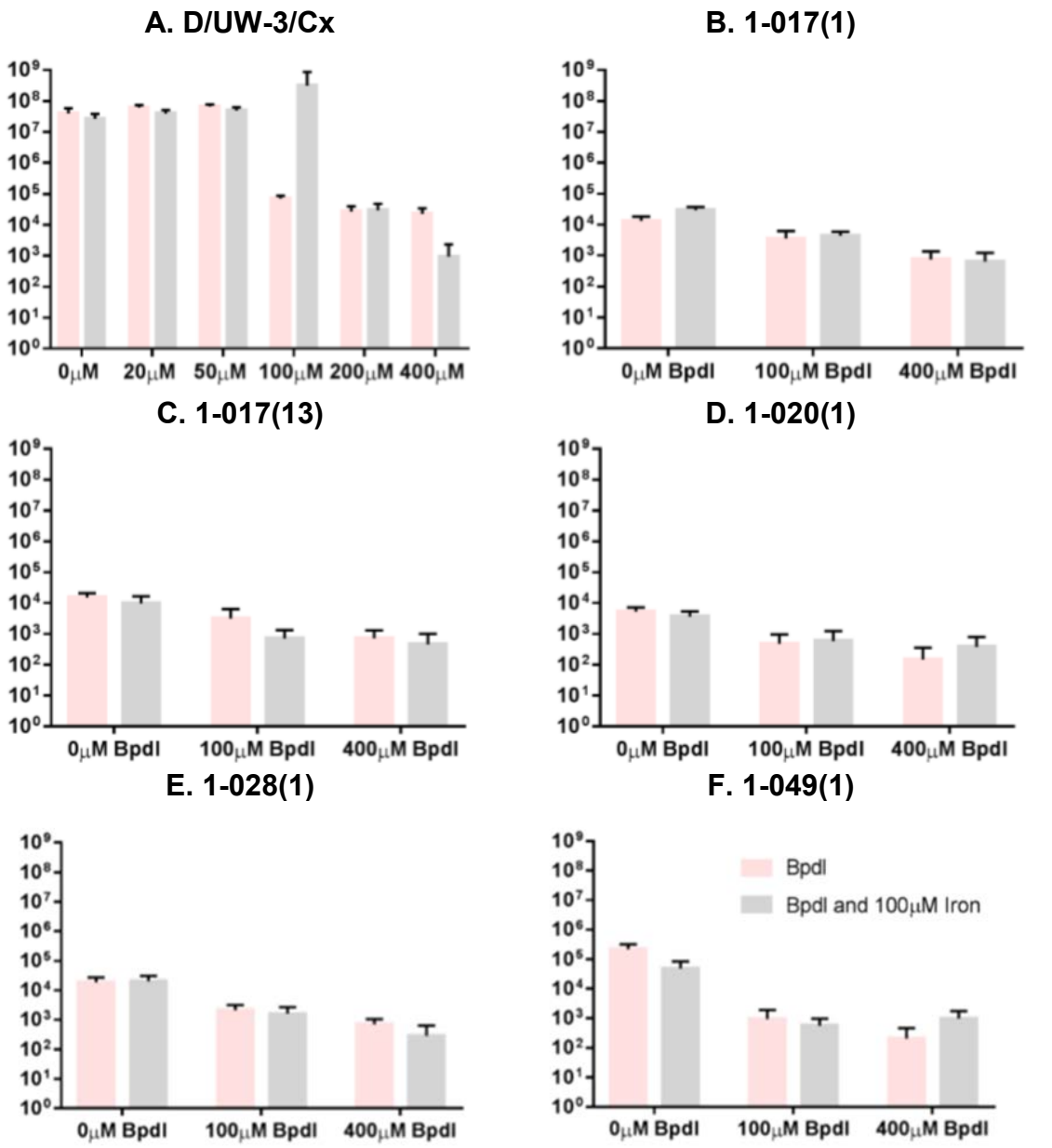


Figure 4.3. Viability of clinical isolates and the type strain in conditions of iron deprivation. This figure shows the amount of infectious progeny produced by five clinical isolates and type strain D/UW-3/Cx in MCF-7 cells treated with Bpdl ± 100 μM iron as $FeCl_3$ for recovery. Each value represents the mean of three experimental replicates with error bars showing the standard error of the mean (SEM).

Treatment with azithromycin during iron deprivation-induced persistence decreases the number of recoverable infectious progeny among clinical isolates

Each of the five clinical isolates and the type strain were cultured in MCF-7 cells and treated with Bpdl, azithromycin (Az), and $FeCl_3$ for recovery. All six of them showed a significant decrease in viability at 44 h PI when treated with azithromycin, Bpdl, or both (**Figure 4.4**). Compared to the untreated control at 44 h PI ($5.4x10^5$ IFU ml^1), D/UW-3/Cx showed an impaired ability to recover from the effects of Bpdl when also treated with azithromycin ($2.9x10^4$ IFU ml^{-1}), even when accompanied by iron supplementation ($5.0x10^2$ IFU ml^{-1}). Compared to their respective untreated controls at 44 h PI (**Figure 4.4**), all five clinical isolates were found to have only slight differences in their resulting viability when treated with the combinations of azithromycin, Bpdl, and $FeCl_3$. For example, the untreated control 1-017(13) culture produced $2.2x10^4$ compared to only $1.5x10^3$ IFU ml^{-1} after treatment and recovery. Analysis of the cultures by confocal microscopy showed morphologies consistent with persistence, as inclusions visible were consistent with persistence after treatment with penicillin; and regular development after allowing for recovery from the drug (**Figure 4.5**).

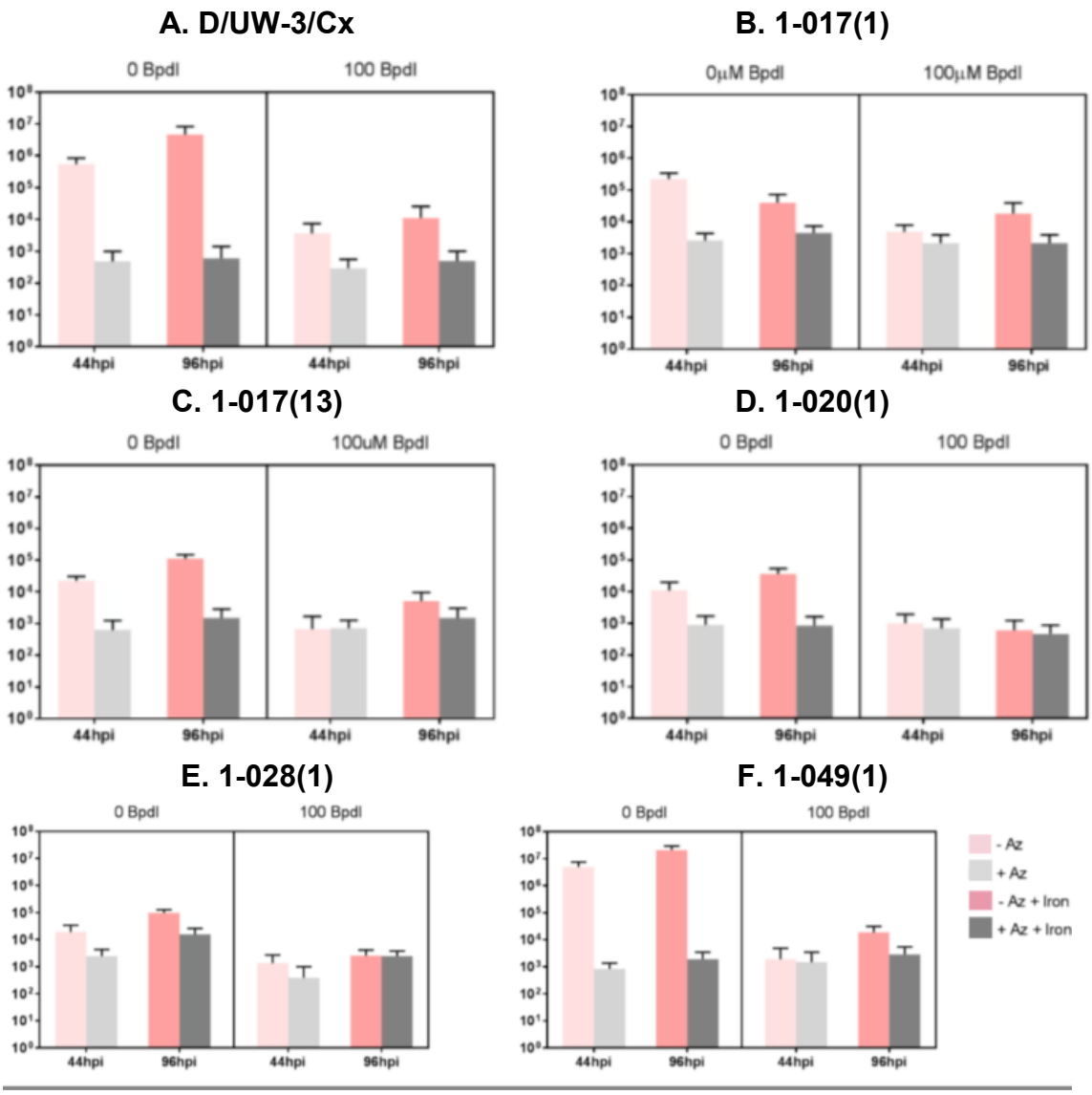


Figure 4.4. Viability of five clinical isolates and the type strain following treatment with bipyridal and azithromycin. The number of infectious progeny produced by five clinical isolates and type strain D/UW-3/Cx in MCF-7 cells treated with Bpdl, Az ± 100 μM iron as $FeCl_3$ for recovery. Each value represents the mean of three experimental replicates and error bars show SEM.

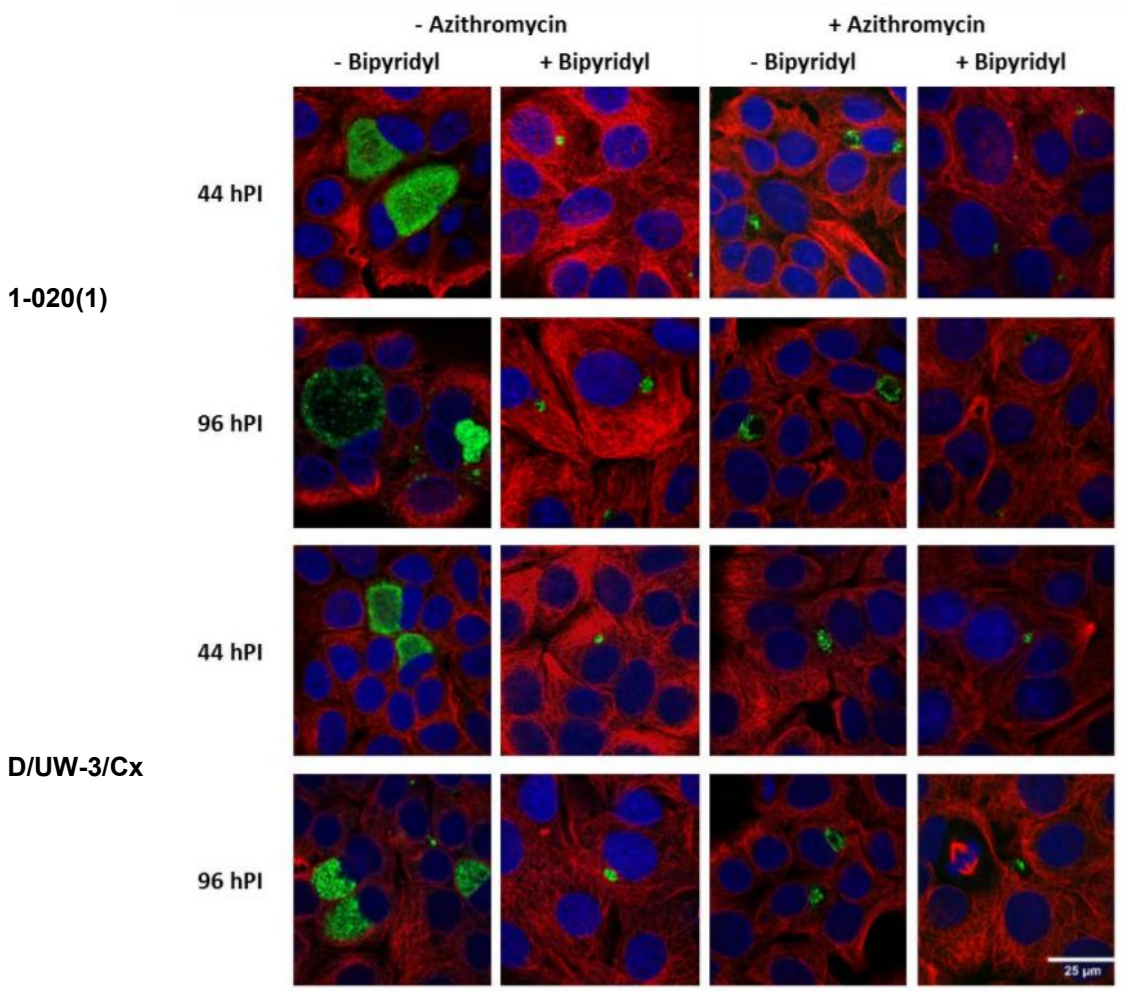


Figure 4.5. Morphological characterisation of a clinical isolate and type strain of *C. trachomatis* after treatment with bipyridal. Confocal microscopy of D/UW-3/Cx and clinical isolate 1-020(1) was performed during *in vitro* iron-deprivation induced persistence. In the above panels, *C. trachomatis* HtrA appears as green, host alpha-tubulin as red, and host cell nucleus as blue. The scale bar indicates 25 µm.

Clinical isolates entered persistence at lower doses of penicillin compared to the type strain D/UW-3/Cx

Several doses of penicillin were used to induce persistence in each strain, which was confirmed by allowing measuring viability before and after recovery from the drug. The infectivity data shown in **Figure 4.6** demonstrates that compared to the untreated controls at 44 h PI, the addition of 0.02 U ml^{-1} penicillin had slightly impacted the development of each clinical isolate, while the 0.05 U ml^{-1} and 1.0 U ml^{-1} doses had rendered them non-cultivable. Conversely, only the 1.0 U ml^{-1} dose of the antibiotic was able to induce persistence in the type strain, which exhibited less pronounced effects than those of the clinical isolates when observed at the lower doses. 1-017(1) and 1-017(13) produced $2.1x10^6$ and $5.5x10^5$ IFU ml^{-1} in their respective untreated cultures, compared to $6.6x10^5$ and $8.7x10^4$ IFU ml^{-1} in the cultures treated with the lowest dose of penicillin. D/UW-3/Cx exhibited only slightly impacted growth at both the lower doses ($5.0x10^7$ IFU ml^{-1} untreated versus $4.1x10^6$ and $9.4x10^4$ at 0.02 and 0.05, respectively), and only became non-cultivable at the 1.0 U ml^{-1} dose. Each of the five clinical isolates and the type strain were able to recover and re-enter (after rescue at 44 h PI) the regular developmental pathway by 110 h PI, and all produced a similar level of infectious progeny, even after treatment with 1 U ml^{-1} of penicillin. Confocal microscopy of penicillin-treated cultures supported this finding. **Figure 4.7** shows each of the six strains at 44 and 110 h PI, grown in the presence and absence of penicillin. At 44 and 110 h PI, in the absence of penicillin, each of the five clinical isolates and the type strain showed morphologies consistent with regular development. Comparatively, the inclusions observed in penicillin-treated cultures at 44 h PI

were significantly smaller and contained enlarged particles, both of which are morphological features of persistent development. Imaging of the cultures at 110 h PI (66 hours after the removal of penicillin from culture) showed inclusions typical of regular growth – indicating that all six strains had reverted from the persistent form into the regular developmental pathway.

