Mobility and detection of antimicrobial resistance genes in *Mycoplasma hominis*, *Ureaplasma parvum, Ureaplasma urealyticum* and *Neisseria gonorrhoeae*

By

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Summary

Mycoplasmas are some of the most structurally minimalist bacteria currently known to science. As urogenital pathogens, they can lead to a variety of adverse outcomes such as spontaneous abortion, pre-term birth and neonatal pneumonia. Their structural simplicity means that they lack most of the targets of antibiotic treatments, and their high degree of genomic plasticity allows them to rapidly adapt to the few treatments that are available. As such, the primary goal of this work was to investigate the mechanisms and detection of tetracycline and quinolone resistance in *Mycoplasma hominis* as well as tetracycline resistance in *Ureaplasma parvum* and *Ureaplasma urealyticum*. In addition, I investigated the effectiveness of utilizing real-time PCR techniques for the clinical detection of urogenital mycoplasmas and their associated antimicrobial resistances, along with that of *Neisseria gonorrhoea* and *Mycoplasma genitalium* due to their international prominence as "superbugs".

An interrogation of the mechanisms behind fluoroquinolone resistance in *M. hominis* demonstrated an environmentally driven mechanism of resistance, mediated by mutations I observed in *gyr*A that preceded mutations in the complementary *par*C gene. Furthermore, I observed a similarly high degree of genomic heterogenicity between the *tet*M genes that mediate tetracycline resistance in *M. hominis*, suggesting that horizontal gene transfer mediated the development of tetracycline resistance in *M. hominis*. These observations were only partially mirrored in my analysis of tetracycline resistant *Ureaplasma spp*. All *Ureaplasma urealyticum* isolates shared a common insertion site for *tet*M that appeared to be part of a Tn916-carried composite integrated conjugal element (ICE), while most *U. parvum* isolates retained a second conserved *tet*M insertion site (but without most of the

expected Tn916-associated genes) and a final *U. parvum* isolate that appears to have acquired a Tn916 insertion event at a unique third somatic position. For all *Mollicutes*, the transition from standard bacterial coding where UGA is stop, and not the main histidine codon, appears to drive mutation in these open-reading frames in *M. hominis* and *Ureaplasma spp*.

Analysing isolates collected as part of the Antibiotic Guardian study, which had to be significantly truncated due to the COVID-19 outbreak and restrictions on sexual health patients coming into hospital, found that *U. parvum* had highest prevalence, followed by *M hominis*, *U. urealyticum*, *N. gonorrhoea* and finally *M. genitalium*. Furthermore, a significantly higher prevalence of *U. parvum* and *M. hominis* in female patients suggested a gender bias for pathogenicity. The in-house qPCR assays used showed 100% concordance with both commercial molecular methods and NHS culture methods for detection of urogenital mycoplasmas, indicating the reliability and accuracy of this assay for use as a rapid diagnostic tool. Furthermore, the high levels of resistance in *N. gonorrhoea*, *M. genitalium* and *M. hominis* observed were identical between SpeeDx commercial assays (not *M. hominis*) and culture-based confirmation. However, the significantly lower viability of *N. gonorrhoea* in culture demonstrated that the molecular methods had a distinctive advantage in diagnosis relative to traditional susceptibility testing methods. Given the increasing prevalence of resistance for all pathogens, continued surveillance to monitor antimicrobial resistance trends is required.

Abbreviations

AMR	Antimicrobial resistance
ARG	Antibiotic resistance gene
ATP	Adenosine triphosphate
BASHH	British Association for Sexual Health and HIV
BLAST	Basic Local Alignment Search Tool
CARD	Comprehensive Antibiotic Resistance Database
CDC	Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CSF	Cerebrospinal fluid
CT	*Chlamydia trachomatis*
EMA	European Medicines Agency
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	*Neisseria gonorrhoea*
HGT	Horizontal gene transfer
HIV	Human immunodeficiency virus
HPV	*Human papillomavirus*
ICE	Integrate conjugative element
IRAS	Integrated Research Application System
LGV	Lymphogranuloma venereum
MBA	*Multiple banded antigen*
MG	*Mycoplasma genitalium*
MH	*Mycoplasma hominis*
MHBM	Mueller Hinton broth medium
MIC	Minimum Inhibitory Concentration
MLST	Multi-locus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSM	Men who have sex with men
NCBI	National Centre for Biotechnology Information
NHS	National Health Service
NICE	National Institute for Health and Care Excellence

ORF	Open reading frame
PCR	Polymerase chain reaction
PHE	Public Health England
PHW	Public Health Wales
PID	Pelvic inflammatory disease
QRDR	Quinolone resistance-determining region
RPP	Ribosomal protection protein
RPR	Rapid plasma reagin
SARA	Sexually acquired reactive arthritis
SNP	Single Nucleotide Polymorphism
STI	Sexually transmitted infection
TPHA	Treponema pallidum haemagglutination assay
UKHSA	UK Health Security Agency
UP	*Ureaplasma parvum*
UU	*Ureaplasma urealyticum*
VAA	Variable adherence antigen
VFDB	Virulence Factors of Pathogenic Bacteria Database
WHO	World Health Organization

Contents

1 Introduction

1.1 Sexually transmitted bacterial infections in the UK

While a key part of healthcare treatment provided by the NHS, there has been a more recent rise in importance of sexual health and subsequently the treatment of sexually transmitted infections. In Wales, only 0.1% of NHS Wales funding is directly allocated to the treatment of sexually transmitted infections (Welsh Government 2023) but 4.7% of NHS Wales funding is spent on urogenital illnesses (excluding infertility) often linked to untreated or poorly treated STIs (Henkel 2021). A further 3.2% is spent on maternity and reproductive health, another sector of healthcare that the literature consistently notes as being impacted by STIs (Ikonomidis et al. 2016; Beeton et al. 2019; Paira et al. 2021). One of the biggest issues facing this important field of medicine is the rapid increase in antimicrobial resistance (AMR) across many of the most common STIs treated in clinical settings. Globally, *Neisseria gonorrhoea* has been observed to demonstrate resistance to first-line treatments in seventy different countries globally (Unemo et al. 2019) with ciprofloxacin resistance in *N. gonorrhoea* of up to 100% of some cohorts observed by the World Health Organization (WHO) (WHO 2019) and significant levels of AMR occurring in other STIs such as *Mycoplasma genitalium* (MG) (Unemo and Jensen 2017) and *Chlamydia trachomatis* (Ikonomidis et al. 2016; Paira et al. 2021).

It is a well-established principle that prevention is better than cure and relating this to STIs, poor sexual health outcomes can contribute to a variety of other conditions. The most well-known links relate to the well-established connections between STIs and the risk of lowered fertility (Beeton et al. 2019; Tang et al. 2020; Paira et al. 2021; Smolarczyk et al. 2021) and adverse pregnancy outcomes (Viscardi 2014; Tang et al. 2020; Ma et al. 2021; Vallely et al. 2021) but also includes less well-known links such as septic arthritis (Chen et al. 2020; Lemoine et al. 2021) and eye disease (Sadhasivam and Vetrivel 2018; Spiteri et al. 2019; Shah et al. 2021), demonstrating that the rapid diagnosis of STIs is a key part of improving the healthcare outcomes of patients (Huntington et al. 2018; Whitlock et al. 2018; Lorenc et al. 2021).

1.2 Pathogens

While it is important to recognize the relevance of many non-bacterial sexually transmitted infections (STIs such as viruses (e.g., Herpes and HIV) or protozoans (e.g., *Trichomonas vaginalis*), the focus of this work shall be on urogenital Mycoplasmas that are linked with the common symptoms of vaginitis and urethritis and avail themselves more readily to resolution with antibiotic therapy, specifically:

- *Mycoplasma genitalium* (MG)
- *Mycoplasma hominis* (MH)
- *Ureaplasma parvum*
- *Ureaplasma urealyticum*

Additionally, while not a focus of this thesis, for the sake of completeness, *C. trachomatis* is one of the most common causes of STIs and thus is described here to outline its lack of inclusion. *Chlamydia trachomatis* is an obligate intracellular Gram-negative bacterium causing some of the most common occurrences of STI and is more colloquially known as just "Chlamydia". *C. trachomatis* can be further sub-divided into biovars and serovars (Faris et al. 2019), with serovars A-C consisting of mainly ocular pathogens (Sadhasivam and Vetrivel 2018). Serovars D-K are the biovar responsible for sexually transmitted variants of the bacterium. One final biovar consists of serovars L1-3, pathogens cause lymphogranuloma venereum (LGV), a sexually transmitted disease, common to men-who-have-sex-with-men (MSM) manifesting as ulcerating lymphadenopathy that produces characteristic "buboes" (Seth-Smith et al. 2021).