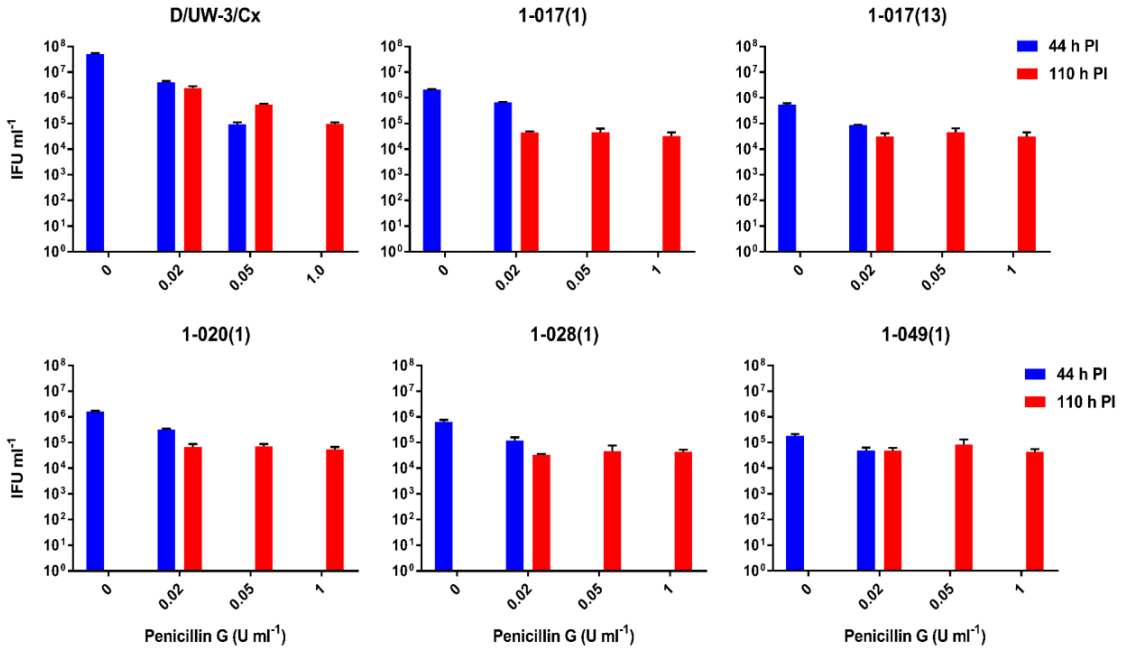
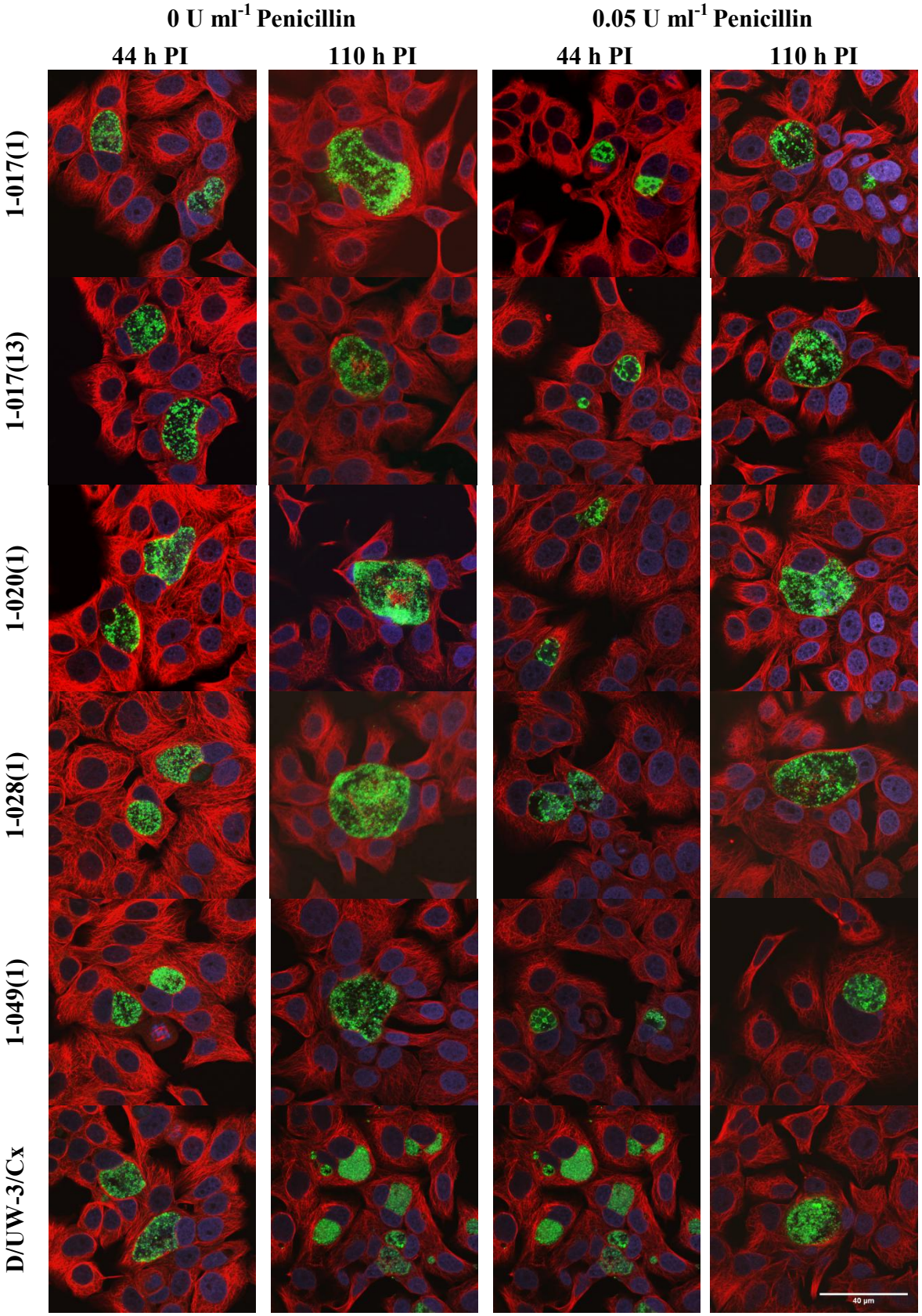


Figure 4.6. Viability of the chlamydial isolates and type strain after treatment with penicillin. Enumeration of viable infectious progeny was conducted by infecting McCoy B cells with serial dilutions of the SPG from each individual culture harvested at 44 and 110 h PI. Data shown represents the mean value of three identical treated wells, each further cultured in triplicate to determine the IFU present after a single experimental condition. Error bars represent the SEM.



(Figure legend and description of Figure 4.7 continued on next page.)

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Figure 4.7. Morphological appearance of clinical isolates and the type strain of *C. trachomatis* during penicillin persistence. Confocal microscopy of the six strains in the presence and absence of penicillin, before and after a period of recovery from the effects of the antibiotic. Chlamydial HtrA appears as green, host alpha-tubulin as red, and host cell nucleus as blue. Scale bar shows 40 μm.

Clinical isolates treated with azithromycin during penicillin persistence showed a dose-dependent decrease in recoverable infectious progeny

To assess whether clinical isolates had altered susceptibilities to azithromycin during persistence, three clinical isolates and D/UW-3/Cx were cultured in MCF-7 cells and treated with both penicillin and azithromycin. Persistence was induced using 0.05 U ml^{-1} of penicillin for the three clinical isolates and 1.0 U ml^{-1} for the type strain. At 44 h PI, **Figure 4.8** shows a complete loss of cultivability for the penicillin-treated cultures, which was recoverable by 110 h PI. The cultivability of the four strains at 44 h PI was also observed to decrease by up to 100-fold lower when treated with azithromycin alone. In the type strain, this effect was also seen, whereby the recoverable infectious progeny decreased in the presence of azithromycin only, with a complete loss of viability at 44 h PI when both azithromycin and penicillin were added. The effects of combining penicillin and azithromycin on the clinical isolates are less clear, however, 1-017(1) did produce more infectious progeny at 110 h PI than 1-049(1), both with and without penicillin.

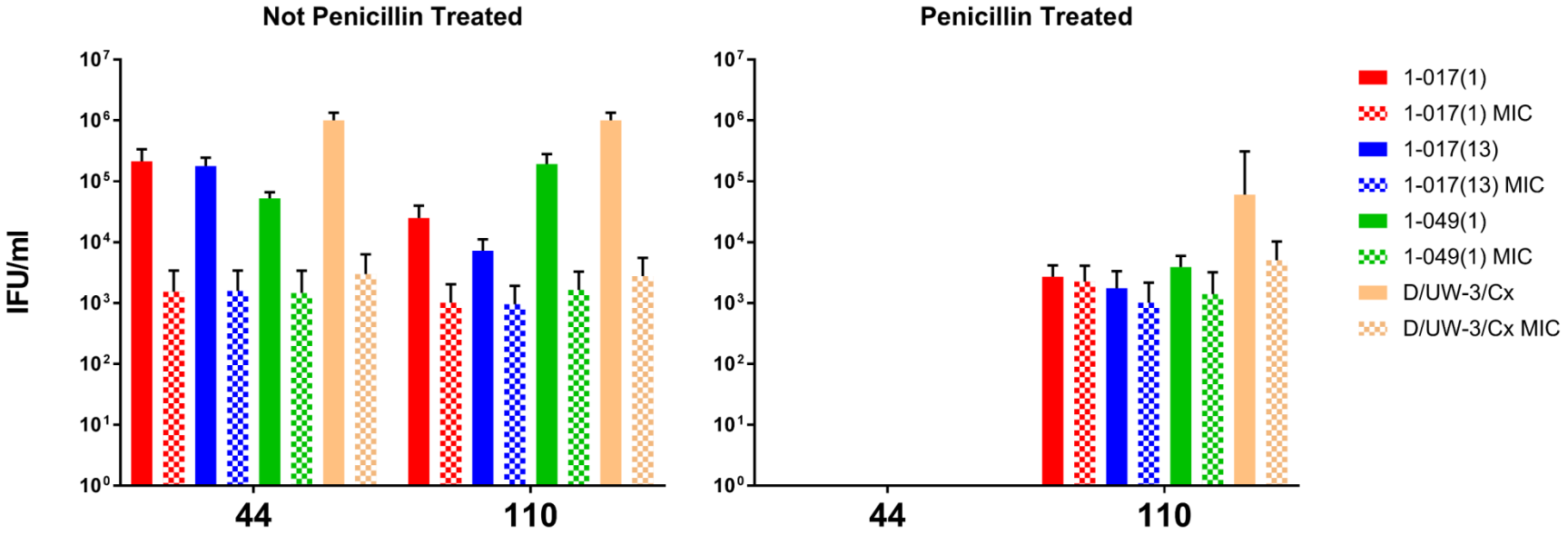


Figure 4.8. Viability of clinical isolates and D/UW-3/Cx after treatment with azithromycin during penicillin persistence. Data in this figure showing the IFU ml^{-1} of each clinical isolate and type strain D/UW-3/Cx. Cultures were infected with *C. trachomatis* before persistence was induced (±benzylpenicillin) and azithromycin was added. Viability was measured at 44 h PI and 110 h PI (± recovery) for each condition. Error bars show standard deviation of triplicate wells.

Discussion

The susceptibility and growth permissiveness of different cell lines to infection by *C. trachomatis* enables examination of the complex host-pathogen relationship, which begins with chlamydial entry, and finishes with chlamydial exit [165, 223, 232, 233]. Such studies frequently find differences between strains in their ability to enter the host cell, and complete their developmental cycle [234]. Conversely, much less is known about how the genotypes and phenotypes of chlamydia isolates from clinical samples impact and relate to the pathogen‟s virulence, and the host‟s defence against infection. The infectivity of the clinical isolates in the present study was observed to be highest in the MCF-7 cell line, despite the long-standing practice throughout the field of using McCoy or HeLa cells for cultivation and isolation of *C. trachomatis*, especially from clinical samples [235-237]. This does however make sense when also considering the viable infectious yields produced in each cell line, which were indeed highest in the McCoy B cell line for each of the strains tested. The ARPE-19 cell line was observed to have a very low susceptibility to infection by, and permissiveness of growth to all strains except D/UW-3/Cx – yet was nonetheless able to be infected by each of the strains. This reinforces the key phenotypic differences between type strains and clinical isolates respectively, and likely reflects the adaptation of D/UW-3/Cx to growth *in vitro*, in this and other immortalised cell lines [238].

Although it has been shown that type strains of *C. trachomatis* have varying levels of susceptibility to the *in vitro* effects of IFN-γ, fewer studies have examined how

different strains respond to penicillin or iron deprivation [23]. In this study, we examined how clinical isolates respond to such conditions. Both treatments were selected on the basis that penicillin has been used consistently by better understand chlamydial persistence [40, 41, 230, 239]; while iron deprivation is a more clinically relevant model when considering the physiology of the female reproductive tract [54, 55, 240]. Penicillin is known to induce persistence by interacting with chlamydial penicillin-binding proteins (PBPs), while host cell-derived iron is essential for chlamydial development [54, 55]. Both points combined raise the possibility that genetic variation among infecting strains could result in differing thresholds at which they divert from the regular developmental cycle into persistence. Because so little is known about the mechanisms by which *C. trachomatis* enters and exits the persistent state, it is also important to consider that the genes encoding PBPs are unlikely to be the only basis for any differences in susceptibility.

To assess the susceptibility of each strain to penicillin, they were treated with a dose range in MCF-7 cells. None of the clinical strains showed any notable difference in their levels of susceptibility, with all entering a viable but non-cultivable state at 0.05 U ml^{-1} of the antibiotic. In contrast, the type strain D/UW-3/Cx remained cultivable up to the maximum dose used, which was 1 U ml^{-1}. A recent study by the Schoborg group into the effects of beta-lactam antibiotics on *C. trachomatis* showed that type strain E/UW-3/Cx entered persistence at 0.02 U ml^{-1} of benzylpenicillin [230]. Although HeLa and MCF-7 cell lines were used by the Schoborg group and the present study respectively, this suggests there may indeed be different susceptibilities among type strains. While our experiments showed no

obvious difference in the susceptibility of clinical strains to the effects of penicillin, a broader dose range may be useful to identify more subtle differences.

Similarly, there were no large observed differences in the responses of clinical isolates to the effects of iron deprivation. In a recent review, Pokorzynski and colleagues postulated a complex system by which *C. trachomatis* may be able to both passively and actively acquire ferrous and ferric iron from within the host cytoplasm by modulating the hosts own iron trafficking pathways [241]. As with other instances of chlamydial persistence, it is possible that strain differences may in turn result in slight differences in the chlamydial porins and siderophores, as well as the inclusion membrane composition itself. Population heterogeneity in such an important metabolic acquisition process may itself be a means by which a subpopulation enters or remains in persistence *in vivo*. Another important consideration of this complex host-pathogen relationship is the cyclically-fluctuating concentration of iron within the female reproductive tract.

The effects of azithromycin were tested on several clinical isolates during both active and persistent development. We observed no differences in the susceptibilities of active and persistent infections, in any of the clinical strains tested. Previous findings by the Caldwell group demonstrate that during IFN-γ-mediated persistence, a serovar D type strain of *C. trachomatis* is significantly more susceptible to azithromycin [228]. However, Wyrick and Knight have shown that a serovar E type strain is less susceptible to the same antibiotic during penicillin-mediated persistence [41]. This highlights again that different inducers of persistence clearly produce different phenotypes, which likely reflects the physiological stress the inducer places upon *C. trachomatis*.

Collectively these findings show that clinical isolates respond to the effects of penicillin, iron deprivation, and azithromycin in a similar but more dramatic way than type strain D/UW-3/Cx. It is known that many inducers of persistence produce similar and overlapping transcriptional and morphological phenotypes (reviewed in [242]). Here, we have demonstrated that clinical isolates of *C. trachomatis* showed subtle variation in their growth and thresholds for entering persistence. While these subtle variations were observed using *in vitro* models, it is plausible that such differences are more pronounced and impactful in the complex *in vivo* environment.

CHAPTER 5: ANALYSIS OF HOST AND CHLAMYDIAL GENE EXPRESSION IN WOMEN TREATED FOR *C. trachomatis* INFECTIONS OF THE UROGENITAL TRACT

Chapter notes

Chapter 5 details a study in my research project which investigated the host and pathogen factors involved in treatment failure. Specifically, I examined the host and microbial gene expression in the endocervical swabs of selected ACTS participants defined using a nested case-control type of study design. It should be noted that the selection of participants for inclusion in this particular study was done independently of their inclusion in any other related or unrelated studies. Thus, their inclusion in a previous investigation(s) formed no part of the selection criteria. They were included based on being cases (repeat infection or treatment failure) and then two of the most closely matched controls (age, contraception and infecting serovar where possible). The key works of this study are presented in manuscript form and will likely be expanded upon or used to accompany other studies conducted using ACTS samples.

Name	Contribution
Mark Thomas	Conducted all experimental components, contributed to the research plan development, performed analysis of the data and drafting of the manuscript for future publication.
Jane Hocking	Contributed to major aspects of study design, assisted with interpretation of the findings.
Peter Timms	Assisted with interpretation of findings.
Wilhelmina Huston	Developed major experimental design components, provided critical review of drafted versions of the manuscript as well as the analysis and interpretation of experimental findings.

Abstract

The intracellular pathogen *Chlamydia trachomatis* is in constant opposition with both its host cell and the coordinated immune response to its presence. In order to survive within its unique environmental niche, it employs a variety of strategies to alter not only its own cellular processes, but those of its host as well. This evasion and modulation of the immune system is thought to contribute to the high rate of asymptomatic infections in the general population. In many cases, the immune system facilitates clearance of the infection, without causing the inflammatory symptoms that lead to a diagnosis. Clinical studies have demonstrated that *C. trachomatis* rarely survives even a single 1 g dose of azithromycin. Non-clearance after antibiotic treatment is commonly attributed to re-infection, newly acquired infections, and treatment failure. Though poorly understood, this complex process likely involves various important host and microbial components and is associated with an increased chance of pathology and sequelae. Thus, non-clearance is a clinically important aspect of chlamydial infection. To improve collective knowledge of the factors involved in infectious clearance, the biological effects of azithromycin on both the human and chlamydial cells of the endocervical microenvironment were examined. A nested case-control study was performed to assess the degree of association between clearance and various host and chlamydial genes relevant to immunity and treatment. A total of 34 ACTS participants were divided into control (n=22) and case (n=12) groupings based on their treatment outcomes. Reverse transcription qPCR was performed using samples from endocervical swabs taken before (T_0) and after (T_1) the participants

received 1 g azithromycin. Analysis revealed that after treatment, the expression of several human genes involved in the immune response to chlamydial infection was lower in women whose infections were not cleared by azithromycin. Specifically, it revealed that levels in the post-treatment (T_1) case group were lower than one or both of the pre-treatment ($T_{0)}$ groups‟ levels of *CXCL9*, *IL8*, *IDO1*, *TNFA*, *IL1A* and *IL10* (*CXCL9* (case: p= 0.024; control: p=0.0038), *IL8* (control: p=0.0152), *IDO1* (control: p=0.0419), *TNFA* (control: p=0.0035), *IL1A* (control: p= 0.0084) and *IL10* (case: p=0.019 and control: p=0.0009)). Conversely, analysis of chlamydial gene expression in the same samples revealed that the levels of *euo*, *omcB*, *ompA*, *htrA* and *trpBA* in the post-treatment (T_1) case group were all significantly higher than the levels of expression of both the case and control groups prior to (T_0) treatment (*euo* (case: p= 0.0240, control: p= 0.0047), *htrA* (case: p=0.0355, control: p= 0.0014), *omcB* (case: p=0.0001, control: p<0.0001), *ompA* (case: p= 0.0046, control: p=0.0001) and *trpBA* (case: p=0.0011, control: p=0.0161)). Collectively, these data suggest that the expression of genes involved in inflammatory processes is downregulated, even when the immune system and antibiotics are unable to facilitate clearance of the pathogen. Secondary to this, the overexpression of chlamydial genes involved in both active and persistent development could be an important feature associated with the host‟s inability to resolve the infection. These findings indicate a degree of correlation between unresolved infections and the altered gene expression of both *C. trachomatis* and its host. However, it is unclear whether there is a causal relationship between these two factors.

Introduction

Localised immune processes in the lower portion of the female reproductive tract serve to defend the upper portions from pathogenic microbes, and facilitate successful reproduction [243]. This requires a constant state of symbiosis between the vagina and the beneficial bacterial flora present within it, which is to the detriment of professional pathogens [140, 244]. A fine balance is maintained by a combination of host and microbial factors, many of which remain to be fully understood. Evidence suggests that not only do the dominant species in healthy women provide a protective effect, but also that changes in their abundance and that of their metabolites are associated with increased incidence of bacterial vaginosis [245]. Studies have long shown that in many women, the vagina is colonised predominantly by lactobacilli [173, 246]. However, more recent evidence suggests that the species of *Lactobacillus* found to be present in the highest abundance can be a crucial factor in predicting dysbiosis and disease [247].

In 2011, Ravel and colleagues used next generation sequencing approaches to identify and define five community state types (CSTs) of the vagina [131]. The five distinct CSTs are defined by the relative taxonomical abundance of the host vaginal microbiome, which has become an increasingly attractive candidate for 16S rRNA sequencing in a number of clinical contexts [140, 248-250]. Importantly, Ravel and contributors identified that while CSTs I-III and V are predominated by lactobacilli (*L. crispatus*, *L. gaserii, L. iners,* and *L. jensenii* respectively), CST IV had no dominant *Lactobacillus* species, and was instead a

taxonomically diverse community, with a higher relative proportion of anaerobic bacteria [131]. While the complex connection between the vaginal microbiome and localised host gene expression has not been completely defined, recent studies have described a number of important symbiotic interactions. Communities of protective *Lactobacillus* spp. have been observed to benefit from the presence of functional and homeostatic levels of proinflammatory cytokines such as IL-6, IL-1β and IL-8; yet the same communities appear to be negatively impacted by dysbiotic levels of the same cytokines [251]. Similarly, these same three cytokines have been seen to be expressed differently in cases of bacterial vaginosis, in the presence or absence of infection by *C. trachomatis* [252]. Finally, the abundance of vaginal lactobacilli has also been shown to have an inverse correlation with symptoms of vaginal dysfunction associated with increased inflammation within the epithelial microenvironment [253]. Dysfunction between the vagina and its resident microbiota is associated with a spectrum of gynecological health and fertility issues in women, which includes bacterial vaginosis, thrush and candidiasis, sexually transmitted infections, pelvic inflammatory disease (PID) and tubal factor infertility (TFI) [254].

Microbial and viral intracellular pathogens have long been understood to elicit particular immune expression profiles, which often serve to starve the parasitic organisms of the nutrients, energy, and metabolic precursors they must scavenge in order to survive [255]. The host cell‟s response to infection by *C. trachomatis* has been well characterised, most recently through novel and exacting RNA sequencing approaches [256, 257]. While these and other studies have provided valuable insights into the host‟s general response to infection, the overwhelming majority of these studies have been conducted using *in vitro* or *ex vivo* models – an

important limitation. Thus, their inherent shortcoming is their lack of translatable clinical and *in vivo* findings. Murine modelling of *C. trachomatis* infection has identified that the innate immune response alone is able to resolve infection [89]. Secondary to this, T_h1 (responses to intracellular pathogens) and T_h17 (signal mediation between the immune system and mucosal epithelium) type immune responses have been shown to promote the successful clearance of the pathogen [258], while a TH2-dominant response to *C. muridarum* infection produced more pathology with decreased clearance [259]. It is unclear how such findings relate to chlamydial infections in women, but it is likely a complex combination of both host and pathogen factors which drive the outcome and pathology.

Collectively, *in vitro* and *in vivo* data appear to suggest that both the innate and adaptive pathways of the human immune system play important roles in the short and long-term clearance of chlamydia. For a sufficiently-developed adaptive response to this intracellular pathogen, it has been proposed that current diagnostic and treatment programs are somewhat prohibitive – as their efficacy and speed does not grant the patient‟s immune system a sufficient duration of exposure for long-lasting protective immunity to develop [260]. In turn, this „arrested immunity hypothesis“ has been proposed as one explanation for the apparent decrease in seroprevalence and seroconversion, during a period in which infection rates continue to rise. Collectively, these points emphasise the importance of understanding how both the innate and adaptive host responses to infection affect the host and pathogen individually, and collectively. A vital component of this approach is characterising the interferon-type pathways, and of particular importance are those which involve the production, inhibition and modulation of IFN-γ [36, 228, 261, 262]. Although the link between this cytokine and both

clearance of and protective immunity to *C. trachomatis* is yet to be completely described, a 2018 study by Geisler and colleagues [263] has again correlated its regulation with protection against newly-acquired infections – the most common cause of incidental reinfection in women after treatment [264].

Chlamydia spp. share a biphasic developmental cycle, in which they infect the host cell, replicate within a parasitiphorous cytoplasmic inclusion, and then leave the host to infect neighbouring cells [236]. The process begins when the infectious and non-replicative chlamydial elementary body (EB) attaches to and enters the host cell, eventually undergoing a transition to the replicative, highly metabolically active reticulate body (RB) form [32, 261]. Further to this, extensive *in vitro* characterisation of *C. trachomatis* has revealed the presence of an alternate developmental form during what is commonly referred to as chlamydial persistence [32, 78, 228, 265]. Outside the clinical context, persistence refers to the inducible, reversible and well-characterised divergent developmental pathway *C. trachomatis* adopts in the presence of a number of exogenous stimuli and environmental conditions (reviewed in depth by Wyrick (2010)[226]). As a stress response, chlamydial persistence may arise *in vivo* under conditions of iron or tryptophan depletion [36, 241, 261], or indeed antibiotics such as penicillin [239]. Studies conducted using *in vitro* models of chlamydial persistence typically show that the pathogen downregulates expression of its late-stage genes involved in differentiation, cell membrane proteins (such as *ompA* and *omcB*) and exiting the host cell; while it upregulates repressors of late genes (such as *euo*) as well as stress-response proteins (such as *htrA*) specific to the stimuli inducing persistence [36, 40, 261, 265]. It has been suggested that this cessation of developmental processes in the presence of these conditions or stimuli is a defence strategy,

which enables the pathogen to outlast the host innate response to infection – presumably when this response is not strong enough to facilitate clearance on its own [30]. Such conditions have indeed been shown to alter the outcome of infection by the respiratory pathogen *C. pneumoniae* [54]. Secondary to this, the role of chlamydial persistence in the complex process of treatment failure also remains to be fully established.

IFN-γ has long been recognised as an important modulator of chlamydial infection in human epithelial cells [32]. While the cytokine is known to cause many downstream effects, its upregulation of indoleamine dioxygenase-1 (IDO1) causes the most profound and studied effects on the *in vitro* growth and development of *C. trachomatis*; all of which can be reversed by the supplementation of tryptophan [49]. As a broad defence against intracellular bacterial and viral pathogens, the upregulation of the catabolic enzyme IDO1 serves to decrease the intracellular stores of tryptophan – an amino acid which *C. trachomatis* must scavenge from its host in order to survive [36, 261]. Not only does IFN-γ serve as a defence strategy to eliminate intracellular pathogens from infected cells, recent findings by Quayle and colleagues suggests it also functions to provide a level of protection in nearby „bystander" cells from secondary infection (chlamydial expansion) [262]. The effects of azithromycin on *C. trachomatis* are known to differ when persistence is induced by stimuli such as IFN-γ and penicillin [41, 230]. Furthermore, host genetics and cell intrinsic factors also appear to be important determinants in how well this particular interferon-type response is able to resolve infection [226, 266]. Finally, the vaginal microbiome has also been shown to play a role in IFN-γ-induced chlamydial persistence [58, 267]. Collectively, it is not implausible that the host immune profile at the time of treatment plays a large – if not the largest –

role in determining whether the combination of antibiotics and immune measures are able to successfully resolve the infection entirely and prevent reinfection from occurring.

Like many modern bacterial pathogens of humans, *C. trachomatis* has evolved alongside with and in opposition to the innate and adaptive branches of our immune system. As evidence which reinforces the intrinsic link between host immune function and microbial communities continues to emerge, it has become increasingly apparent that *C. trachomatis* infection is also likely at the mercy of these, and other crucial factors. Recurrent chlamydial infections are a serious cause for concern, yet the mechanisms by which such clinical instances arise remain poorly understood. To address this, we compared the host and chlamydial gene expression in women treated for infections with azithromycin, using a matched case-control study design, at two intervals. Cases were defined as women who tested positive for *C. trachomatis* urogenital tract infections after treatment, while controls were defined as women who tested negative for infection after treatment. At the first interval, we examined whether host gene expression in the endocervical microenvironment was associated with the clearance of urogenital *C. trachomatis* infections by therapeutic intervention. Also, at this interval, the expression of several chlamydial genes involved in either active or persistent development were also analysed and compared, to see how the type of development impacted treatment. For the second interval, we used follow-up swabs from participants who exhibited a recurrence of infection after treatment (case participants only) to compare how the host and chlamydial gene expression profiles at this timepoint differed to those taken at the time of treatment (for both the case and control groups).

Methods

A nested case-control study to investigate the host and pathogen expression profiles in women receiving treatment for *C. trachomatis* urogenital tract infections

All samples for this study originated from participants in the Australian Chlamydia Treatment Study (ACTS). The inclusion criteria used to assess eligibility for both ACTS and the present study can be seen in **Figure 5.1**. The participants in this study were all women who had tested positive for infection (T_0), were treated with 1g azithromycin, and had been assigned a treatment outcome based on follow-up testing (T_1). A 2:1 nested study design was used to sample a total of 47 matched participants from the ACTS cohort, controlling for age, contraceptive usage and type, and the serovar of the infecting strain. Control subjects (n=30) were defined as women who had tested positive before treatment (T_0) and negative after treatment (T_1).Case subjects (n=17) were defined as women who had tested positive both before (T_0) and after (T_1) treatment. In addition to their case-control status, other data collected and made available for this analysis included their vaginal microbiome CST, infectious load (IFU ml^{-1}), and serology [162]. Prior to some statistical analyses, the case group was further divided based on data from ACTS which showed that their positive follow-up test was the result of either a newly acquired infection (NI), or treatment failure (TF). The final 34 (22 controls;

12 cases) samples were included for analysis on the basis of RNA quality and measurement of *GAPDH* expression levels by RT-qPCR measurement.

Samples used in the present study

When chlamydia-positive women were recruited by ACTS, the attending clinician performed a speculum examination to assess their gynecological health. During this examination, the clinicians also used a break-point floc swab to sample the mucosal surface of the patient‟s endocervix. The tip of each swab was placed into a sterile, labelled, 2 ml cryovial tubes containing RNA-later, and stored at -80°C until processing. The first swab samples of all the participants in the study were collected immediately prior to treatment with azithromycin and are henceforth referred to as the “primary swabs” by this study. Participants in both groups underwent re-testing between 28 and 56 days after treatment, and a second set of swabs was collected by clinicians from the participants with a positive result (cases). These are henceforth referred to as the “post-treatment swabs” by this study.

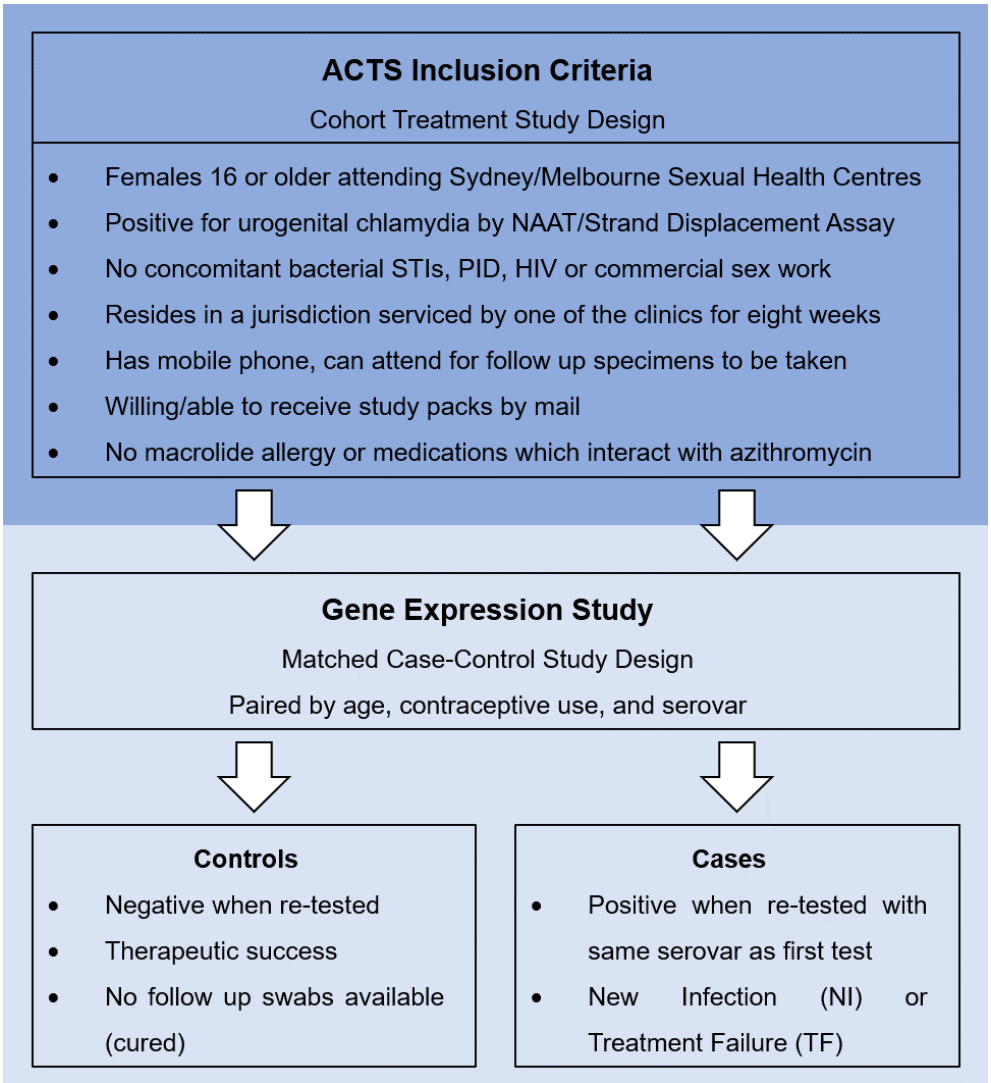


Figure 5.1. Criteria and details used during (dark blue) the recruitment stage of ACTS and (light blue) group selection for this nested case-control study.

RNA extraction and complementary DNA synthesis

Samples were thawed completely and vortexed to resuspend the contents. Total RNA was then extracted and purified from each sample using the Purelink™ RNA Mini Kit (Thermo Fisher Scientific), in accordance with the manufacturer's instructions for liquid samples. A volume of 30 µl of RNase/DNase-free ultrapure dH_2O (Life Technologies) was used to elute the total RNA, and contaminating gDNA was removed by treatment with RNase-free DNase I (Thermo Fisher Scientific). The concentration and purity of each RNA sample was assessed using the NanoDrop™ One/One C UV-Vis spectrophotometer (Thermo Fisher Scientific). Complementary DNA (cDNA) synthesis with random hexamer priming was conducted using the SuperScript™ III First Strand Synthesis Reverse Transcription kit (Thermo Fisher Scientific). Because of the low quantity of total RNA in a high proportion of the samples, 8 µl of total RNA was used as the template for synthesis in each reaction. All cDNA samples were diluted 1:10 in RNase/DNase-free ultrapure dH_2O (Life Technologies) for use in RT-qPCR.

Measuring relative human gene expression levels in cDNA samples

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was performed to determine the expression levels of each gene of interest relative to the geometric mean of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and phosphoglycerate kinase 1 (*PGK1*) cDNA levels. Each of the genes assessed by RT-qPCR can be seen in **Table 5.1**. The oligonucleotide sequences used to amplify, and measure cDNA were (unless otherwise stated) created using the NCBI Primer

BLAST Tool, in accordance with the principles of primer design (**Appendix IV**). Each of the primer pairs used were assessed by conventional PCR and agarose gel electrophoresis to ensure only a sole product (of the correct size) was produced in each reaction. The efficiency of each reaction was then assessed using serial ten-fold dilutions of either cDNA from HeLa cell total RNA, or a mixture of cDNA from samples which were not to be analysed. Each reaction tested had an efficiency of between 90 and 110%. The mean cycle threshold (Ct) value of each of the 46 participant cDNA samples was calculated from technical duplicates, which were repeated or excluded if they differed by more than one standard deviation.

Table 5.1. Human genes selected for expression analysis by RT-qPCR.