Testing for *C. trachomatis* is always undertaken when patients present with urogenital, anorectal, and ocular symptoms, and for any known relevant sexual contacts of confirmed infected persons (Meyer 2016). As there is no national screening programme for *C. trachomatis*, and screening does not occur at time of a cervical smear or routine pregnancy screening, periodic testing only occurs opportunistically when other infections are ruled out. Current first line treatments for *C. trachomatis* infections are 100mg doxycycline for 7 days or 1g azithromycin for 1 day followed by 500mg of azithromycin for 2 days, with doxycycline contraindicated for pregnant patients. Treatment failure in patients fully complying with antibiotic regimes due to resistance is incredibly rare, with most treatment failures relating to a failure to abstain from sexual intercourse during treatment (Chow et al. 2020). As such,

while this pathogen is of clinical relevance in the study of STIs as a field, it is not relevant to this work's particular focus on the investigation of rapidly developing antimicrobial resistance in STIs.

The other commonly tested bacterial STI that is outside the scope of this thesis is also devoid of reported antimicrobial resistance but is described here for completeness. *Treponema pallidum* is a spirochaete bacterium that causes syphilis (including bejel and yaws). Syphilis is transmitted only amongst humans, primarily via sexual contact or vertically from pregnant mother to baby (Patel et al. 2018; Forrestel et al. 2020). *Treponema pallidum pallidum* causes syphilis, *T. p. endemicum* causes nonvenereal endemic syphilis or Bejel, which produces inflammation of the skin and mucosal tissues. *T. p. pertenue* is a tropical infection of the skin causing skin lesions, fatigue, and misshapen scars (Beale and Lukehart 2020). Although the three subspecies produce distinctly different symptoms, they are morphologically and serologically indistinguishable, requiring molecular techniques to determine the differences.

Syphilis is diagnosed predominantly when diagnosed opportunistically as a dual target in HIV screenings, or during antenatal screening, with the most common diagnostic methods utilising serology followed by the treponema-specific testing for Treponema pallidum haemagglutination assay (TPHA) and rapid plasma reagin (RPR) (Terzi et al. 2020). If necessary, a PCR test of primary syphilis ulcer swabs can be used to detect fingerprint amplification sites of the *Treponema pallidum pallidum* genome (Zhou et al. 2019). Penicillin is an effective first-line treatment for syphilis at all stages of the disease, with doxycycline and ceftriaxone equally effective as second line treatments in the event of patient allergies to penicillin (Ghanem et al. 2020). Azithromycin is the only clinically prescribed treatment of note that syphilis is observed to develop resistance to (Walker et al. 2019), and as such is not recommended as a treatment. As with *C. trachomatis*, syphilis is an STI of increasing importance to broader health policy, with a rise in infections amongst the Welsh population (PHW 2019), and as such should be surveyed both generally and for antimicrobial resistance as a precautionary measure. Nonetheless, due to the current lack of rapid antimicrobial resistance, it was not a focus of my research into antimicrobial resistance.

1.2.1 *Neisseria gonorrhoeae*

Neisseria gonorrhoea (or simply Gonorrhoea) is a component of the clinical aspects of this work due to its role as a highly resistant "superbug". Gonorrhoea is a sexually transmitted infection (STI) caused by the bacterium *Neisseria gonorrhoeae*, referred to interchangeably as gonococcus or GC (Unemo et al. 2019). The *Neisseria* genus is a group of gram-negative diplococci bacteria with the two species of most clinical significance (*Neisseria meningitidis* and *N. gonorrhoea*) causing bacterial meningitis and gonorrhoea respectively. As a species, *N. gonorrhoeae* is a non-sporulating obligate aerobe that infects predominantly mucosal tissues, producing symptoms of itching, pain, and discharge in the affected oral, anal, or urogenital areas (Lovett and Duncan 2019). This normally arises from the host immune response rather than any inherent component of GC, but the necessary adhesion to epithelial cell membranes has been linked to tissue damage in cases of long-term infection (Dillard and Seifert 2001; Yu et al. 2019). UKHSA report that gonorrhoea is significantly increasing in prominence as an STI, which is particularly worrying for a pathogen already considered a superbug by the WHO (2018). UKHSA have reported a significant rise in rates of Gonorrhoea of 50% from 2021-2022 (UKHSA 2022,2023), with commensurate rises in antimicrobial resistances (UKHSA 2022).

In men, inflammation of the penile urethra causes a narrowing and stiffening of the urethral lumen which in turn leads to symptoms of burning sensation and generalized pain. This inflammation can extend further along the male genital system and lead to epididymitis (Sivaraj et al. 2021). In women, GC causes inflammation in the uterus, cervix, vagina, rectum and pharynx that in turn leads to vaginal discharge, lower abdominal pain, and dyspareunia (Sweet and Walker 2011), with common complications of untreated GC being pelvic inflammatory disease and scarring of the fallopian tubes (Darville 2021), in turn leading to ectopic pregnancy. Another complication arising from GC is vertical transmission (unclear if this is transplacental or via contact with the birth canal), leading to premature birth and/or neonatal conjunctivitis (Vallely et al. 2021).

The pathogenesis of *N. gonorrhoeae* begins with the adherence of *N. gonorrhoeae* to the outer membrane of its host epithelial cells. *N. gonorrhoeae* achieves this using a diverse set of extracellular type-IV pili (Prister et al. 2019) and porin proteins (Deo et al. 2018) to interact with prominent structures on the host cell's surface. Once the initial pathogen cells have

adhered to the host epithelial cells, *N. gonorrhoeae* overcomes the local microbiota to form microcolonies and potentially microfilms, scavenging nutrients for this process from the cell surface and surrounding microbiota (Quillin and Seifert 2018). The exact composition of nutrients required in laboratory culture varies, but the lysed horse blood used in laboratory GC culture includes iron, glucose and phosphates (Thayer et al. 1965), equally required by i*n vitro* pathogenic colonies. Other sources of nutrients include intracellular resources such as zinc as *N. gonorrhoeae* secretes the outer membrane protein *Tdfj* to bind to and deactivate S100A7 (Maurakis et al. 2019), preventing the host cell from sequestering zinc away from host pathogens.

A key aspect of the *N. gonorrhoeae* lifecycle is the evasion of the immune response while adhered to the host membrane. The secretion of oligosaccharides facilitates one such method of evasion by avoiding covalent attachment of C3b following complement activation (Quillin and Seifert 2018) an opsonin or something that facilitates engulfment by phagocytes. It also provides a barrier to the final stage of complement activation leading to pathogen cell lysis via formation of the membrane attack complex (Russell et al. 2019). However, there is evidence to suggest that GC can utilise phagocytes to aid in distribution of infectious bacteria. GC releases outer membrane vesicles such as oligosaccharide fragments and peptidoglycans that trigger toll-like receptors and recruits leukocytes and neutrophils to phagocytose GC (Quillin and Seifert 2018). To successfully use macrophages and neutrophils as a vector it has been observed to trigger actin-skeleton rearrangement, creating intracellular vesicles around the diplococci colonies that protect them both from the macrophage and detection by the wider immune system. This further benefits the pathogen by providing a vector for transmission between sexual partners via transmission of purulent exudate.

Another vital mechanism to the survival and propagation of GC is its ability to adapt its pathogenesis to evade and overcome both the host's immune system and antimicrobial treatments. GC has been shown to not only evade but actively suppress the host immune response through transforming growth factor-β (TGFβ) cytokine production in BALB/c mouse vaginal cells (Liu et al. 2014), preventing the development of T helper 1 and T helper 2 cells that would facilitate a further immune response. Inversely, GC has been found to downregulate the expression of efflux pumps genes when colonizing cervical cells as compared to urethral cells (Ma et al. 2020), highlighting the need for complex regulatory

mechanisms to aid in balancing the benefits of antimicrobial resistance and the fitness costs of expending resources on additional (and potentially unnecessary) secondary cellular structures. Two GC strains of international concern (ST-7363 and ST-1901) has been observed to have rapidly become resistant to third generation cephalosporins through recombination with another *Neisseria* species bacteria, but also independently and simultaneously develop fluoroquinolone resistance through point mutations in topoisomerase genes. The varied mechanisms by which GC can adapt to a variety of selection pressures emphasizes the need for rapid and precise assays for the detection of these adaptations.