ID	Gene name	Description
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Housekeeping gene involved in the regulation of intracellular ATP stores.
PGK1	Phosphoglycerate kinase 1	Housekeeping gene involved in the glycolysis pathway.
IDO1	Indoleamine 2,3-dioxygenase	Degrades cellular tryptophan as a defence against intracellular pathogens.
IRF1	Iron regulatory factor 1	Transcriptional regulator of IFN and IFN-inducible genes.
CXCL9	Chemokine (CXC motif) ligand 9	Involved in lymphocyte chemoattractant activities for infected cells.
FTH1	Ferritin heavy chain 1	Involved in iron regulation by trafficking iron in and out of the cell.
IL6	Interleukin-6	Proinflammatory cytokine involved in stimulating a localised immune response.
IL8	Interleukin-8	Chemoattractant cytokine responsible for recruiting phagocytic cells.
IL1A	Interleukin-1-α	Cytokine with roles in producing localised inflammation.
TNFA	Tumour necrosis factor-α	A cytokine which regulates other immune cells and systemic inflammation.
IL10	Interleukin-10	An anti-inflammatory cytokine regulating localised inflammation.
IFNG	Interferon gamma	Soluble cytokine that elicits responses to intracellular infections.

Measuring relative chlamydial gene expression levels in cDNA samples

As with the human gene expression profiling, RT-qPCR was used to determine the level of expression of five chlamydial genes of interest, relative to the amount of chlamydial *16S rRNA* cDNA in each sample. All six genes analysed by RT-qPCR are shown in **Table 5.2**. The oligonucleotide sequences used to amplify and measure chlamydial *16S rRNA* [268], *euo* [269] and *ompA* [270] cDNA were from previously published studies, while those used to amplify *htrA, trpBA* and *omcB* cDNA were designed using the NCBI Primer Blast tool. The genome of *C. trachomatis* type strain D/UW-3/Cx (accession number NC_000117) was used as the template for primer design (**Appendix V**); however, primers were only selected if they amplified at the very least each of the serovars in the present study. Each of the primers used were validated by conventional PCR and agarose gel electrophoresis, before the efficiency of each primer set was determined using serial ten-fold dilutions of either cDNA from *C. trachomatis*-infected MCF-7 cells, or a mixture of cDNA from samples previously excluded from analysis. Each reaction had PCR efficiencies of between 90 and 110%. As with the host gene expression, the mean cycle threshold (Ct) value of each of the 46 participant cDNA samples was calculated from technical duplicates, which were repeated or excluded if the mean Ct value had a standard deviation greater than 1.0.

Table 5.2. Chlamydial genes selected for expression analysis by RT-qPCR.

ID	Gene name	Description
16S rRNA	16S ribosomal RNA-encoding gene	Normalisation gene encoding 16S rRNA subunit of *C. trachomatis*.
omcB	Outer membrane protein B	Membrane protein greatly upregulated during active development.
htrA	High-temperature requirement (protein) A	A serine protease with potentially other functions related to protein accumulation, degradation, etc.
euo	Early upstream open reading frame	Early gene repressor of late gene expression.
ompA	Outer membrane protein A	Upregulated during active development, especially late in the cycle.
trpBA	Tryptophan synthase BA operon	Encodes the functional subunits for chlamydial tryptophan synthase.

Data analysis and statistical testing

Participant demographic data was compiled in Microsoft Excel 2010, and the statistical tests described in the results section were conducted using the IBM SPSS software suite. Raw RT-qPCR data was exported from the Rotor-Gene Q Series software platform and stored and transformed using Microsoft Excel 2010. For each sample, the mean Ct value of each gene of interest was subtracted from the geometric mean of *GAPDH* and *PGK1*, to obtain the delta cycle threshold (ΔCt) value. These ΔCt values were used in a negative log base 2 transformation and arbitrarily multiplied by 100 (for ease of graphical comparison). This equation can be expressed as $100 \times 2^{-\Delta Ct}$. The mean of each gene of interest were graphed in Graphpad Prism 7, along with the standard error of the mean. Statistical

comparisons were also conducted using Graphpad Prism 7, with the Mann-Whitney U used test to determine whether any significant differences between two groups existed, and the Kruskal-Wallis test used to calculate the significance of differences in three or more groups.

Results

Statistical comparison of the case and control groups‟ demographic data showed both groups were similar with no significant differences in the variables used in the matching of participants

The participant demographic data was compiled for comparison, to ensure that no confounding factors were evident between the case and control groups. Age, contraceptive information, infecting serovar, and the CSTs of participants were not found to be significantly different between the two (**Table 5.3**). The mean repeat-positive day (at which the participant was determined to still be infected after treatment) was different, owing to the control group inherently having null values for this variable, as they were successfully cured by treatment.

Table 5.3. Participant demographic data comparing the case and control groups.

			Control	Case	Significance
Count			22	12	
Age		Mean (±SD)	24.09 (2.67)	23.50 (3.96)	0.605[1]
Condom Use		Frequency (% within group)	11 (50)	5 (41.7)	0.641770[2]
Oral Contraceptive Pill Use		Frequency (% within group)	14 (63.6)	8 (66.7)	0.860[2]
Serovar	E	Frequency (%)	12 (54.5)	6 (50)	
	F	Frequency (%)	9 (40.9)	5 (41.7)	0.895[2]
	K	Frequency (%)	1 (4.5)	1 (8.3)	
Intrauterine Device Use		Frequency (% within group)	4 (18.2)	4 (33.3)	0.320[2]
Community State Type	I	Frequency (%)	5 (23.8)	7 (58.3)	
	II	Frequency (%)	9 (42.9)	2 (16.7)	0.197[2]
	III	Frequency (%)	6 (28.6)	3 (25.0)	
	IV	Frequency (%)	1 (4.8)	0 (0)	
Repeat positive day		Mean (±SD)	0 (0)	40.83 (11.10)	0[3]

[1] Two-tailed t-test for equality, equal variances assumed

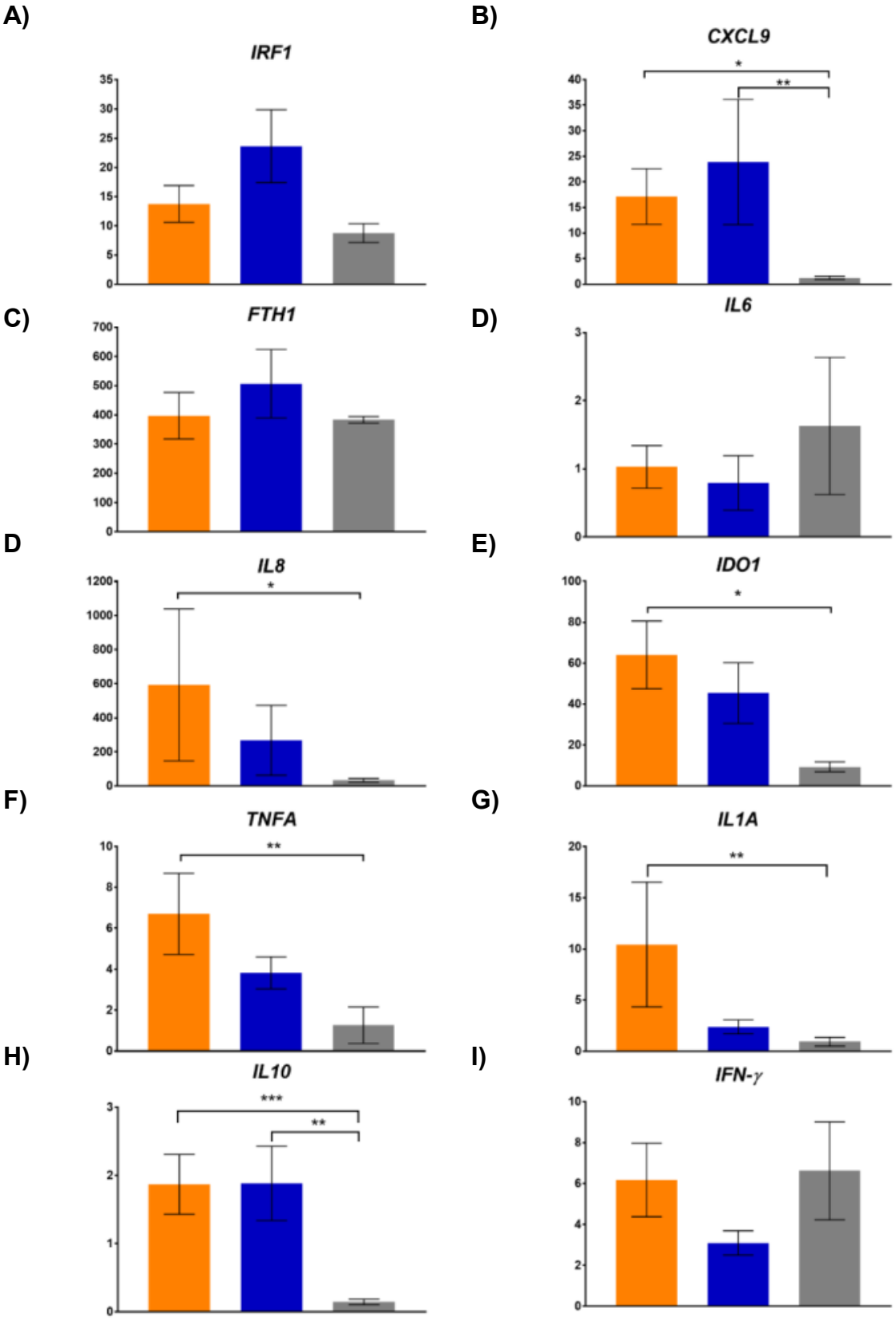
[2] Two-sided asymptotic significance by Pearson Chi-Square testing

[3] Not determined as controls had no repeat positive days by definition of the group

The expression of host genes involved in localised and host-cell mediated responses to intracellular pathogens is significantly lower than on day one if treatment fails to resolve infection

RT-qPCR analysis of the participant samples was performed to determine the expression levels of *IRF1, FTH1, CXCL9, IL6, IL8, IDO1, TNFA, IL10, IFNG* AND *IL1A* (**Figure 5.2**). During a classic immune response to chlamydial infection, most of these genes are expected be upregulated respective to their non-infected state; primarily in response to the presence of the intracellular parasite within its epithelial host cell [255]. When comparing only the samples from the primary swabs of both the case and control groups, *IRF1*, *CXCL9* and *FTH1* were the only genes observed to be higher in the case group than in the control group – but not at a level that was statistically significant. *IL6* and *IL10* were approximately equal in the two groups, while *IL8*, *IDO1*, *TNFA*, *IL1A* and *IFNG* were all observed to be lower in the case group – but once again there was no significant difference. Our analysis showed higher levels in seven of the ten human genes tested, as *IL6*, *IFNG* and *FTH1* were not observed to be different between any of the groups. *CXCL9* expression (**Figure 5.2B**) was lower in the post-treatment swab group than both the primary swab groups, which was statistically significant (p=0.024 and 0.0038, respectively). *IL8* (**Figure 5.2E**) appeared lower in the post-treatment swab group than both the case and control primary swab groups but was only found to be significantly lower than the control primary swab group (p=0.0152). Both *IDO1* and *TNFA* (**Figure 5.2F-G**) were also observed to be

significantly lower in the post-treatment swab group than only the control primary swab group (p=0.0419 and 0.0035, respectively). *TNFA* approached a statistically significant difference in the case primary swab and post-treatment swab groups; post-treatment swab group expression being lower (p=0.0543). *IL1A* was most highly expressed by the control primary swab group (**Figure 5.2H**) but was only significantly higher than the post-treatment swab group (p= 0.0084) and not the case primary swab group (p=0.2787). *IL10* expression (**Figure 5.2I**) was almost identical in the control and case primary swab groups, which were both significantly higher than the post-treatment swab group (p=0.0009 and 0.019). The case (primary and post-treatment) participants were also divided into the subgroupings "New Infection" (NI) or "Treatment Failure" (TF) on the basis of their clinical outcomes (**Appendix VI**). No significant differences were observed in any of the ten genes, for each of the five groups (control, NI (primary swab), NI (post-treatment swab), TF (primary swab), TF (post-treatment swab)), after these subgroupings were performed.



(Figure legend and description of Figure 5.2 continued on next page.)

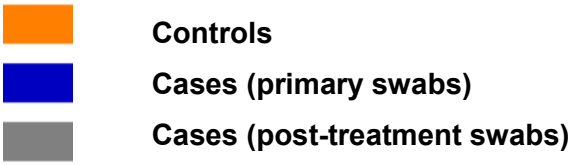


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Figure 5.2. Endocervical gene expression in women who were treated for chlamydia with azithromycin. Women treated with 1g azithromycin as part of ACTS (n=34) were retrospectively assigned case-control groupings on the basis that follow-up testing had shown them to be either negative (controls; n=22) or positive (cases; n=12) for chlamydia. Endocervical swabs were collected from: both the control group (orange) and the case group (blue), prior to the provision of treatment; and exclusively from women in the case group at the same time their (positive) follow-up test was collected. The expression levels (shown on Y-axis as transformed relative expression) of ten human genes were measured by means of RT-qPCR, using RNA purified from each swab. Data shown in each panel is representative of the mean expression value ±SEM for the gene named in the panel title, relative to the geometric mean of *GAPDH* and *PGK1*, for each of the three groups. Statistical significance is denoted by * ($p \leq 0.05$), ** ($p \leq 0.01$) or *** ($p \leq 0.005$).

Chlamydial gene expression analysis suggests that infections present after treatment were a combination of phenotypically stressed and actively replicating sub-clusters

Five chlamydial genes of interest were assayed by RT-qPCR to investigate how their expression differed at the different intervals of treatment. Outside tissue culture models, chlamydial infection is thought to be a heterogeneous mix of infected cells, in which reside *C. trachomatis* at various stages of its own developmental cycle. The asynchronous and temporal nature of this cycle means there is likely to be an approximately similar number of particles present as each of the developmental forms; with a mixture of the gene expression profiles characteristic of each stage of the cycle. With the exception of *trpBA*, each of the genes measured were approximately equal between the control and case primary swab groups (**Figure 5.3**). However, the post-treatment swab group was observed to have significantly higher expression of all five genes than both the primary swab groups. o*mpA* is upregulated during active infection, especially in the mid-late stages of development, and was significantly higher in the post-treatment swab group (**Figure 5.3A**) than the control (p= 0.0001) and case primary swab (p= 0.0046) groups. *euo*, a repressor of late-gene expression, is most highly expressed during the early stages of development, as well as during persistence. We observed that *euo* (**Figure 5.3B**) was highest in the post-treatment swab group, exceeding both the control (p= 0.0047) and the case primary swab groups (p= 0.0240). Similarly, *omcB* expression is highest during late development, and is

downregulated during the early stages, as well as during persistence. Comparisons (**Figure 5.3C**) showed that *omcB* expression was once again highest in the post-treatment swab group, compared to both the control (p= <0.0001) and the case primary swab groups (p= 0.0001). While the product of *htrA* has been suggested to serve as a multifunctional protein, it is upregulated by stress and as typical chlamydial development progresses. Our findings (**Figure 5.3D**) suggest it is expressed at a much higher level in the post-treatment swab group than both the control and case primary swab groups (p= 0.0014 and 0.0355). *trpBA* (**Figure 5.3E**) is dramatically upregulated during persistence and was seen to be highest in the post-treatment swab group, when compared to both the control (p= 0.0161) and case (p= 0.0011) primary swab groups. When comparing the groups using the NI and TF subgroupings of the case participants, several genes were observed to be significantly different among the five groups (**Appendix VII**). *ompA* was significantly higher in the TF (post-treatment swab) group than in the control primary swab group. e*uo* was significantly higher in the NI post- treatment swab group than the control group (p < 0.0001), the NI primary swab group (p = 0.0005) and the TF primary swab (p = 0.0011). *omcB* expression was significantly lower in the control group than both the NI and TF post-treatment swab groups (p=0.0014 and 0.0290, respectively), which were also both higher than their respective primary swab group means as well (p=0.0021 and 0.0072). The only significant difference in *htrA* expression was between the control and NI post-treatment swab groups; the latter of which was higher than the control group (p=0.0201). The mean expression values of *trpBA* were not seen to be significantly different among any of the five groups (**Appendix VII**).