Diagnosis of GC can be undertaken by culture-based analysis, in clinic microscopy, or molecular based diagnostic platforms such as qPCR. Direct microscopy requires enough discharge and/or pus to be a viable diagnostic technique, supplemented with the use of Gram-staining, and as such has a weak positive-predictive value (measuring true positives) of approximately 67% (Hall et al. 2019). This type of diagnostic technique can be utilized in theory with any non-urine sample, but in practice is rarely used with cervical samples due to the potential for confusion with commensal bacterial flora and the typically asymptomatic nature of these infections (Meyer and Buder 2020). Detection of male urethral gonococcal infection is usually undertaken with first-catch urine samples (Meyer and Buder 2020), which refers to the first part of the urine stream passed. Culture-based analysis requires sufficient sampling of discharge to be undertaken effectively, normally using a combination of urogenital, anorectal, or pharyngeal swabs (Wilson et al. 2021). These swabs can then be used to inoculate GC-selective plates containing a balance of antibiotics in addition to the base of agar and lysed horse blood. These include antimicrobials for the inhibition of both Gram-positive and -negative bacteria such as vancomycin and colistin, as well as amphotericin B as an anti-fungal measure and trimethoprim as an anti-*Enterobacteriaceae* (Thayer et al. 1965). Samples incubated on these plates for 24-48 hours at 37^{o}C with supplemental CO_2 typically produce smooth, round, and shiny colonies. Overall, these series of techniques are further supplemented with tests to discern any antimicrobial resistance, increasing the accuracy of testing to 98.6% sensitivity and 91.4% specificity (Hall et al. 2019).

Modern techniques involve the use of NAATs such as qPCR to amplify fingerprint targets of the GC's genome, with antimicrobial resistance detection being built into a multi-target assay (Gaydos and Melendez 2020). The use of NAATs such as PCR analysis generally have a

sensitivity and specificity of 95% and >99% respectively (Meyer and Buder 2020). These values mean that only 5% of false negatives are generated, and only generated less than 1% of false negatives. These values are significantly more reliable and sensitive than the older culture-based methods, particularly for pharyngeal swabs, which would otherwise be difficult to analyse effectively (Cornelisse et al. 2017). In addition, more advanced NAATs can simultaneously identify antimicrobial resistance (AMR) mutations (specifically for ciprofloxacin) but these are not available in routine clinical practice. GC is increasingly considered a pathogen of interest by a variety of national and international health bodies due to both its commonality and its broad swathe of established and developing resistances. In England and Wales, the annual GRASP survey (UKHSA 2022) details increasing levels of antimicrobial resistance between 2017 and 2021. Tetracycline has long been considered an ineffective treatment option for GC, with resistance increasing from 48.5% to 75.2% over this period and ciprofloxacin resistance increasing from 36.4% to 46.9% across this same period. Of all the isolates tested 0% were resistant to ceftriaxone, the only antibiotic surveyed to have this result. However, the report does note that there was an increase in ceftriaxone-resistant GC isolates referred to UKHSA in its capacity as a reference laboratory. This increase from 0 to 9 resistant isolates from 2017 to 2021 is marginal in isolation but indicates the potential for ceftriaxone resistant isolates to become a significant proportion of future sample sizes. While not a member of the class *Mollicutes*, GC as a pathogen mirrors the *Mollicutes* STIs in many ways – GC is a fastidious bacterium that has adapted to scavenge nutrients from the host cell that would otherwise be synthesized by other bacteria, produces inflammatory symptoms that can mirror *M. genitalium* and *U. spp*., and can develop antibiotic resistance rapidly. As such, it is included in many commercial multiplex assays that also detect *M. genitalium* and *U. spp.*, although this is tempered by the lack of uniformity between accepted resistance thresholds (CLSI 2011) between commercial kits (Beeton and Spiller 2017) in turn ironically contributing to the development of antimicrobial resistance, due to the inappropriate administering of treatment. As such, more data and evidence are required to understand the impact of these and other drivers of resistance in Ureaplasma, and as such these bacteria were a component of my studies into the rates of antibiotic resistance in a clinical setting.

1.2.2 *Mycoplasma genitalium*

Mycoplasma genitalium (MG) is a pathogenic *Mollicutes* characterised by a highly reductive genome and microbial structure, most prominently lacking a cell wall (Fraser et al. 1995; Razin et al. 1998; van der Schalk et al. 2020). Most symptoms produced by MG are immunologically mediated, arising from the body's response to the initial extracellular colonisation of the outer cell structure (Razin et al. 1998; Citti et al. 2010; Taylor-Robinson and Jensen 2011). This manifests as urethritis in men, producing discharge and a burning sensation while urinating (Hamasuna et al. 2007; Hadad et al. 2011; Cox et al. 2016). Additionally, there is evidence to support the link between chronic non-gonococcal urethritis, epididymitis and proctitis caused by MG and infertility in men (Huang et al. 2016; Tjagur et al. 2018; Paira et al. 2021), with similar links between MG-mediated chronic pelvic inflammatory disease and infertility in women (Ma et al. 2021; Tantengco et al. 2021b).

A recent study into the epidemiology of MG and *Trichomonas vaginalis* (Perry et al. 2023) found that MG more prominent in male patients than female patients and was most prominently detected in male rectal samples. Furthermore, Perry and colleagues observed a trend of MG/HIV co-infection in these rectal samples, which agrees with earlier evidence of such a relationship (Khaw et al. 2018). A recent Welsh study (Spiller et al. 2020) found that the overall carriage of MG in sexual health patients presenting at STI clinics was low, with MG carriage slightly lower in female patients (2.7%) than male patients (3.5%). Interestingly, the study found no link between MG bacterial load and clinical symptoms, although an overall higher bacterial load was observed in male patients. An inference that can be drawn from these studies is that male patients are consistently more at risk of MG infection than female patients, and both identify points at which MSM patients were particularly at risk – Spiller and colleagues note that the only fluoroquinolone/macrolide dual-resistant isolate was identified in an MSM patient, and Perry et al. note the higher prevalence of MG/HIV co-infection risk in MSM patients.

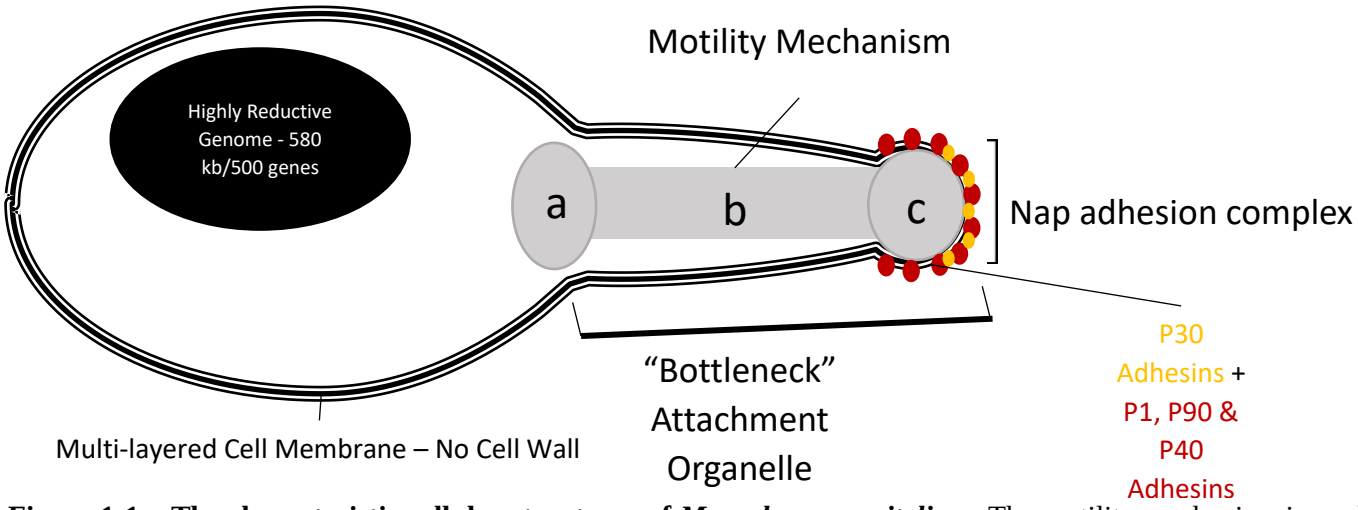

Figure 1-1 – The characteristic cellular structures of *Mycoplasma genitalium*. The motility mechanism is made up of three distinct protein complexes, whereby force is generated at the bowl complex (a), transmitted through the paired plates (b) and modulated by the Nap adhesion complex (c). The structure of the motility mechanism pushes against the cell membrane, creating the characteristic "flask" shape of the cell. Image created in Microsoft Office PowerPoint 2016.

While these infection rates are relatively low when compared to established superbugs such as GC, the treatment of MG and other mycoplasma infections is complicated by the hesitance of clinicians to prescribe doxycycline-based treatments due to their chelating effects in infants and developing foetuses (Sánchez et al. 2004; Pöyhönen et al. 2017), although this consensus has shifted more recently (Gaillard et al. 2017; Kim et al. 2018; Ansari et al. 2021). Another factor is its simplistic cell structure and subsequently parasitic lifecycle, due to its inability to produce most vital respiratory molecules. MG will not grow without the presence of progesterone in most animal models and scavenges glucose from both its host cell and surrounding microflora (Taylor-Robinson and Jensen 2011), although notably not arginine or urea. This underpins the use of phenol red as the chemical mechanism for a colour-change indicator (red to yellow) in MG agar plate cultures, but also limit the metabolic pathways that can be targeted by clinical treatments.

A key identifying characteristic of MG is its cellular structure, a "flask shaped" structure with a prominent "bottle neck" protrusion. This bottleneck terminates in the Nap, a tip-like organelle structure that mediates much of MG's adhesive qualities. This Nap structure is composed of adhesin proteins such as MgPa and P110 (or MgpC) that mediate adherence to epithelial cells (Mader et al. 1991) along with the accessory proteins MG218 and MG317 (Yueyue et al. 2022). Another prominent component of infection is MG's mobility, which could be considered abnormal for such a reductive genome. While in principle, the idea of

such a reductive bacterium retaining motility seems unusual, it is not unhear-of that a bacteria with such a small genome would make use of genetic pleiotropy (Rendueles and Velicer 2020). The source of MG's motility is interlinked with its adhesive qualities, as the adhesin proteins P140 and P110 have been shown to also have a dual role as cytoskeletal proteins (García-Morales et al. 2016) in tandem with the specialized motility proteins MG200 and MG386 (Pich et al. 2006), highlighting the genetic pleiotropy required of such a minimal genome. P140 (or MgPa and MgpB) and P110 (MgpC) are the major adhesins of MG (Yiwen et al. 2021) and as such are common targets for molecular testing due to their importance (Braam et al. 2018; Sweeney et al. 2020; van der Schalk et al. 2020). These adhesins interact with both the external host cell and each other to form a lipoprotein complex that anchors the bacterial cell to the host cell surface (Aparicio et al. 2018). GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) is another protein speculated to moonlight as an adhesin and one that has been shown to bind MG to host cell mucin proteins (Alvarez et al. 2003), providing MG with a broad mechanism of adherence.

The highly variable mechanisms that mediate the adhesive quality of MG is one of the key aspects of its pathogenesis (Mader et al. 1991; Unemo et al. 2018), with a high degree of antigenic variation of the cell adhesin structure used to evade adaptive measures of the host immune system. The antigenic variation displayed by MG adhesion proteins is primarily mediated through the presence of homologous repeat regions in MgPa gene clusters (Hakim et al. 2021). These highly repetitive repeat regions can make up between 4% of the total MG genome (Iverson-Cabral et al. 2007; Wood et al. 2020) and have been observed both *in vitro* and *in vivo* to undergo wide-scale recombination in response to immunologically mediated selective pressures seen in clinical cases (Wood et al. 2020) that are absent in *in vitro* cultures. These variations have been reported to be mediated by the enzymatic machinery that regulates the overall DNA recombination process in MG (Hakim et al. 2021), likely an adaptation in light of the simplistic genome of MG. Genes implicated in this phenomenon include the *Ruv*A/B family of DNA enzymes and the Uvr family of DNA-binding proteins (Burgos and Totten 2014), indicating the variation occurs as part of the routine DNA replication pathway of MG in turn conserved across eukaryotic cells.

While MG normally is observed to adhere to the membranes of host cell and historically was exclusively considered an extracellular bacterium, with the development of techniques such

as confocal laser scanning microscopy we have gained the ability to observe intracellular host cell invasion in low percentages of MG infection cases (Jensen et al. 1994). Along the mechanisms of motility and adhesin-mediated evasion of the immune system mentioned previously, cellular localization in host epithelial cells is another shield against immune clearance and the means by which a more permanent infection can be established (Mcgowin et al. 2009). While this phenomenon is thought to be rarer than extracellular host cell adhesion, it is thought to contribute to clinical cases of chronic and recurrent infection that do not clear with first-line antibiotics (Pinto-Sander and Soni 2019; Soni et al. 2019).

The immune response that causes symptoms in MG infections are mediated through the detection of pathogen-associated molecular patterns (PAMPs), small molecular motifs that can be discerned by the immune system to belong to an infection. In the case of MG, toll-like receptors 1, 2, and 6 are the main mediators of the subsequent secretion of pro-inflammatory cytokines and chemokines (Dehon and Mcgowin 2017). Because of the shared serological effects of antigens shared between MG and *M. pneumoniae*, it has been theorized that patients with previous *M. pneumoniae* respiratory infections may be susceptible to more severe MG symptoms (Hakim et al. 2021; Vizarraga et al. 2021), but in practice this has not been observed in animal models and is rarely recorded in medical cases. Nonetheless, the antigenic variation of surface proteins coupled with the variation in host immune system has been demonstrated in animal models (Wood et al. 2013) to be a contributing factor to abnormally heavy or light symptoms in MG infections.