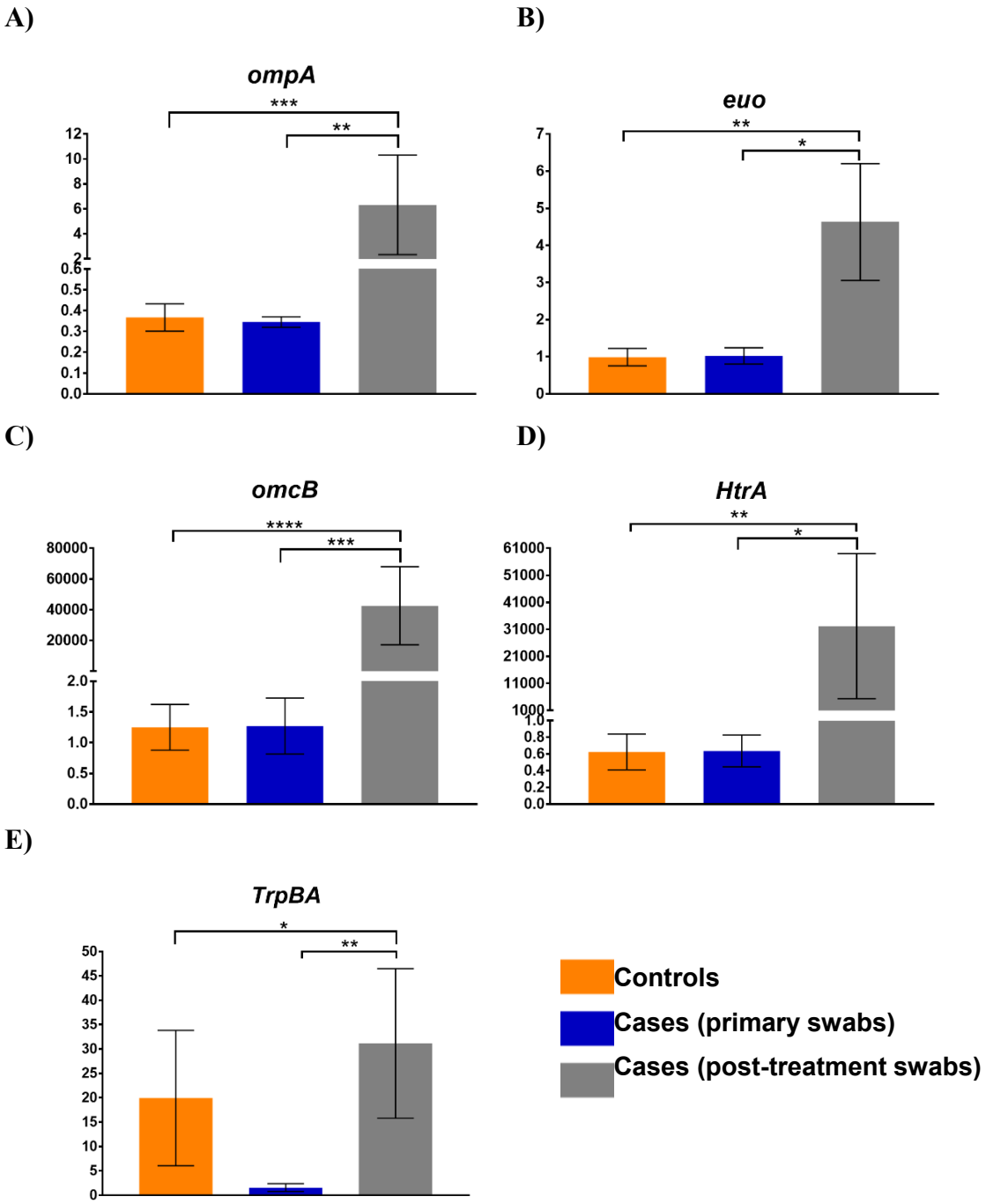


Figure 5.3. RT-qPCR analysis of chlamydial gene expression in women who received 1 g azithromycin as treatment for *C. trachomatis* urogenital tract infections. Chlamydial gene expression (shown on Y-axis as transformed relative expression) was measured in the endocervical swab samples of control (primary swabs (orange)) and case (primary swabs (blue); post-treatment swabs (grey)) groups of the study. Panels show the mean expression levels ± SEM of *C. trachomatis ompA* (A), *euo* (B), *omcB* (C), *htrA* (D) and *trpBA* (E) normalised to *Ct* 16S rRNA expression. Statistical significance is denoted by * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.005$) and **** $p \leq 0.001$).

Table 5.4. Significance of observed differences in expression of human genes in the control and case groups.

	Control vs Case (primary swabs)	Control vs Case (post-treatment swabs)	Case vs Case (primary vs post-treatment swabs)
Gene		**Adjusted P Values[1]**	
IRF1	0.1749	>0.9999	0.1958
CXCL9	0.9246	0.024 *	0.0038 **
FTH1	0.609	>0.9999	>0.9999
IL6	>0.9999	>0.9999	>0.9999
IL8	0.5111	0.0152 *	0.5805
IDO1	>0.9999	0.0419 *	0.0808
TNFA	>0.9999	0.0035 **	0.0543
IL1A	0.7857	0.0084 **	0.2787
IL10	>0.9999	0.0009 ***	0.0019 **
IFNG	0.9782	>0.9999	>0.9999

[1]Adjusted P values returned by using the Kruskal-Wallis test to compare the mean expression levels of each gene between the three groups, with significant differences denoted by * ($p \leq 0.05$), ** ($p \leq 0.01$) and *** ($p \leq 0.005$).

Table 5.5. Gene expression data divided into the three original groups to show the mean[1], SEM and count (N)[2].

	Controls			Case (primary swab)			Case (post-treatment swab)		
Gene	**Mean**	**SEM**	**N**	**Mean**	**SEM**	**N**	**Mean**	**SEM**	**N**
IRF1	13.75	3.13	22	23.64	6.23	12	8.76	1.61	11
CXCL9	17.13	5.44	22	23.86	12.23	12	1.23	0.31	11
FTH1	397.16	79.66	22	506.55	117.06	12	383.32	11.29	11
IL6	1.03	0.31	21	0.79	0.40	12	1.63	1.00	9
IL8	593.56	445.24	22	268.08	205.06	12	33.74	10.67	11
IDO1	63.99	16.58	21	45.37	14.83	12	9.29	2.40	9
TNFA	6.71	1.98	21	3.82	0.78	12	1.27	0.89	10
IL1A	10.43	6.10	22	2.38	0.69	11	0.93	0.44	10
IL10	1.87	0.44	21	1.88	0.54	12	0.15	0.04	7
IFNG	6.18	1.80	22	3.09	0.59	12	6.62	2.39	11
ompA	0.37	0.07	20	0.35	0.03	12	6.32	3.99	7
euo	0.99	0.23	20	1.02	0.22	12	4.63	1.57	5
omcB	1.25	0.37	20	1.27	0.46	12	42636.47	25434.04	11
htrA	0.62	0.21	20	0.64	0.19	12	32178.84	26834.90	9
trpBA	19.94	13.89	22	1.58	0.80	12	31.14	15.33	11

[1] All mean values listed for human genes are relative to the geometric mean of *GAPDH* and *PGK1,* while the mean values of the chlamydial genes listed are relative to chlamydial 16S rRNA gene expression levels.

[2] Where N does not equal the full amount of that particular group, the data was unable to be reproducibly measured in duplicate to within a SD of 1.0 cycle.

Discussion

It has become increasingly clear that the vaginal microbiome and endocervical transcriptome are key factors in treatment and clearance of *C. trachomatis* infections of the urogenital tract [271]. Less clear, however, is the degree to which they each individually impact treatment outcomes, and how the human and microbial genotypes involved could be used to predict the efficacy of treatment for individualised clinical approaches [77]. Historically, evidence has highlighted the complexity of the relationship which exists between *C. trachomatis*, the host's immune response to infection, and the pathological outcomes which result from this interaction [272, 273]. Particular attention has been paid to how *C. trachomatis* responds to tryptophan depletion by IFN-γ, and how this is influenced by other beneficial vaginal microbial and their metabolites [32, 50, 60, 257]. In the present study, we looked at how the immune profiles of women treated for chlamydia with azithromycin related to their treatment outcomes. Specifically, we examined whether the endocervical transcript levels of genes relevant to chlamydial infection differed between the women who were successfully treated, and those who experience reinfection or treatment failure. In addition to this, we examined the chlamydial gene expression at the time of treatment, as well as in cases where treatment had not resolved the infection.

The endocervical swab samples used in this follow-up study were from Australian Chlamydia Treatment Study (ACTS) participants. ACTS was a comprehensive longitudinal study which examined the factors involved when using azithromycin to treat women with *C. trachomatis* infections of the urogenital tract [162]. The

subselection of participants used in the present study was made using matched-pairing to control for age, contraceptive usage at baseline, and serovar of the primary infection. The first grouping (case vs control) of participants was informed by the results of their follow-up clearance test. Participants who retested negative for infection (controls) were therefore sampled only once – at the time of treatment. Conversely, those who retested positive for infection (cases) were sampled a second time, so that a post-treatment swab sample could be obtained for analysis. Further to this, sub-grouping of the cases by the probable cause of their positive retest (reinfection vs. treatment failure) was also performed for some investigations. It should therefore be considered that the gene expression data generated for the case group likely reflects a mixture of participants who experienced re-infection from several possible sources, and actual therapeutic failure. Furthermore, it is not an unreasonable assumption to make that within the constraints of this study, the host gene expression profiles of the time-of-treatment controls (n=22) represent an immune microenvironment which is conducive to the clearance of chlamydial infection following the administration of azithromycin. A major limitation of this study is that by not conducting post-treatment swabs of the control group, we have no insight into their localised immune expression after treatment, in the absence of *Chlamydia* spp. This could have provided valuable data to set the "baseline" for non-infected individuals, which would have been compared to the post-treatment case samples we did obtain to see whether untreated infections cause shifts in gene expression (compared to uninfected individuals). Building on this, it could also be a consideration in future studies to look at the immune expression profiles in case participants after their second round

of azithromycin – provided the antibiotic is used to treat non-resistant infections (which have not been identified clinically).

To investigate how the expression of human genes in the endocervix at the time of treatment was related to the outcome of treatment, we employed RT-qPCR to compare the expression levels of ten human genes of interest. Each was normalised to the geometric mean of *GAPDH* and *PGK1* gene expression levels, both of which have been previously shown to be stable and suitable housekeeping genes for assaying endocervical cell gene expression [274]. Comparison showed no significant differences in gene expression between the case and control group samples taken at the time of treatment. While this may suggest that the expression levels of the genes tested do not correlate with the treatment outcome, the sample size of our study (controls = 22, cases = 12) and frequently-high standard errors of the means must be considered in this interpretation. Conversely, comparison of the two time-of-treatment groups with the post-treatment case group revealed that several genes associated with promotion and modulation of the localised inflammatory response were significantly lower in the post-treatment case group. Specifically, the post-treatment case group showed significantly lower levels of *CXCL9, IL8, IDO1, TNFA, IL1A* and *IL10* than the time-of-treatment control group, and significantly lower levels of *CXCL9* and *IL10* than the time-of-treatment case group. These differences reinforce the notion that using azithromycin to treat chlamydial infections may impact the patient‟s ability to resolve future challenges, and develop chlamydia-specific immunity [89, 260]. In the type-II interferon pathway, *IFNG* expression is upregulated to produce higher levels of the pro-inflammatory cytokine IFN-γ for localised secretion. IFN-γ has been demonstrated to in turn upregulate the expression of both *IDO1* (which encodes a tryptophan-

catabolising enzyme [49]) and *CXCL9* (which encodes a $CD8^+$ T-cell attractant chemokine [275]). The role of *IDO1* in chlamydial infection has been investigated in great depth over the years, and it is readily apparent that its enzyme product is crucial for clearance of the pathogen [36, 45, 50, 226, 257, 261-263, 266]. Furthermore, when Darville and colleagues examined the interferon pathways of *IFN-α/β* receptor-deficient female mice infected with *C. muridarum*, they found that *CXCL9* may enhance clearance when there is no type-I interferon pathway signaling [276]. *CXCL9* has also been associated with the ascension of infection from the cervix to the endometrium – yet the impact this has on treatment and clearance is unclear [277]. The difference in expression levels of these two genes that we observed between the control and post-treatment case groups possibly reflects the degree to which treatment limits the ability of the host's immune system to confer a lasting immunity to, which in turn has the potential to leave these women more susceptible to reinfection.

Production of IL-10, IL-6 and IL-8 by immune and epithelial cells are known to be increased by chlamydial infection. The expression of *IL10*, which encodes the anti-inflammatory cytokine IL-10, has previously been associated with chlamydial infection and iron deprivation by the host cell [278]. *IL10* expression has also seen been shown to be upregulated in PBMCs by *C. trachomatis* infection [279], which in turn reduces the local expression and production of proinflammatory cytokines. Finally, IL-10 has been demonstrated to have a role in antigen presentation by T cells, whereby its production modulates the ability of dendritic cells, in a murine model, to assist in clearing the infection [279]. Thus, it is plausible that the observed reduction in expression compared to the time-of-treatment control group likely represents either lower numbers of actively replicating *C. trachomatis*, or a

decreased T-cell presence at the site of infection and sampling, following treatment with azithromycin. *IL6*, the gene which encodes the largely proinflammatory cytokine IL-6, was not observed in our study to be significantly different in the post-treatment case and time-of-treatment control groups, while *IL8* was. In humans, *IL8* is expressed by various epithelial, endothelial and immune cells to produce and secrete IL-8; a chemokine which promotes the migration of neutrophils to the site of infection via a chemotactic gradient. Studies using *in vitro* models of chlamydial infection have shown that infected cultures increase their expression of *IL8* when *Chlamydia* are actively replicating, as their metabolic requirement for some host-derived lipids stimulates host cell production of inflammatory prostaglandins [280, 281]. Combined, these effects have been shown to be dependent upon Nucleotide-binding oligomerization domain 1 (NOD1) signaling, as well as the presence of IL-1α [282, 283]. Interestingly, the post-treatment case group expression levels of *IL1A*, which encodes this proinflammatory cytokine, were also significantly lower than the time-of-treatment control group. Combined these data suggest that either the infected cells are not showing the same magnitude of innate response to infection, or perhaps other dampening and modulation of these responses has occurred. It remains unclear why of the two proinflammatory interleukins (*IL8* and *IL6*) we assayed only *IL8* was significantly different; when they both share NFκB and NOD-mediated induction pathways and are upregulated by chlamydial infection [284]. Cell culture studies have shown that IL-6 levels are different in cells infected with *C. trachomatis* after the induction of chlamydial persistence [285]. More recently, Huston and colleagues showed that IL-6 production in *Chlamydia*-infected epithelial cells is influenced by the presence of peripheral blood mononuclear cells

(PBMCs), as well as chlamydial stress proteins [286]. This could suggest that the apparent disparity between *IL6, IL10* and *IL8* resulted from a higher proportion persistent *Chlamydia* in the post-treatment case group, which decreased the expression of *IL8* and *IL10*, but not *IL6*. Such a hypothetical scenario could also explain the apparent fewer differences in gene expression levels between the case groups, before and after treatment. If reinfection or treatment failure resulted from higher proportions of *Chlamydia* being in persistence at the time of treatment (first sample point), it is plausible to suggest that the host gene expression profile would be less likely to change if they remained persistent after treatment (second sample point). Alternatively, *IL6* may be elevated (or not decreased) post-treatment due to other non-chlamydial factors, such as concomitant microbial issues or host genetics.

The transcriptome of *C. trachomatis* in conditions favourable and permissive to typical development has been previously examined in detail [28, 36, 257, 287]. Studies have also demonstrated the effects that various environmental conditions and stimuli have on the pathogen‟s transcriptome, and ability to complete its typical developmental cycle [27, 40, 62]. Because the majority of studies examining the chlamydial transcriptome have been conducted using *in vitro* models, we selected a small panel of five chlamydial genes with the intention of using them to evaluate whether the infecting strain was mostly undergoing typical or persistent development – and how this related to treatment outcome. The measured levels of *C. trachomatis* 16S rRNA were used to normalise the expression levels of each of the four chlamydial genes of interest, as it is expressed throughout each stage of development [28, 36]. The late-gene repressor *euo* was selected on the basis that it has been reported to be overexpressed

(relative to other genes) by up to 30 times during persistence [36, 261]. Both *ompA* and *omcB* were selected for their relative expression during the late stage of typical development [28, 36]. *htrA* was included as some evidence suggests it is expressed at higher ratios to late genes such as *ompA* and *omcB* in some models of persistence [265]. Finally, *trpBA* was used as it is tightly regulated and an established transcriptional indicator of chlamydial persistence in urogenital strains [59, 62].

Of the five genes of interest measured, none showed any significant differences in expression levels between the time-of-treatment case and control groups. While not statistically significant, the difference in *trpBA* expression levels between the two time-of-treatment groups is interesting for the fact that the control group (n=22) appears to indicate high levels of expression in at least some of the participants. This is not mirrored by the case group, which showed low levels of expression consistent with what is to be expected of a bacterial stress response gene. Overall, the high degree of similarity between both the time-of-treatment groups observed during this study is not surprising, when considering the complexity of *in vivo* infection. In particular, the asynchronous developmental cycle of the pathogen observed *in vitro*, and the heterogeneity likely to result from both the host cell and the infecting strain [78]. Conversely, chlamydial gene expression in the post-treatment case group was observed to be significantly different to both the time-of-treatment groups, for each of the five chlamydial genes measured. The higher levels of *euo*, *trpBA* and *htrA* in the post-treatment group may suggest a higher proportion of the chlamydial population were in persistence at the time of sampling, as they have previously been observed to be expressed at higher levels in stressed and persistent *in vitro* cultures [36, 62, 261,

265]. The concordant increase in expression of both *ompA* and *omcB* – which are generally markers of active chlamydial development – may be an artefact of greater overall transcription or could also represent a larger overall infectious population with synchronised development.

While limited by the sample size and genes selected for analysis, the present study has provided insight into how gene expression in both humans and *C. trachomatis* differs after treatment with azithromycin. Our results show that a number of genes involved in the promotion and regulation of inflammation appear to be down-regulated after treatment, despite the re-occurrence of infection. As some of these genes are involved in the pathology, clearance of and immunity to urogenital tract infection by *C. trachomatis*, it may be important to conduct a larger and more extensive study of exactly which immune pathways are being altered through the course of treatment, as well as whether there is an underlying genetic basis for this in humans. Our results also suggest that genetic markers of chlamydial persistence may be important factors in recurrent infection, and treatment of diagnosed infections. Expanding on both the human and *Chlamydia* aspects of clinical therapy may help to inform and develop personalised treatment strategies and improve current treatment practice.

CHAPTER 6: GENERAL DISCUSSION AND FUTURE DIRECTIONS

Scope and purpose of this research project

Infertility is defined as the inability to achieve pregnancy by means of unprotected sexual intercourse, over a period of no less than 12 months [288]. In women, this has been attributed to a number of infectious diseases including gonorrhea, chlamydia and tuberculosis [289, 290](reviewed in [291]). Studies have shown that infection-associated infertility is often the result of a tissue-damaging inflammatory response by the immune cells of the reproductive tract [292]. Current evidence suggests that the microbiota of the female reproductive tract contribute significantly to gynecological health and infertility [127]. Not only do these "beneficial" bacteria promote a healthy epithelium within the vagina, they also contribute to localised immune homeostasis, and protect against pathogenic microbes [293]. Thus, microbial pathogens such as *C. trachomatis* appear to play an important part in not only disease progression, but also the complications which may arise [292]. Tubal factory infertility (TFI) is a pathological condition, exemplary of this infection-microbiota-host relationship, in which the immune-mediated occlusion of the fallopian tubes has been demonstrated to have a direct effect on female fertility [294]. The occurrence of TFI has been associated with *C. trachomatis* infections within the urogenital tract [70, 290]. The fact that such infections are readily treatable is an important part of what makes this unique pathogen an attractive target for clinical and experimental research; which could ultimately translate into strategies aimed at reducing the prevalence and social burden of infectious infertility among women.