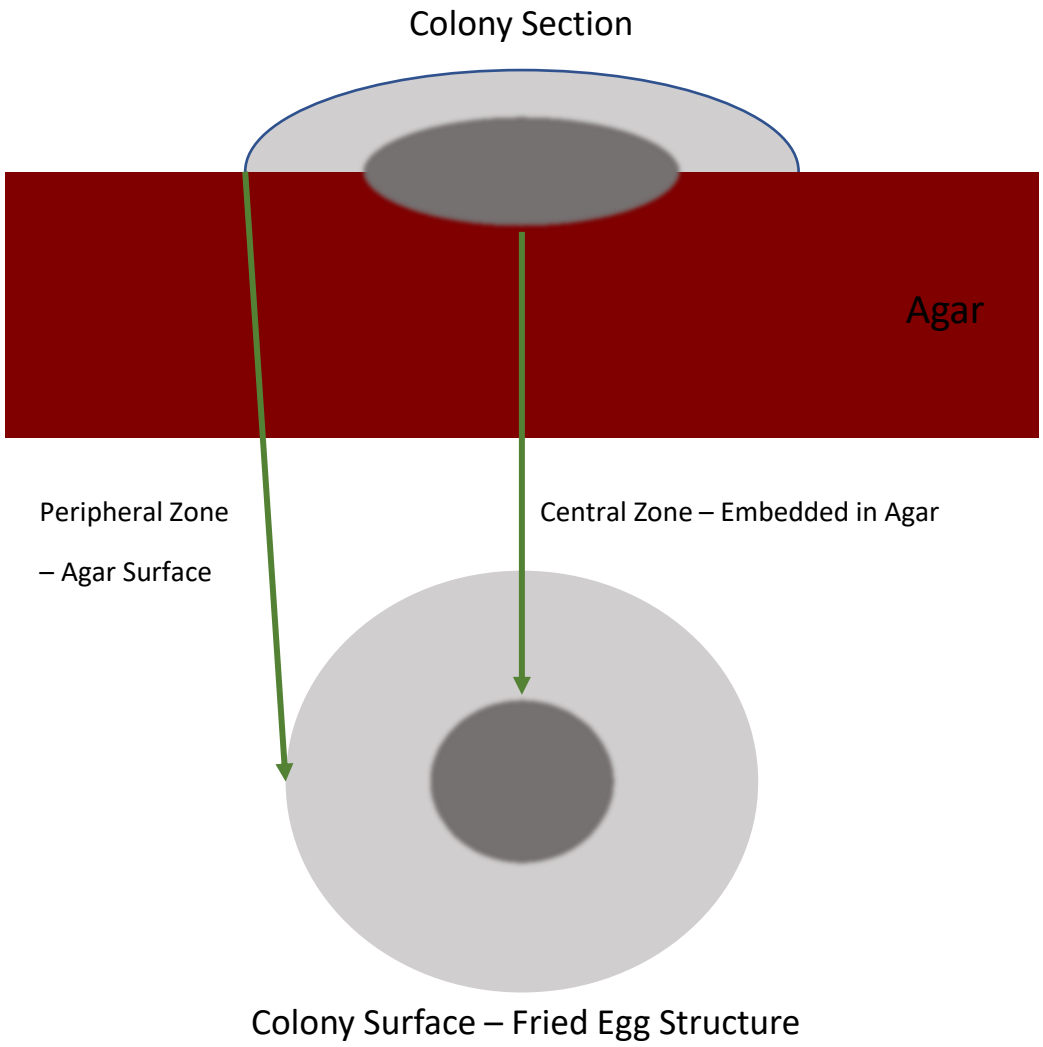

Figure 1-2 - The colony structure of *Mycoplasma genitalium* cultured in agar. Visible growth (under microscope) can be achieved in approximately a week but can often take 2-4 weeks depending on the viability of the bacterium.

When agar media has been inoculated with MG, it colonises the surrounding area via binary fission and forms characteristic "fried-egg" colonies of bottle-shaped cells in agar culture when observed both by eye and using a microscope (Taylor-Robinson and Jensen 2011). The formation of these colony structures in agar is due to the aggregation of nutrients within the agar medium, prompting more accelerated growth in the colony section more able to access the agar. As these cells are pushed out from growth occurring within the colony, bacterium die off and become sparser in concentration, creating the visible gradient of cells observed. This colony structure is notably only observed in oxygenic agar culture, as culturing MG on agar in the presence of 5% CO_2 will cause the colony to burrow deeper into the agar, creating

a clear spherical colony structure that lacks the density gradient observed in grown aerobically (Tully et al. 1983).

In addition to agar-based culture, there are well-established techniques for the cultivation of MG in cell-line cultures, typically Vero cell-lines (Taylor-Robinson and Jensen 2011). While this technique is well-established and has been optimised for working directly with first-void urine (Hamasuna et al. 2007), the culture times are, the given culture times are prohibitively long for usage in diagnostics – two weeks for the initial cultivation of MG inoculated on Vero cells, and additional time for pre- and post-inoculation processing. As such, cell-culture based methods would not be useful in a clinical context and indeed are superseded by agar-based and broth-based methods.

There are also established techniques for the culturing of MG in broth media, broadly in line with the original protocols devised by Tully and colleagues during the initial species identification of MG (Tully et al. 1983). While a variety of commercial broth media exist, the standard media initially outlined by Tully and colleagues is SP-4 media, utilizing pH-based colour change indicators such as cresol red or phenol red to produce colour-change in the growth media in the presence of MG metabolites excreted during growth (Waites et al. 2023). Both indicators shift from a dark red to a bright yellow as the waste products of MG glycolysis cause the broth media to become more acidic, providing a simple method for visually detecting the growth of a viable culture of MG.

One downside of this method of detection is that the pH range of these indicators is much broader than the optimal pH range of MG broth culture. In line with its fastidious culture requirements the broth culture of MG is ideally made up to a pH of 6.95 +/- 0.01, while the colour change of both phenol red and cresol red is much broader. Colour indicators reliant on pH can often be deceiving to the untrained eye and suggest high levels of growth, when in fact the pH of the broth has often shifted to the point of cytotoxicity, rendering it unviable for further investigation. Nonetheless, if the culture is not needed for further investigation and only a simple diagnosis by culture is required, broth media is usually preferrable to agar culture media due to the sharply reduced cultivation time.

Even though culture techniques are available for the cultivation of MG, they function on a timescale unviable for clinical diagnosis. In optimal conditions and with specialist knowledge,

MG strains have been long established to have a culture time of about a week (Tully et al. 1983). However, when conducted in a clinical diagnostic laboratory setting, most healthcare providers list up to a fortnight for a conclusive diagnosis. In this context, the only method that can be utilized expediently is the use of molecular methods, but while there are a variety of commercially available assays that have been individually validated for laboratory use, there is no common standard for MG NAATs. As such, diagnostic variance can and does exist between clinics and national surveillance systems, notwithstanding the overall high accuracy of NAATs as a diagnostic tool.

MG has in recent years has been further scrutinized due to its wide-ranging antimicrobial resistance qualities as a “superbug” (Unemo and Jensen 2017; Machalek et al. 2019; Sweeney et al. 2020), and its established links with a variety of adverse outcomes (Pinto-Sander and Soni 2019; Gnanadurai and Fifer 2020), while the antimicrobial resistance encompasses both inherent conserved and variable induced resistance to main-line treatments (Bradshaw et al. 2017; Unemo and Jensen 2017; Braam et al. 2018; van der Schalk et al. 2020). The status of MG as a superbug has been precipitated by inconsistent prescription of antibiotics leading to rapid development of AMR (Soni et al. 2019), in turn due to a lack of rigorous testing parameters that lead to incomplete treatment and resulting outcomes (Birger et al. 2017), and coupled with a lack of information on many aspects of its resistance mechanisms and wider genomic structures. Due to the pressing need to fill significant gaps in our understanding of MG, it was chosen to be explored further in this thesis.

1.2.3 *Mycoplasma hominis*

Mycoplasma hominis (MH) is a species of bacteria of the *Mollicutes* class, inclusive in the colloquial term "genital mycoplasmas", but this also includes the *Ureaplasma* genus of bacteria. While normally a harmless commensal genitourinary colonizer, this organism can produce bacterial vaginosis, pelvic inflammatory disease and urethritis in patients (Woodhall et al. 2018; Vodstrcil et al. 2021), while also being linked to infertility (Boujemaa et al. 2018; Ma et al. 2021).

It is difficult to ascertain the epidemiology of M. hominis as an STI, due to the reticence of clinicians in Europe to test for its presence (Horner et al. 2018). Nonetheless, there are some studies conducted in different countries which can be used to infer the dynamics of MH infections in sexual health patients. A meta-analysis by Moridi et al. (2020) found that in Iran, women presenting with genitourinary tract infections were more likely to carry *M. hominis* than male patients with genitourinary tract infections, and was more likely to be detected in infertile patients than fertile patients. Shao et al. (2021) found in Shenyang, China that both *M. hominis* infections and *M. hominis* co-infections with *U. urealyticum* were significantly more prevalent in female patients and highest amongst the 20-24 and 25-29 age cohorts, which the study attributes to sexual activity. While it is difficult to draw concrete conclusions from studies conducted on patients subjected to different treatment regimens and clinical systems, these findings do emphasize the role of *M. hominis* as a causal agent of urogenital diseases.

When looking at other pathophysiology, *M. hominis* can cause the induction of pre-term birth (Ansari et al. 2021) in pregnant women and cause severe respiratory dysfunction (Waites et al. 2009) or meningitis (Gwon et al. 2020) in newborn children if MH is transmitted via normal vaginal birth. MH is well-established to have links with infertility in both sexes (Skiljevic et al. 2016; Boujemaa et al. 2018; Ma et al. 2021; Paira et al. 2021), as well as forming ascending infections in pregnant women that can lead to chorioamnionitis, spontaneous abortion and preterm labour. Furthermore, infants born with MH infections frequently develop respiratory inflammation, requiring ventilation and a regime of antibiotics to regain lung function. This disease is further complicated by the nature of its biological structure, and how it limits what drugs can be used to treat it. While broadly considered a commensal bacterium in healthy adult patients, the established association of MH with failure in organ transplant patients

(Spiller 2017; Givone et al. 2020), hyperammonaemia and pre-term birth mean (Waites et al. 2009; Meygret et al. 2019) that it is still important to develop fast and effective methods of treatment for the disease in at-risk patients.

While much of MH's resilience is via the loss-of-function mutations that eliminate vectors of treatment, the genome of MH has been refined by selective pressures to also contain a variety of virulence factors that aid it in its pathogenicity. The variable adherence associated (*Vaa*) antigen is a key adhesin structure of MH (Morris 2019; Chalker et al. 2021), as well as a primary target of phylogenetic analyses of MH (Boujemaa et al. 2018; Roachford et al. 2019; Durrant et al. 2020; Yiwen et al. 2021). The *Vaa* gene mediates the synthesis of an approximately 49kDa multiple-banded membrane lipoprotein, with the exact size and molecular composition demonstrating high levels of allelic variability. This variability is in turn shown be mediated by genomic variation of homologous "cassette" modules of genomic regions (Brown et al. 2014), such that there are large differences between certain strains but these have not been defined as different serotypes to date. These homologous arrangements of these cassettes can be used to fingerprint strains and aid in outbreak tracing (Boujemaa et al. 2018). Equally, phase variation can occur in a poly-A region at the start of the conserved sequence that causes a frame shift and truncating that can "turn off" the major surface antigen (Brown et al. 2014). Boujemaa et al. 2018 created a multi-locus sequence typing (MLST) scheme using house-keeping genes (*gyrB*, *tuf*, *fts*(Y), *uvr*(A), *gap*) combined with putative virulence genes (p120', *vaa*, *lmp*1, *lmp*3, p60), which they refer to as multi-virulence-locus sequence typing (MVLST) loci. Whereas, Jironkin et al., (2016) generated three proposed 7-locus MLST schemas for *M. hominis* based on pan-genome gene frequencies.

MH utilizes a variety of surface proteins to evade the host immune system, adapting their expression and specificity using regulated mechanisms of controlled variation, namely antigenic variation of genes. Antigenic variation in the context relates to the modulation of repeat regions of DNA in genes such as the *vaa* gene and other surface protein lipoproteins, The ability to type MH strains using genomic fingerprinting is hampered somewhat not only by the variable nature of virulence genes such as the *vaa* gene, but also by MH's ability to undergo horizontal gene transfer, integrating entire gene clusters of functional genes such as resistance genes (Dordet-Frisoni et al. 2019; Meygret et al. 2019; Berglund et al. 2020), from other strains or even species of bacterium. This variation is not only a persistent hinderance

to phenotyping, but also a means of genomic variation that allows MH to overcome immune responses in a relatively short amount of time.

Due to its minimal gene structure, MH cannot generate energy via the usual pathways such as oxidative phosphorylation or the Krebs cycle. Instead, MH utilizes a biological pathway known as the arginine-dihydrolase or arginine deamination pathway. This pathway combines arginine with N-dimethyl-arginine to form citrulline, catalysed via arginine deiminase and N-dimethylarginine dimethyl-aminohydrolase. Citrulline is then converted to carbamoyl-phosphate and ornithine by ornithine carbamoyl transferase. Finally, ATP can then be generated via carbamate kinase on carbamoyl phosphate. Mediated by an integrated membrane protein called an arginine-ornithine antiporter (*arc*(D)), this metabolic process requires no ATP for arginine acquisition, making it an efficient alternative to more common metabolic pathways. When culturing MH in broth medium this pathway is also the means of qualitatively assessing growth, as the resultant basic pH change in the growth medium can be tracked by a colour-change pH indicator, usually phenol red.

Broth culture is the most common method of culturing MH, as the techniques and necessary reagents have well-established international standards (Waites et al. 2012), and unlike agar-based culture techniques these reagents can be acquired commercially. SP4 media is the most

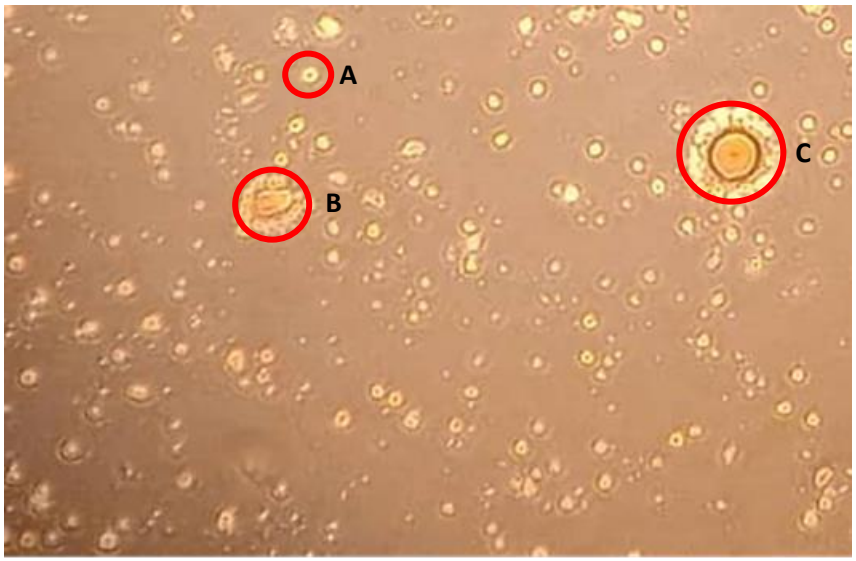

Figure 1-3 – ***Mycoplasma hominis* colonies observed visually with optical microscopy.** The cell circled in red denote the progression of the *M. hominis* colony from a small colony of uniform density (**A**) to a partial colony structure (**B**) and the characteristic "egg-fried" colony structure (**C**) observed in mature colonies. Image taken by author at 100x magnification on selective agar (Mycoplasma Experience Ltd.) with *M. hominis.* strain DF145.

commonly cited media used, but other alternatives such as laboratory-prepared modified Hay-flick's mycoplasma broth (MHMB) (Razin and Hayflick 2010) and Mycoplasma Liquid

Medium® (Mycoplasma Experience® Ltd, Reigate, UK) are available for use. In anoxygenic conditions growth will occur within 24 hours, typically turning the broth from yellow/orange to a deep red, providing an easy method of detection. However, as with broth culture methods for MG, this colour change occurs in the presence of toxic metabolites that can kill the MH present in the culture and prevent further cultures or other investigations.

In comparison, while agar-based culture techniques are not usually used for diagnostic purposes due to the slow growth of Mycoplasma agar cultures, they offer a means to examine cellular morphology that broth culture lacks. The formulation of agar culture usually starts with the addition of arginine (an obligate metabolite) (Schimke et al. 1966) and is usually followed by the addition of selective antimicrobials such as penicillin, vancomycin and amphotericin B (Parija 2023) to control for fungal and bacterial contaminants that might inhibit MH growth. Additionally, a few commercially available formulations are available. These reagents are typically in the form of agar supplements (Merck, Dorset, UK; Oxoid, Basingstoke, UK) but can also supplied as pre-prepared agar plates (Mycoplasma Experience® Ltd, Reigate, UK). If successfully cultured, MH will form small clear spherical colonies within 3-5 days after inoculation (Cardot Martin et al. 2021), with varying density across the sphere that creates the classic "fried-egg" appearance. These colonies mirror MG's colony morphology, with the two distinguishing features being that MH is significantly quicker to culture overall, and the agar colonies of MH are larger and more easily identified visually. Nonetheless, MH's similar colony structure to MG means that without specific training, there could be a misidentification. This notably could result in incorrect treatment, due to MH's inherent resistance to azithromycin (Pereyre et al. 2002) and the frequency with which doxycycline is contraindicated in many categories of patients. Due to this and the time taken for culturing MH on agar, the preferred diagnostic assay is real time or quantitative PCR.

M. hominis is long-established to be resistant to erythromycin and as a microbe requires a variety of specialist techniques and resources to facilitate viable *in vitro* growth (Waites et al. 2012), it relatively difficult to culture *in vitro* (Spiller 2017) and thus detect (CLSI 2011), increasing the chance the disease is treated empirically and thus without full knowledge of any antimicrobial resistances. Detection options are limited in practice to the use of NAATs

(Pereyre et al. 2009; Grosjean et al. 2014; Citti et al. 2018) such as qPCR (Férandon et al. 2011; Jironkin et al. 2016; Givone et al. 2020), but as mutations conferring quinolone resistances can arise from a single point mutation (Pereyre et al. 2002; Meygret et al. 2018; Yang et al. 2020b), resistance can occur spontaneously in isolates regardless of lineage and therefore genomic analysis of resistant isolates for novel genomic variation is the most reliable way of identifying these resistant strains.