The purpose of this research project was to examine the relationship between the host and microbial factors of chlamydial infection, and female fertility (**Figure 6.1**). Aim 1 of this research project was to investigate the possible link between the reproductive tract microbiota, the expression of several host genes relating to infection and immunity, and female fertility. I also examined the chlamydial factors of infection, in the context of antibiotic treatment, which may relate to complications such as infertility. This proved difficult by design, as is perhaps best highlighted by data from women in Columbia which suggests that approximately 94% of infections are able to be resolved by the localised immune response without complications [295, 296]. It is also understood that this may occur either spontaneously, or in combination with the effects of the antibiotic treatments used [103]. Thus, it is generally accepted that chlamydial infections which are not cleared by the immune system in combination with antibiotics represent a small fraction of the total number of infections within the total population. Although treatment failure is known to occur, it has yet to be determined how the developmental and stress phenotypes of *C. trachomatis* impact its clearance when antibiotic treatments are used. For this reason, Aim 2 (**Figure 6.1**) of this research project was to assess the *C. trachomatis* developmental and stress-response phenotypes of several clinical isolates. For my third and final aim, I wanted to investigate the relationship between the host and pathogen factors related to treatment. Summarily, **Figure 6.1** describes the key factors identified, as they relate to the aims of the research project.

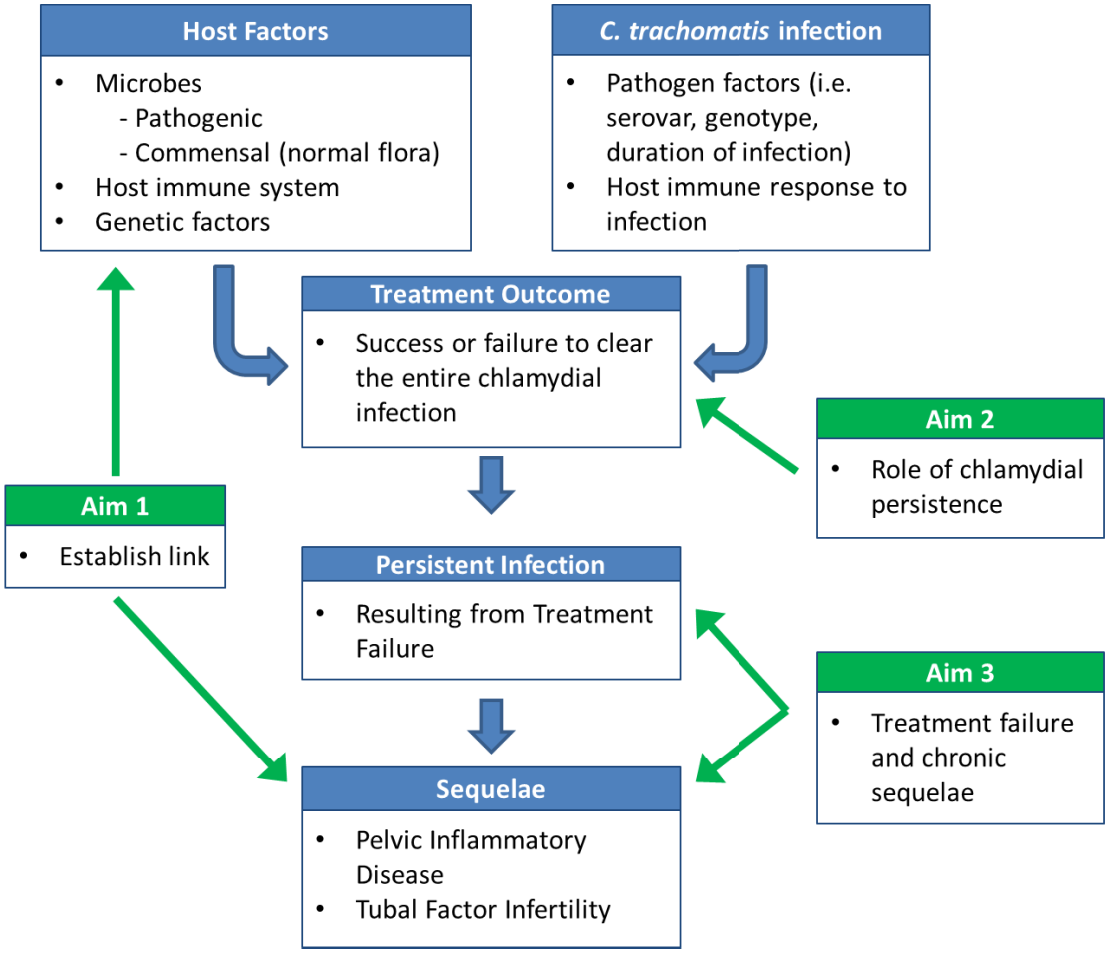


Figure 6.1. Flowchart describing the factors being investigated and aims of the project. The relationship between some of the important microbial and pathological factors in the context of *C. trachomatis* infections of the urogenital tract. Each of these factors and how they relate to the overall process of infection are indicated by boxes with blue arrows and heading bars. A brief summary of each of the three research aims and their links to the identified gaps in knowledge are indicated by boxes with green arrows and heading bars.

Aim 1

Establishing a link between the microbial communities of the female reproductive tract, immune gene expression, and fertility

Modern research combining advanced DNA sequencing methods and statistical modeling aims to explore the association between profiles or combinations of microbial populations, and biomarkers of health and disease [128, 293]. While the early works of Ravel *et al* [297] certainly examined this association in the vagina in greater detail than any previous studies, they did not focus on the biological processes underlying gynecological dysbiosis. Thus, the first aim of my thesis was to examine the relationship between the microbes found in the female reproductive tract, fertility, and immune gene expression. The intention of this work was to characterise the vaginal, endocervical and endometrial microbiomes of a group of Australian women, who were participants in a retrospective case-control fertility study. This "bigger picture" look at whether certain microbial populations were associated with infertility was performed in conjunction with another set of experiments. In these, I investigated the endometrial expression levels of human genes important to bacterial immunology and reproductive pathologies, to evaluate their relationship with female fertility and the concomitant microbial compositions. There were three main findings from this body of work (see Chapter 3) which address Aim 1 of this research:

- Incongruence between the microbiota of the upper and lower FRT.
- *TNC* over-expression was observed among women with a history of miscarriage.

- *Ureaplasma* spp. were overrepresented in the vaginal and cervical microbiota of infertile women.

The human vaginal microbiome is likely to be an important factor in chlamydial infection and clearance [298] as well as infertility in women [273]. It has also been shown that microbial communities dominated by certain *Lactobacillus* spp. may provide protective effects against sexually transmitted pathogens such as *C. trachomatis* [299]. This protection offered by beneficial bacteria is in part mediated by the maintenance of their microenvironment at conditions ideal to their growth, but far less permissible to the growth of bacterial pathogens such as *C. trachomatis* [300]. In the present study, we did not identify a link between the distinct microbial community profiles (or community state types) of the vagina and fertility or case-control status. One plausible explanation for why no association between CST and infertility was observed is also an important limitation of the study. Infertility among women is often further classified as being of either infectious (ININF) or non-infectious (nININF) origins [291]. Importantly, women with nININF and ININF have been shown to have different proportions of *Lactobacillus*-dominated CSTs, except where *L. iners* is the dominant species [301]. As no such causative distinction was made between the infertile (case) participants during the recruitment stage of this study, it is unclear:

- What proportion of the total number of cases would be considered nININF;
- What proportion of the total number of cases would be considered ININF;
- How many of the ININF cases resulted despite having protective CSTs;
- The proportion of ININF cases in which TFI is the underlying cause of infertility.

While the case-control study of the female reproductive tract microbiota described within this thesis did not identify any significant association between CST and fertility status, it is worthwhile to consider the possibility that this observation was at least in part due to the incongruent microbiota we observed between at the different sites of the FRT. Additionally, this study was retrospective by design, so it should be highlighted that fertility status of participants in the study may not have been concurrent with the microbiota sampled at each of the sites. Community variation between these sites was a key finding of the study, as it suggested that although the vaginal microbiome was a reasonable predictive indicator of the cervical microbiota, it was not predictive of the endometrial microbiota. This should be considered an important outcome, given that the aim of the research was to establish a link between host and microbial factors, and fertility. Thus, I believe it is reasonable to suggest that incongruence between the vaginal and endometrial microbiota especially, may have both clinical and experimental implications in the context of female fertility. The role of the endometrium and its microbiota in female fertility and pregnancy is complex and poorly understood [302]. Evidence from the limited number of studies which have examined this relationship suggest that the endometrial microbiota relatively stable [303] with temporal changes, and may affect implantation of the embryo [185].

It is understood that in some cases, chlamydial infections ascend beyond the endocervix [242] and into the upper portions of the female reproductive tract. This is likely in part due to the environmental conditions of this niche; which has fewer protective bacteria, making it more conducive to chlamydial development [300]. In the context of the finding discussed above, it could be argued that just because a woman is found to have a “protective” vaginal microbiome does not mean that *C.*

trachomatis (or other microbial pathogens) can‟t or won‟t be found at other locations such as the endometrium, after overcoming the host‟s protection. It would therefore be desirable for future investigations similar to the ones comprising Aim 1 to look at multiple sites, especially when conducting surveillance for sexually transmitted pathogens known to ascend past the cervix.

The human immune system serves to protect us against both endogenous and exogenous threats to our health. Additionally, it is known that its ability to detect and eliminate bacterial pathogens can be affected by polymorphisms in the human genome affecting specificity, as well as regulation of expression [304]. Interestingly, the human immune system has also been shown to be modulated by the microbiomes [305] which establish themselves early after partum [306]. *Lactobacillus* spp. which dominate the vaginal microbiome in many women are understood to assist in immunity and actively protect against pathogens of the female reproductive tract [299]. This has been attributed to their secretion of bioactive metabolites into the vaginal microenvironment [254, 307]. One such metabolite, lactic acid, has long been studied for its role in vaginal pH, gynecological disease and chlamydial infection [173, 300]. Shown to have a crucial role in vaginal eubiosis, lactic acid has also been shown to affect the local immune balance, by dampening the production of inflammatory modulating-gene products [308]. Despite this, the gene expression data generated by this research project showed no difference in the limited number of genes tested. To address the shortcomings of this limited investigation, a more comprehensive assessment of the gene expression may reveal differences relating to the variations in microbiota observed at these key sites in the female reproductive tract. Additionally, performing a similar study using a prospective cohort design, rather than

retrospective, may offer more valuable insight concurrent with fertility status of participants.

TNC is a human gene which encodes the extracellular matrix protein, tenascin-C (TNC). TNC has various tissue-specific roles throughout the body [309], but was originally thought to be an extracellular matrix remodeling protein which existed primarily to modify cell adhesion and migration [310]. Recent evidence also suggests it may play an important role in modulating the immune response and protecting against the effects of localised inflammation [311]. In addressing aim 2, we identified that *TNC* was overexpressed in women who had experienced at least one miscarriage, compared to those who had not. While the fact that this was a self-reported data point collected in questionnaire format could be considered a minor limitation of this finding, there is nothing to suggest any of the participants had a reason to or did report inaccurate details. While TNC is known to have roles in embryogenesis [312] and the formation of uterine fibroids [313], little is known about its involvement in miscarriage. As ECM remodeling and dysregulation has been associated with an increased risk of miscarriage [314], our observations potentially present another interesting avenue of investigations into fertility and pregnancy. As with many other analogous processes, the role of TNC as both a regulator and regulatory target of other components of the immune system is likely highly complex and may be microenvironment-specific [315].

The third and final key finding of the aim addressed in **Chapter 3** of this thesis was that sequences read corresponding with *Ureaplasma* spp. were overrepresented amongst infertile women (cases) in the case-control microbiota study. Specifically, four case participants and one control were observed to have sequence reads which mapped to *Ureaplasma* spp., either in the vagina (four cases, one control), cervix

(four cases, one control), or both (three cases, one control). Though the small sample size of both the study and this group of women with *Ureaplasma* are clear limitations of interpreting these findings, it is nonetheless an interesting result. *Ureaplasma parvum* and *Ureaplasma urealyticum* are facultative anaerobic pathogens of the female reproductive tract and are associated with a spectrum of urogynaecological diseases and complications (reviewed in [316]). Additionally, *Ureaplasma* spp. have been associated with adverse pregnancy outcomes [200] including premature birth [317] and chorioamnionitis [201]. *Ureaplasma* have also been associated with infertility [318] and are suspected by some to be a causative agent of PID in women [319, 320]. Thus, while it should be considered a key finding of this work, its implications as well as the relationship between *Ureaplasma* colonisation and female fertility remain unclear.

Aim 2

Establishing whether the developmental and stress phenotypes of C. trachomatis clinical isolates are associated with the outcome of treatment

Genomic evidence suggests that the genus *Chlamydia* has evolved and adapted to each of its hosts, including humans, wild and domesticated animals [321, 322]. In order to survive and proliferate within its unique intracellular niche, *C. trachomatis* has evolved mechanisms to overcome the host immune response. In addition to this, it has developed strategies to compete with the local commensal flora and survive within its intracellular niche [323, 324].

Cases of antibiotic treatment failure are rare and poorly understood [114]. In the literature, apparent cases of treatment failure have often been suspected to be the result of reinfection, rather than antibiotic resistance [325]. Although chlamydial persistence has been proposed as a possible mechanism that *Chlamydia* spp. use for their survival [326], its involvement in treatment failure has yet to be determined. My interest in both chlamydial treatment failure and persistence stemmed from the gap in knowledge about the microbial and host factors involved in treatment outcomes. Based on this, I made the second Aim of my thesis to assess the role of chlamydial persistence in the context of how it related to treatment. It was my hope that these investigations would help improve the collective understanding of not only how *C. trachomatis* responds to stress and antibiotics, but also whether such a response could have implications for treatment. Fortunately, Dr. Willa Huston‟s collaboration in ACTS granted me access to a selection of clinical isolates. My objective for this project was to use an *in vitro*

persistence model to measure and compare the responses of these clinical isolates to both penicillin and azithromycin. From the research undertaken to address Aim 2, which is detailed and discussed in **Chapter 4** of this thesis, there emerged three key findings:

- Clinical isolates were more susceptible to environmental stress than the laboratory strain.
- Subtle variations between the cultivated strains observed *in vitro* may not sufficiently reflect that of *in vivo* infections.
- Cultivation of all strains in the McCoy B murine fibroblast cell line yielded the most EBs after 44 hours of growth.

Collectively, these findings reinforce the complexity of the chlamydial infection process, and provide some insight into the importance of both clinical and experimental research in improving our understanding of this unique pathogen.

After comparing multiple mammalian cell lines, we were able to demonstrate that chlamydial growth was greatest in the McCoy B cell line. This cell line also proved to be the most conducive to cultivation of the clinical isolates. In the absence of viable and ethical research alternatives, most research into this obligate intracellular pathogen has been conducted using lab-adapted type strains, cultivated in either immortalised mammalian cell lines, or animal models – most commonly mice [327]. *In vitro* methods, especially very recent advances in the genetic manipulation of *Chlamydia* spp., have contributed to our current knowledge of this unique bacterium [328]. From this, researchers have gained valuable insight into the unique host-pathogen interactions during infection, as well as a reasonable understanding of the developmental biology of *Chlamydia*.

Importantly, they have helped inform our understanding of chlamydial persistence – a somewhat unique topic and feature of chlamydial biology.

Chlamydia is often misconstrued by young Australians as a sexually transmitted infection (STI) that is of little cause for concern. This is likely at least in part due to the public‟s perception of the “silent STI” as well as the availability and simplicity of antibiotic treatments. *C. trachomatis* infections of the urogenital tract are associated with a spectrum of serious diseases and sequelae, among which are pelvic inflammatory disease (PID), tubal factor infertility (TFI), and ectopic pregnancy. Azithromycin and doxycycline are routinely used to treat uncomplicated cases of *C. trachomatis* urogenital tract infection and are typically successful [113]. Although it is a small number, the 4% of patients that experience treatment failure is an important figure for two reasons. The first being that treatment failure and reinfection are correlated with an increased risk of chlamydial diseases and their sequelae [273]. Secondly, there exists very little data which provides any substantial insight or sound explanation for clinical observations such as these.

Chlamydial persistence *in vitro* is the collection of developmental phenotypes that *C. trachomatis* and other species of the genus exhibit, when cultivated in conditions that cause stress to the developing organisms. Exposure to sub-optimal environmental conditions and specific exogenous stimuli has been shown to induce chlamydial persistence. Studies examining gene and protein expression of *C. trachomatis* during persistence suggest the pathogen‟s response varies between different inducers – differences which reflect the metabolic process affected.