MH is not screened for in sexual health by the NHS, and so the information required to determine optimal antimicrobial treatments must be derived from research on MH and MG (as the most relevant similar pathogen) along with the established CLSI/US guidelines on its detection (CLSI 2011). Considering this, treatment should normally be a first course of doxycycline, unless contraindicated in which case clindamycin or levofloxacin should be considered. Due to the risk of resistance, any treatment regime should be paired with a concurrent test for antibiotic resistance to avoid contributing to the well-documented rise in antibiotic resistance in MH (Rideau et al. 2019; Yang et al. 2020b). Analysis methodology is not consistent or well-regulated in many ways, as MH is not currently recognised as a pathogen by European clinical organisations (Horner et al. 2018), and therefore detailed information on the treatment of MH is not fully available. This reluctance to engage in regular MH screening is commonly cited as being due to lack of evidence of MH as a pathogenic STI, as well as a reluctance to potentially contribute to increasing levels of AMR in the wider microflora. This statement is tempered with an acknowledgement of a lack of relevant evidence for regular testing rather than a consensus of evidence against such a course, based on a perceived lack of studies using accurate molecular testing and issues around controlling for co-infections that acts as confounding variables.

While not routinely screened for by clinicians, there is a growing body of evidence to support the pathogenicity of MH in humans. Case reports have established the role of MH in neonatal lung infections (Che et al. 2022), neonatal meningitis (Gwon et al. 2020; Nohren et al. 2020; Ansari et al. 2021; Yeung et al. 2021; Che et al. 2022) and a variety of serious illnesses developed by immunocompromised patients which mainly concerns transplant patients (Smibert et al. 2017; Spiller 2017; Gerber et al. 2018; Nowbakht et al. 2019; Vecchio et al. 2021) but also other illnesses such as hyperammonaemia syndrome (Nowbakht et al. 2019). There is also broad acknowledgement of MH's co-relation with bacterial vaginosis as a

contributory pathogen (Dessì et al. 2019), and both broad evidence (Waites et al. 2009) as well as direct case studies (Givone et al. 2020; Ansari et al. 2021; Che et al. 2022) demonstrating its pathogenicity in neonates. Dessì et al. (2019) reviews the mechanisms of symbiosis between MH and *Trichomonas vaginalis*, noting the relative rarity of symbiosis between two obligate human pathogens. They describe previous research conducted by the group (Fiori et al. 2013) linking the severity of *T. vaginalis* symptoms to the upregulation of proinflammatory cytokines such as Il-1β by an MH infection (Sabo et al. 2020). The symbiosis between these two bacteria is also posited to exacerbate symptoms of vaginal dysbiosis, with *M. hominis* aiding in the adhesion (Hinderfeld and Simoes-Barbosa 2020) of *T. vaginalis* to host cells and subsequent formation of biofilms.

Proinflammatory cytokines not only aid in the colonization of host cells by *T. vaginalis*, but may contribute as a risk factor for progression of virally-associated cervical cancers such as HPV and HIV (Klein et al. 2020). This is caused by structural and genomic changes in host cells that lead to the development of cancers, well-established to be induced by chronic inflammation (Archer et al. 2020). Nonetheless, the exact mechanisms by which MH-mediated inflammation damages host cells and induces oncogenesis have been speculated in reviews (Garbas et al. 2021; Tantengco et al. 2021b) as relying on statistical association and longstanding precedent rather than a substantiated mechanism. As such, more direct evidence is required before a definite link can be established, but the overall ability of *M. hominis* to cause chronic inflammation and the link between that and diseases such as prostate cancers (Archer et al. 2020) suggests that *M. hominis* can affect long-term damage on patients if not treated promptly and effectively. A consistent topic across many of these reviews and studies is the simultaneous consistency of association between *M. hominis* and lack of concrete evidence, which this thesis hopes to provide.

1.2.4 *Ureaplasma spp*.

Ureaplasma as a clade (referred to as *Ureaplasma spp*. in this work) are a subgroup of *Mollicutes*, exhibiting many features shared with Mycoplasmas, with some key characteristics particular to the genus. Like all *Mollicutes*, ureaplasma exhibit a refined and simplified genomic structure and as such exhibit a parasitic lifestyle, using host nutrients and resources in place of the cellular machinery required to produce these components. The main distinctive property of ureaplasma is the ability to metabolise urea via intracellular urease to produce ATP, hence their genus designation (Thirkell et al. 1989; Biernat-Sudolska et al. 2017). *Ureaplasma spp*. STIs are composed of two different species, *Ureaplasma parvum* (UP) and *Ureaplasma urealyticum* (UU), but as these species share over 95% genetic identity between their very compact genomes it is common for the two species to be referred to interchangeably as *Ureaplasma spp*. Nonetheless, there are distinct conserved genetic variations which demarcate the two species, with multi-locus sequence typing (MLSTs) relying on well-established loci such as the multiple banded antigen (MBA) (Teng et al. 1994; Knox et al. 1998; Glass et al. 2000) and a variety of virulence genes.

Between the two species, UP and UU variants are comprised of 14 serovar variants shared between two species. UP includes “serovars” 1, 3, 6 and 14 and UU comprises the remaining 10 serovars (Zheng et al. 1995; Paralanov et al. 2012; Sweeney et al. 2017b), with suspicion that some serovars have a greater capacity for pathogenicity, although this is not fully understood. Attempts to try and correlate putative MLSTs with serovar categories has been inconclusive and many times this is caused by frequent horizontal gene transfer (Xiao et al. 2011; Silva et al. 2019). Although occasionally compartmentalised dual infection by two different serovars within twin neonate hosts without shared genomic determinants (Beeton et al. 2016b), suggesting horizontal gene transfer is sporadic and not necessarily commonplace.

As ureaplasma usually exist as a commensal, it is usually constrained to adhesion and replication on the epithelial layer of the urogenital tract, where for most of the population ureaplasma are harmless components of the microbiome (Garcia-Castillo et al. 2008; Pandelidis et al. 2013; Rowlands et al. 2021). However, ureaplasma can induce immune responses in hosts that present as a variety of symptoms and diseases. In men, pathogenic UU infections produce symptoms of urethritis and/or prostatitis (Beeton et al. 2019), while in

women UP infection can manifest as bacterial vaginosis and endometritis (Beeton and Spiller 2017; Sweeney et al. 2017a) as well as a contributor to infertility (Beeton et al. 2019). More serious symptoms are produced by ascending infections of *Ureaplasma spp*. in pregnant mothers, leading to premature birth and stillborn births (Biernat-Sudolska et al. 2017), as well as neonatal pneumonia and meningitis in neonates (Waites et al. 2009; Beeton and Spiller 2017). In addition, immunocompromised patients such as neonates and transplant patients can develop life-threatening symptoms from dangerously high serum ammonia levels (Matson and Sonetti 2019; Smith et al. 2019), which can lead to delirium, swelling on the brain and eventually death.

While the adhesin complement of U. spp. has not been fully characterized, surface proteins known as multiple banded antigens (MBAs) have been identified as one key component of virulence (Aboklaish et al. 2016; Sweeney et al. 2017b) and are used in many serological testing assays. Immunoglobulin protease A (IgA) has been speculated as another component of *U. spp*. virulence, as has the ammonia by-product of urea hydrolysis components of *U. spp*. cell surface structures (Biernat-Sudolska et al. 2017; Silva et al. 2019). Phospholipases were also speculated to act as virulence factors but following investigation (Paralanov et al. 2012) were determined not to be a significant component in virulence. These factors seem to only contribute to the immune-based response that forms the basis of *U. spp*. symptoms, as well as the more severe pathogenicity demonstrated in immunocompromised patients such as neonates and transplant patients. This is further complicated due to the structural refinement of *Ureaplasma* physiology rendering most antibiotics ineffective. Due to the lack of cell wall structures and its parasitic metabolism as mentioned in previously discussed *Mollicutes*, treatment for *U. spp.* is constrained in practice to doxycycline as a first-line treatment with azithromycin as a second-line treatment, but the use of azithromycin is only advised if the infection is determined to not be azithromycin-resistant (Horner et al. 2016).