Failed clearance of chlamydial infections usually results in the detection of the same infecting strain after completion of the antibiotic therapy. As previously mentioned, this clinical phenomenon has been attributed to both reinfection and treatment failure [162]. The caveat to treatment failure is that many such instances are likely the result of reinfection by a sexual partner, who may be unaware they are infected. Currently, it is unclear what process is involved when *C. trachomatis* does survive treatment. Antibiotic resistance has been all-but ruled out [120, 121, 123, 329], suggesting that a heterogenetic trait or temporal developmental strategy may, perhaps by chance, allow a small sub-selection of *Chlamydia* to survive. Chlamydial persistence is a developmental response to stress exhibited by the genus *Chlamydia* (reviewed by [242, 326]. It is characterised by a broad range of transcriptional, metabolic and morphological changes, each of which differ depending on the inducer of persistence [36, 43, 265]. Some *in vitro* studies have shed light on how persistence may affect *Chlamydia*'s susceptibility to azithromycin and other antibiotics [126], but they have not addressed how this may occur, or how likely their models are to reflect real infections in women. Despite only slight variations in their genomes, we observed subtle differences between the developmental and stress-response phenotypes of our clinical isolates, and our laboratory strain. None of these were associated with treatment outcomes, but we gained some insight into how clinical isolates grow in various cell lines, as well as how they respond to stressors such as antibiotics and iron deprivation. As mentioned previously, it is unclear whether the subtle differences we observed *in vitro* reflect the magnitude of difference likely to occur *in vivo*. This is a common and inherent limitation of models using immortalised cell lines to culture

intracellular pathogens, as the complexity of the endocervical and vaginal microenvironments are incredibly difficult to recreate.

Aim 3

Analysis of host and chlamydial gene expression in women treated for **C. trachomatis** *infection of the urogenital tract*

Chlamydial infection has been associated with a spectrum of urinary and reproductive tract diseases in both men and women. The adverse outcomes associated with chlamydial infection are thought to be mediated by the immune response to infection, rather than by the pathogen itself, although the involvement of bacterial genotypes has not been excluded as a contributing factor [330]. While the relationship between infection, immunity, disease and sequelae is not completely understood, dysfunction and/or dysregulation of the host's immune system is central to most theories of chlamydial disease progression [331]. As previously discussed, there exists an intrinsic relationship between the host cell and its and intracellular pathogen. While the interactions between *C. trachomatis* and its host are generally well understood, less is known about the biological processes which facilitate and occur during infection [332]. Further, it has not yet been determined how gene expression by the host cell and *C. trachomatis*, at the time of treatment with antibiotics, influences the outcome of treatment. Because of this, I decided to investigate how both the host and chlamydial gene expression levels associate with or appear related to the outcomes of antibiotic treatments.

Azithromycin treatment activates chlamydial stress responses at a transcriptional level

Penicillin and gamma interferon are two of the most extensively studied inducers of chlamydial persistence. The effects of azithromycin on *C. trachomatis* have also been examined, with one study even suggesting the antibiotic itself may induce chlamydial persistence [333]. Using a selected group of ACTS participants in a nested case-control study design, I investigated whether the expression of several human and chlamydial genes (important to *Chlamydial* biology and treatment) before and after treatment showed any association with treatment outcome. With some limitations, our gene expression data appears to indicate that there was no clear difference between the case and control groups at the time of treatment. We did however observe that when *C. trachomatis* does survive azithromycin (in cases of treatment failure), there were significant and important increases in its expression of genes known to be associated with both typical and stressed development. It is unclear whether such an observation is likely to reflect a mixed population that has expanded after the subsidence of azithromycin. Furthermore, the modulation of the immune system by azithromycin may also be an important consideration in interpretation of this finding. It is also unclear whether the effects of azithromycin on the vaginal microbiome have an impact in subsequent challenges or clearing the non-resolved infection [298].

Conclusion

The goal of this project was to examine interactions between the female reproductive tract, its microbiome, and the bacterial pathogen *C. trachomatis*. The localised immune system of the female reproductive tract is in constant interaction with the commensal microbes which reside in this unique anatomical niche. Modulation or ablation of this crosstalk can lead to a spectrum of gynecological dysbiosis and diseases, many of which are caused by opportunistic and professional pathogens alike. *C. trachomatis* must overcome these defences in order to establish an infection, using the specialised virulence genes from its highly conserved genomic arsenal. From the work in this project, we can hypothesise that both infertility in women and chlamydial treatment failure are outcomes stemming from profiles of contributing factors and interactions between human and microbial processes. By better understanding the human factors involved in infertility and treatment failure, we may identify important predisposing factors such as genetic polymorphisms that increase an individual's risk of adverse outcomes or infertility. Ultimately, research such as the present study aims to reduce the incidence and burden of treatment failure as well as improve fertility-related health outcomes.

Future directions

This project combines finding from several related studies, which each focused of one aspect of the overarching research problem. To expand upon the observations made during these studies, there are several potential avenues for future investigations. The intimate relationship between the human microbiome and immune system has gradually become better understood [293]. Fertility is an important aspect of gynecological health, and the study Chapter 3 was conducted to improve our knowledge of how the microbial communities of the female reproductive tract are associated with the ability to conceive. A limitation of the design used was the sample size, owing to the difficulty involved in recruiting participants to studies examining very personal, private and often emotional aspects of their lives and bodies [334]. Increasing the number of participants in both the control (n=16) and case (n=15) groups for future is an important consideration, especially when considering that microbial community diversity and proportion may vary significantly even between women of the same clinical classification. Longitudinal sampling of the anatomical sites selected should also be considered, as evidence suggests that microbial population stability may be an important component of infectious infertility [152, 335-337]. While the appropriate ethical concerns would need to be addressed, it may also be more relevant to the research question if the microbial populations of women were looked at over an extended period of time – as has been done recently by similar investigations [152, 336]. Recently, several groups have looked at and highlighted the importance of the endometrial microbiome in fertility and the progression of pregnancy[302]. Despite this, it has yet to be determined how these important

factors are involved in fertility or how they relate to achieving natural or assisted pregnancies. Reflecting this, endometrial microbiota should be considered for sampling, using an ethical and clinically-appropriate method, especially if implantation and pregnancy outcomes are being examined [185, 338]. The human immune response to infection is regulated by a complex collection of interconnected signaling pathways. Many of these are likely to be positively or negatively regulated during pregnancy, to facilitate development and survival of the embryo and fetus. Because of this, it is suggested that the expression of much broader range of human genes are investigated in future endeavours. This could be achieved using RNA-seq, an RNA microarray, or a commercial RT-qPCR gene panel analysis kit. The number of samples, stability of RNA, sample type, cost and level of technical skill required should all be considered when selecting the most suitable technology. Additionally, profiling the gene expression of the microbes in tandem with the host may provide a degree of insight into the relationship between inflammation, the growth and development of lactobacilli, as well as virulence and pathogenesis of selected bacteria-of-interest, such as *C. trachomatis*. Measuring bacterial metabolites such as lactic acid and *D*-lactate is another recommendation, as comparing the levels of bactericidal and other metabolites with those of human and chlamydial gene expression may improve our knowledge of how the pathogen survives *in vivo* under stressful conditions[56].

Clinical isolate studies are relatively scarce yet could provide important information about the strains of *C. trachomatis* which actively infect the community. Although the findings discussed in Chapter 4 of this thesis provide a degree of insight into using *in vitro* methods to study this complex pathogen, there are more questions that need to be addressed. Though subtle differences in growth

and stress responses were seen, none were associated with treatment outcomes. Expanding the number of clinical isolates from cases of treatment success and failure may be helpful in associating treatment outcomes to its developmental phenotypes or genotypes [330]. Given the degree of similarity between the isolates‟ development in the penicillin and iron deprivation persistence models, it may seem attractive to seek alternative explanations for the mechanisms of treatment failure. Because of this, it would be remiss of me not to remind the reader that current scientific opinion supports the idea that chlamydial persistence likely plays some role in antibiotic treatment outcomes [326]. Alternative and complementary models of chlamydial persistence, such as interferon-gamma, glucose deprivation, or biologically relevant bacterial metabolites (lactic acid, etc) should also be considered for future clinical isolate studies. Not only for their physiological relevance to the niche environment inhabited by *C. trachcomatis*, but also because their simplicity of measurement *ex vivo* could enhance other studies of the female reproductive tract pathogens. The high level of genetic identity among the previously encountered strains of *C. trachomatis* also presents opportunities to use next generation sequencing technologies to locate and characterise the chlamydial genes and polymorphisms associated with treatment outcome. As before, these genetic approaches could accompany studies of chlamydial persistence, to better understand the processes involved in this bacterial survival strategy.

The avenues for investigation stemming from Chapter 5 of this thesis are similar to those recommended for Chapters 3 and 4. Specifically, it may be beneficial to look at treatment outcomes and fertility using similar study designs, as well as drawing larger amounts of participant and experimental data (sub-investigations) from

them. Arguably the most important improvement that can be suggested for improving building upon this project‟s findings are to increase sample sizes as much as possible. This may in turn increase the likelihood that significant associations or differences are visible and not masked by intra-individual differences in gene expression. As was mentioned for the data in Chapter 3, it would be prudent to include considerably more human and chlamydial genes for analysis and comparison, ideally using an alternative transcriptomic quantitation strategy. The inclusion of genes potentially involved in chlamydial persistence should be considered prime candidates in any host-pathogen gene expression analysis study, to enhance our understanding of chlamydial developmental biology [339] as well as implications for antibiotic treatment.

Ultimately, a better understanding of the host and microbial factors involved in dysbiosis and chlamydial infection of the female reproductive tract will help to improve gynecological health and fertility, and develop better diagnostic and treatment strategies for intracellular pathogens.

APPENDICES

Appendix I

Appendix I. Supplementary table showing the read counts from 16S rRNA gene amplicon sequencing before filtering, after filtering and after taxonomic assignment.

Sample ID	Raw read pairs	Human sequences filtered, assembled, filtered at q20	Assigned by QIIME	Assigned by STIRRUPS
WH004.A	196356	107379	107908	103015
WH004.E	43	17	17	5
WH005.A	207003	119276	119286	117322
WH005.E	161	15	15	13
WH007.A	311123	190018	190014	189112
WH007.C	247648	129013	129011	128533
WH007.E	439	74	74	35
WH008.A	192366	123399	123378	123175
WH008.C	293754	177005	177020	176626
WH008.E	586	90	90	88

WH009.A	273576	133672	133502	132993
WH009.C	264242	134604	134403	133951
WH009.E	530	136	136	127
WH010.A	245481	148035	148034	147224
WH010.C	158363	97210	97200	96643
WH010.E	461	46	46	36
WH011.A	228559	130167	130220	125423
WH011.C	274417	146310	146374	144339
WH011.E	845	31	31	24
WH012.A	180376	100484	100511	99860
WH012.C	269728	156411	156436	155495
WH012.E	742	96	97	62
WH014.A	261930	156555	156632	155988
WH014.E	282	71	71	68
WH016.A	217337	117814	117849	114822
WH016.C	188319	111902	111955	110403
WH016.E	85	26	25	8
WH017.A	234319	123837	123835	122796
WH017.C	271272	151955	151950	150971
WH020.A	173004	99132	99140	98489

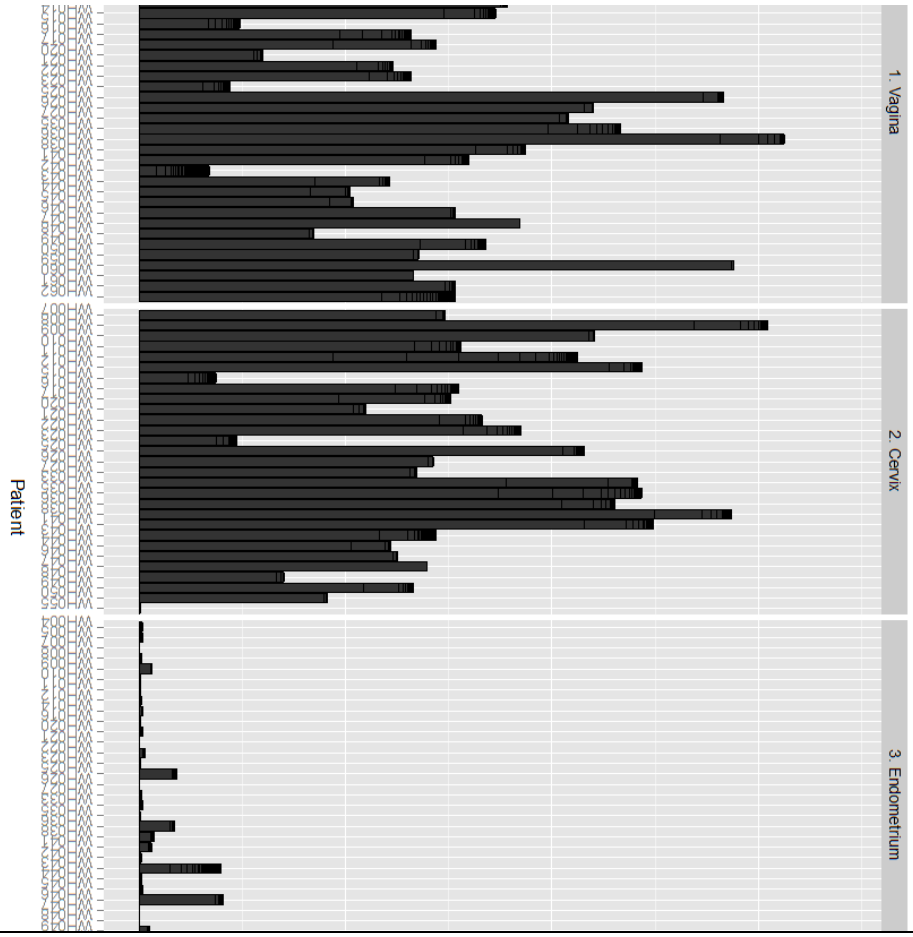
WH020.C	181807	105611	105611	105073
WH020.E	1254	250	251	237
WH021.A	44893	28386	28379	28293
WH021.C	318733	198583	198504	197974
WH021.E	178	29	29	10
WH022.A	210574	130320	130255	129562
WH022.C	247521	144907	144849	143921
WH022.E	1541	455	455	448
WH023.A	9215	5504	5532	1109
WH023.C	14069	8084	8112	694
WH023.E	1130	254	253	214
WH025.A	332231	201274	201271	199789
WH025.C	402194	246712	246672	243107
WH025.E	124463	21307	21304	11181
WH026.A	132405	29333	29333	29303
WH026.C	147779	35043	35043	34996
WH026.E	19998	1859	1859	182
WH027.A	157769	33745	33745	33685
WH027.C	158761	32332	32333	32157
WH027.E	27697	5407	5410	4414

WH035.A	233767	80025	80030	79403
WH035.C	149254	52075	52073	50515
WH035.E	10114	340	340	129
WH036.A	134665	45150	45152	45073
WH036.C	111372	42731	42735	42686
WH036.E	79208	29867	29863	29490
WH038.A	143663	51881	51884	51624
WH038.C	122865	45384	45389	45020
WH038.E	83820	28096	28089	27344
WH041.A	139505	44491	44486	43863
WH041.C	195649	52971	52968	52401
WH041.E	38792	14803	14798	13897
WH043.A	132517	53088	53090	51096
WH043.C	288519	116620	116595	109916
WH043.E	204284	49707	49645	27659
WH044.A	116152	23448	23430	22417
WH044.C	165008	38664	38638	37854
WH044.E	9190	468	468	30
WH045.A	148663	80715	80710	79486
WH045.E	17273	3040	3038	2722

WH048.A	124558	35531	35560	34694
WH048.C	149581	44465	44504	43305
WH048.E	10141	227	227	135
WH049.A	146451	50702	50699	49083
WH049.C	108815	35822	35836	34468
WH049.E	28632	5441	5441	5260
WH050.A	154346	29259	29259	29230
WH050.C	117171	23243	23243	23233
WH050.E	140045	24854	24851	24447
WH061.A	104920	18977	18974	18620
WH061.E	23808	1657	1656	1419
WH062.A	203361	103060	102862	88578
WH062.E	92288	37991	38027	21480
WHB1N	126	49	49	28
WHB2N	374	129	129	47
WHB3N	153	17	17	10
WHB4N	315	11	11	10
WHB5N	328	125	125	7
WHB6N	68	29	29	19
WHB7N	672	115	114	102

WHB8N	183	33	33	14
WHB9N	12	2	2	1
WHB10N	565	77	77	45
WHB11N	375	61	61	52

Appendix II



Appendix II. The relative proportions of all taxonomic assignments in the samples grouped on the basis of the anatomical sites (vagina, cervix or endometrium).

Appendix III. Reaction conditions and oligonucleotide sequences used in RT-qPCR analysis of samples from the endometrial tissue specimens of women in the case-control fertility study.

Gene name	GenBank accession ID	Oligonucleotide sequence 5'→ 3'	Annealing temp (°C)	Amplicon size (bp)	Efficiency (%)
Glyceraldehyde-3-phosphate dehydrogenase	NM_002046	Sense: GGATTTGGTCGTATTGGGCG Antisense: AGGGATCTCGCTCCTGGAAG	52	220	92
Peptidylprolyl isomerase A	NM_021130	Sense: GAGGAAAACCGTGTACTATTAGC Antisense: GGGACCTTGTCTGCAAAC [295]	54	113	86
Interleukin-6	NM_000600	Sense: ACCCCCAGGAGAAGATTCCAAAG Antisense: TCACCAGGCAAGTCTCCTCATTG	52	251	92
Interleukin-8	NM_000584	Sense: GCTCTGTGTGAAGGTGCAGTTTTG Antisense: ACCCAGTTTTCCTTGGGGTCCAG	54	203	92
Syndecan-1	NM_002997	Sense: GGAGCAGGACTTCACCTTTGAAAC Antisense: GTCCTTCTTCTTCATGCGGTACAG	58	226	92
Interluekin-1-alpha	NM_000575	Sense: CATCCTGAATGACGCCCTCAATC Antisense: ATCTCAGGCATCTCCTTCAGCAG	54	228	102
Tenascin-C	NM_002160	Sense: CGACGTGTTTCCCAGACAGA Antisense: GTGGCTTGTTGGCTCTTTGG	54	143	90
Tumor necrosis factor (alpha)	NM_000594	Sense: CTTCTGCCTGCTGCACTTTG Antisense: CTCAGCTTGAGGGTTTGCTAC	54	157	90

Appendix III

Appendix IV

Appendix IV. Oligonucleotide sequences used during host gene expression analysis by RT-qPCR.

ID	Accession number	Sense (5‟ → 3‟)	Antisense (5‟ → 3‟)	Amplicon Size (bp)
GAPDH	NM_002046.5	CTCTCTGCTCCTCCTGTTCG	GACCAAATCCGTTGACTCCG	112
PGK1	NM_000291.3	TGGAGCTCCTGGAAGGTAAAG	AGTTGACTTAGGGGCTGTGC	101
IDO1	NM_002164.5	GCAAGAACGGGACACTTTGC	TGGGTTCACATGATCGTGGATT	100
IRF1	NM_002198.2	CAAATCCCGGGGCTCATCTG	CTGCTTTGTATCGGCCTGTG	145
CXCL9	NM_002416.2	CAGGCTCAAAATCCAATACAGGAG	TACTGGGGTTCCTTGCACTC	124
FTH1	NM_002032.2	CCAGAACTACCACCAGGACTCAG	GGTCAAAGTAGTAAGACATGGAC	101
IL6	NM_000600.4	ACCCCCAGGAGAAGATTCCAAAG	TCACCAGGCAAGTCTCCTCATTG	92
IL8	NM_000584.3	GCTCTGTGTGAAGGTGCAGTTTTG	ACCCAGTTTTCCTTGGGGTCCAG	92
IL1A	NM_000575.4	CATCCTGAATGACGCCCTCAATC	ATCTCAGGCATCTCCTTCAGCAG	102
TNFA	NM_000594.3	CTTCTGCCTGCTGCACTTTG	CTCAGCTTGAGGGTTTGCTAC	90
IL10	NM_000572.3	TACGGCGCTGTCATCGATTT	ACTCATGGCTTTGTAGATGCCT	110
IFNG	NM_000619.2	ATTGGAAAGAGGAGAGTGACAG	CACTCTTTTGGATGCTCTGGTC	105

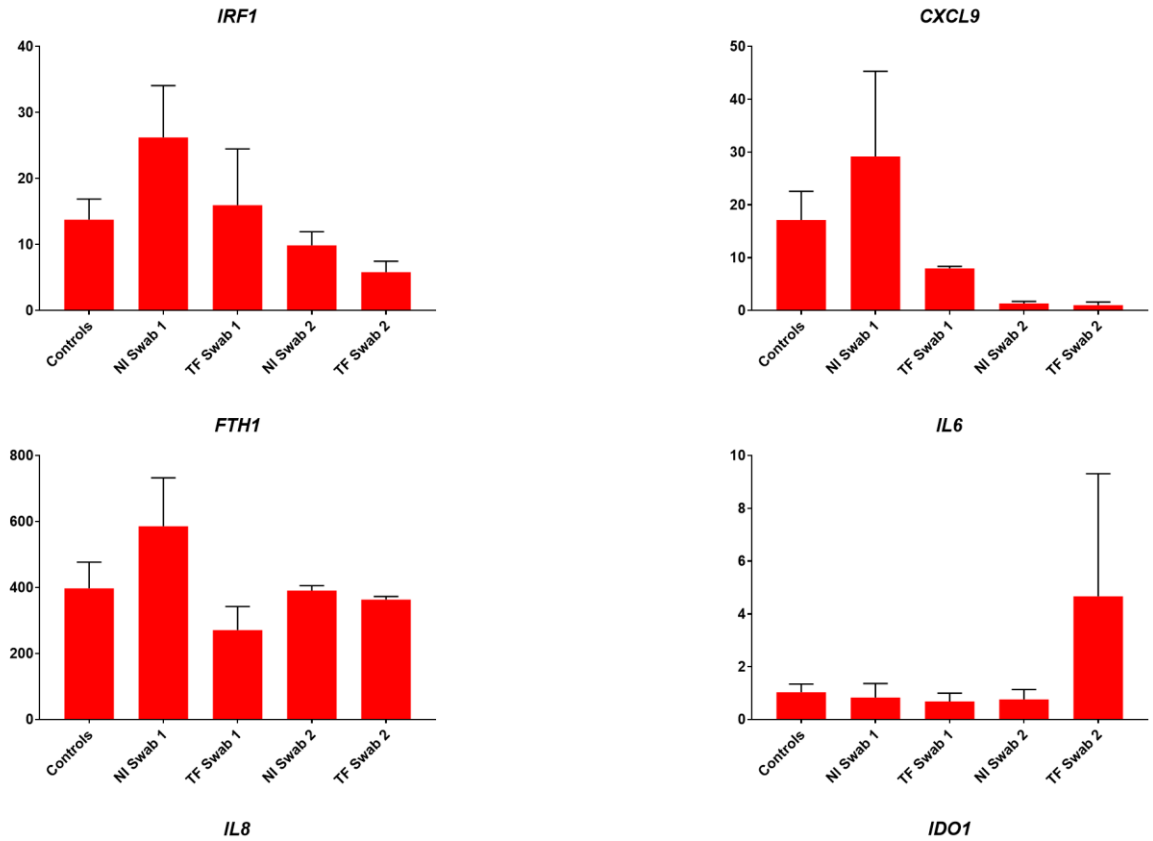
Appendix V

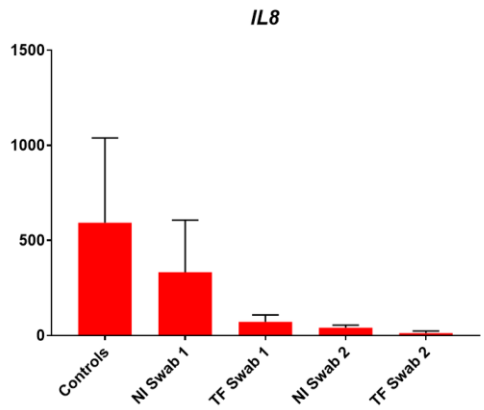
Appendix V. Oligonucleotide sequences used during chlamydial gene expression analysis by RT-qPCR.

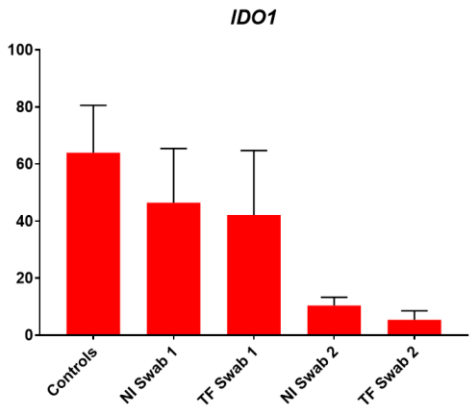
ID	Sense (5' → 3')	Antisense (5' → 3')	Amplicon Size (bp)	Reference
16S rRNA	GGAGAAAAGGGAATTTCACG	TCCACATCAAGTATGCATCG	173	[268]
omcB	GTAAGAGCACAAACTCCTGG	GCACATATGAGTAGCAGCAA	129	
htrA	GACTGGGCTATTGCTATTGG	ATGGCAGCATCTGTTTGAAT	131	
euo	TCCCCGACGCTCTCCTTTCA	CTCGTCAGGCTATCTATGTTGCT	263	[269]
ompA	TGCCGCTTTGAGTTCTGCTT	GTCGATCATAAGGCTTGGTTCAG	75	[270]
trpBA	GCATTGGAGTCTTCACATGC	ACACCTCCTTGAATCAGAGC	258	

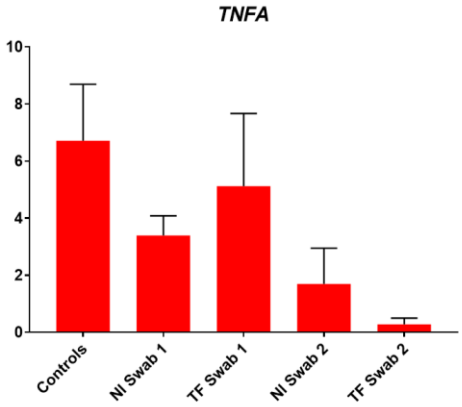
Appendix VI

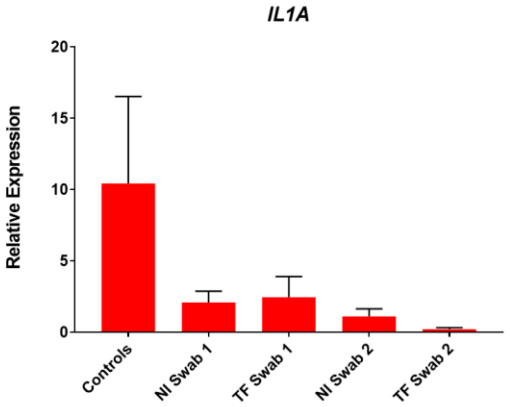
Appendix VI. RT-qPCR plots of the ten human genes of interest after subdivision of the groups. This data shows the comparison of the control, case NI primary and post-treatment, and case TF primary and post-treatment swab groups. Displayed are the mean expression values relative to the geometric mean of *GAPDH* and *PGK1*. Error bars show the standard error of the means, with statistical significance denoted by * ($p \leq 0.05$), ** ($p \leq 0.01$) and *** ($p \leq 0.005$).

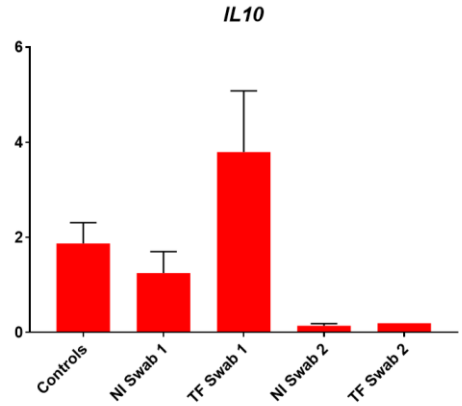


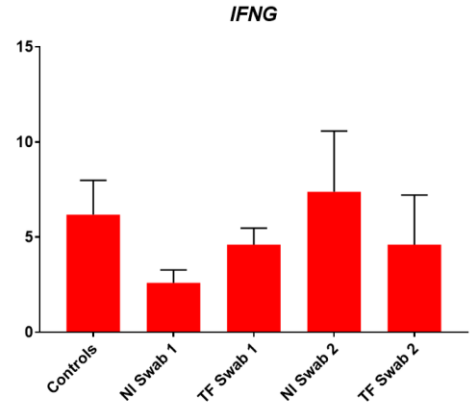

IL8
1500
1000
500
0
Controls
NI Swab 1
TF Swab 1
NI Swab 2
TF Swab 2


IDO1
100
80
60
40
20
0
Controls
NI Swab 1
TF Swab 1
NI Swab 2
TF Swab 2


TNFA
10
8
6
4
2
0
Controls
NI Swab 1
TF Swab 1
NI Swab 2
TF Swab 2

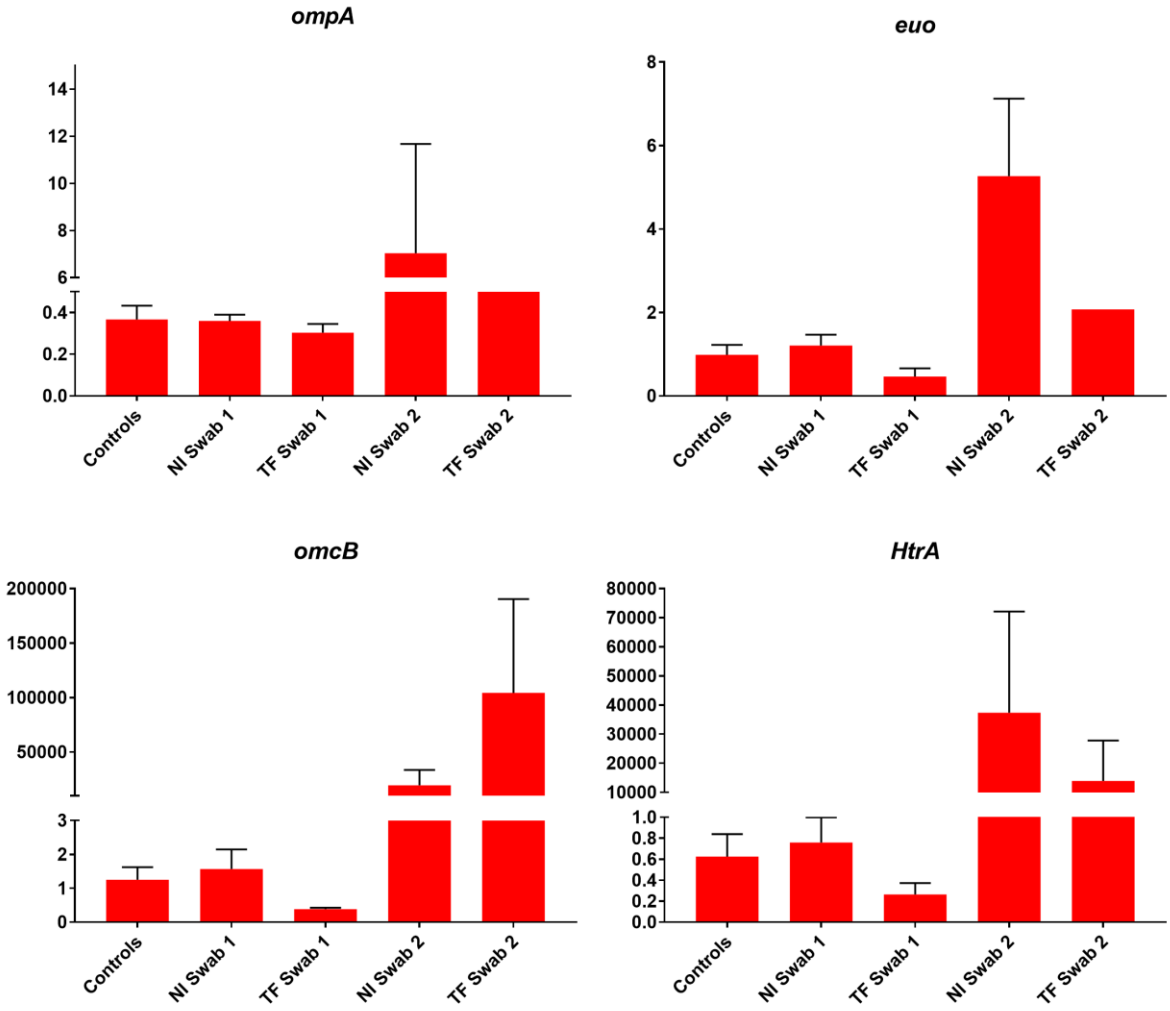

IL1A
Relative Expression
20
15
10
5
0
Controls
NI Swab 1
TF Swab 1
NI Swab 2
TF Swab 2

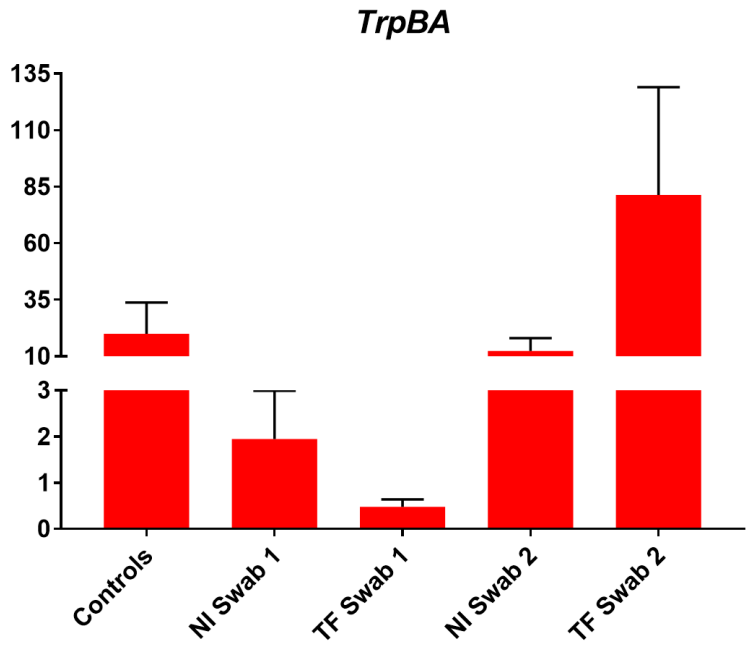

IL10
6
4
2
0
Controls
NI Swab 1
TF Swab 1
NI Swab 2
TF Swab 2


IFNG
15
10
5
0
Controls
NI Swab 1
TF Swab 1
NI Swab 2
TF Swab 2

Appendix VII

Appendix VII. RT-qPCR plots of the five chlamydial genes of interest tested compared after subdivision of the case group. This appendix shows comparison of the expression levels in the control, case NI primary and post-treatment, and case TF primary and post-treatment swab groups. Displayed are the mean expression values chlamydial *ompA* (A), *euo* (B), *omcB* (C), *htrA* (D) and *trpBA* (E) relative to the expression levels of the *C. trachomatis* 16S rRNA gene. Error bars show the standard error of the means, with statistical significance denoted by * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.005$) and **** $p \leq 0.001$).


ompA
14
12
10
8
6
0.4
0.2
0.0
Controls
NI Swab 1
TF Swab 1
NI Swab 2
TF Swab 2
euo
8
6
4
2
0
Controls
NI Swab 1
TF Swab 1
NI Swab 2
TF Swab 2
omcB
200000
150000
100000
50000
3
2
1
0
Controls
NI Swab 1
TF Swab 1
NI Swab 2
TF Swab 2
HtrA
80000
70000
60000
50000
40000
30000
20000
10000
1.0
0.8
0.6
0.4
0.2
0.0
Controls
NI Swab 1
TF Swab 1
NI Swab 2
TF Swab 2


TrpBA
135
110
85
60
35
10
3
2
1
0
Controls
NI Swab 1
TF Swab 1
NI Swab 2
TF Swab 2

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