In addition, there is a debate as to the relevancy of *U.* spp. as a pathogen in the treatment of sexual health, with BASHH guidelines determining that approximately 25% of urethritis are linked to a positive *Ureaplasma* diagnosis (Horner et al. 2016) but as *U.* spp. is a common commensal bacterium in the urogenital tract, it has been posited that it cannot be determined to have a causal link to symptoms of urethritis and/or PID unless a significantly high load is detected (Horner et al. 2018). This is further complicated by the lack of feasible diagnostics

available to a clinical setting (Sprong et al. 2020), mainly due to the difficulties in culturing such a fastidious bacterium (Waites et al. 2012). While much improved in recent years, a lack of species specificity is cited as one of the main reasons that routine testing is not recommended in asymptomatic patients as a clinical measure (Horner et al. 2016; Horner et al. 2018), along with a lack of evidence describing the causal link between *U. spp.* and diseases such as urethritis.

Because of the reticence of European clinicians to screen and treat Ureaplasma infections (Horner et al. 2018), studies into the epidemiology of Ureaplasma infections in directly applicable geographies are limited. A study by Lesiak-Markowicz et al. (2022) found significantly higher levels of *U. parvum* (5.4%) in asymptomatic male patients than *U. urealyticum* (2.4%), while in a Brazillian study (Carneiro et al. 2020) found that amongst female patients undergoing routine screening via cervical pap smears, 14.9% of patients tested positive for *U. parvum* and 4.2% of patients tested positive for *U. urealyticum*. In both cases while there were no overt symptoms present in either cohort, both Ureaplasma infections were associated with cytological abnormalities suggestive of potential cervicitis, emphasizing the need for routine surveillance in asymptomatic patients. The study by (Shao et al. 2021) previously discussed also surveyed *Ureaplasma urealyticum* infections in Shenyang, China and found significantly higher levels of positive infections in female patients, and a Moroccan study conducted by Karim et al. (2020) found that out of 1053 women with symptoms associated with Ureaplasma infections (leucorrhoea, pelvic pain/dyspareunia, pruritus, menorrhagia, metrorrhagia, or dysuria), 25.4% were positive for either *U. urealyticum* (12.1%), *U. parvum* (7%), or a Ureaplasma co-infection (6.3%). Karim and colleagues identify age as a risk factor, with patients below the age of 30 at particular risk of symptomatic Ureaplasma infection due to increased sexual activity, in agreement with Shao et al. (2021) and their analysis of *M. hominis* risk factors.

Recommendations against routine surveillance by the European STI Guidelines Editorial Board are tempered by the acceptance of UP infections being correlated with non-gonococcal urethritis when present in sufficiently high bacterial loads, albeit at levels that have not been sufficiently calibrated for clinical use (Strauss et al. 2018). As with many other *Mollicutes* bacteria, *Ureaplasma* infections are a known risk in transplant procedures (Gerber et al. 2018; Matson and Sonetti 2019; Nowbakht et al. 2019) and their links to fertility are well-established

and well-studied (Knox et al. 2003; Mihai et al. 2011; Huang et al. 2016; Beeton et al. 2019; Paira et al. 2021). Furthermore, higher-than-normal *Ureaplasma* bacterial loads have been correlated with HPV infections (Amorim et al. 2017; Ye et al. 2018) and have been observed to increase the clearance time of HPV infections (Kim et al. 2019), which in turn is an established cause of cervical cancers in female patients. While many bacteria are labelled as pathobionts based on weak correlations that have not corrected for confounding variables (Jochum and Stecher 2020), this is not the case with *Ureaplasma parvum* or *Ureaplasma urealyticum*. While treating it as a first-call pathogen would sometimes be incorrect due to its presence as a commensal in many patients, there is a large body of evidence linking it to chronic or recurrent urogenital conditions, especially those investigations that have already excluded traditional pathogens such as GC or MG that may share similarities in symptoms or lifecycles. This work therefore seeks to explore the pathogenicity and incidence of *Ureaplasma spp.* in symptomatic patients as well as its ability to adapt to microbial treatments.

1.3 Antimicrobial Treatments

MG, MH and *U. spp*. exhibit highly refined structures that lacks many of the structures targeted by antimicrobials (Beeton and Spiller 2017). The lack of a cell wall precludes the use of drugs such as beta-lactams and glycopeptides (Beeton and Spiller 2017; Bradshaw et al. 2017; van der Schalk et al. 2020; Yang et al. 2020b), an absence of folic acid enzymes likewise precludes sulphonamides and trimethoprim (Spiller 2017) and MH is well established to be naturally resistant to erythromycin (Pereyre et al. 2002).

Novel variants of pre-existing treatments are available that work to overcome these structural immunities, but progress is slower than resistance development and treatments are often restricted to last-line usage due to fears of resistance development – of note, only two antibiotic treatments approved since 2017 have novel mechanisms of action (Shrestha et al. 2022), assessed by Shrestha and colleagues to be due to the financial risk of developing antibiotics likely to be outdated soon after a costly and time-consuming rollout. Of the two treatments they identified, Lefamulin is already an established veterinary treatment for respiratory mycoplasma infections but has only been regulated for clinical usage in recent years (File et al. 2021; Spiller-Boulter et al. 2021), while meropenem is ineffective against mycoplasmas due to the lack of a cell wall. In addition to production bottlenecks, there are a cohort of treatments that while technically effective, are unviable, restricted, or otherwise unutilized in a clinical setting. The established antibiotic Pristinamycin is reserved only for multi-resistant MG infections due to fears of resistance development (Read et al. 2018), which has been observed in some clinical cases (Durukan et al. 2020; Palich et al. 2021). Spectinomycin is another antibiotic that is noted to be effective at treating multi-resistant strains of *M. genitalium* (Falk and Jensen 2016), but requires daily intramuscular injections and is unviable as mono-treatment due to recurrent issue with resistance development (Yang and Ke 2020). Similarly, Daptomycin has been noted as an effective anti-mycoplasma treatment in cell cultures (Strieker and Marahiel 2009), but its full efficacy as a clinical treatment is not well-established and current NICE guidelines proscribe a relatively significant monitoring regime that restricts its practical usage.

Looking at more theoretical sources of treatment, Melittin is an antimicrobial peptide derived from bee sting venom that has observed antimicrobial activity against mycoplasmas (Ko et al.

2020), but not at sub-lethal levels that would be safe for clinical use (Askari et al. 2021) – current efforts are being made to reduce its cytotoxicity without impacting its antimicrobial efficacy.

With alternative sources of treatment either impractical, flawed or still currently being developed, the current treatments available for the treatment of pathogenic *Mollicutes* infections are sharply limited. This small group of drugs mainly target the protein synthesis and genomic regulation of pathogens, and can be narrowed down to fluoroquinolones, lincosamides/macrolides and tetracyclines (Wang et al. 2016) (Figure 4). This limited collection of antimicrobials have been the focus of works attempting to analyse the broader landscape of pathogenic *Mollicutes* treatment (Viscardi 2014; Chernov et al. 2018; Roachford et al. 2019; Ahmadi 2021), as well as the focus of concern regarding the development of antimicrobial resistance (Unemo et al. 2019; WHO 2019; Derbie et al. 2020; Truong et al. 2022). As such, these forms of treatment have also formed part of the focus of this thesis, along with the clinical applications of their usage and the impact of resistance development against these treatments in a clinical setting.

Table 1-1 - Clinically relevant antibiotics and their effectiveness against urogenital mycoplasmas. Columns in red are ineffective treatments for Mycoplasma infections due to inherent immunity, while antibiotics listed in orange are potentially viable treatments but could be ineffective for a particular medical case due to the presence and/or emergence of antimicrobial resistance.

Potential Antibiotics for Treatment of Mycoplasmas						
Inhibition of Cell Wall Synthesis		Inhibition of Nucleic Acid Synthesis			Inhibition of Protein Synthesis	
Beta-Lactams	Polypeptides	Antifolates	RNA Synthesis Inhibitors	Quinolones	30S Inhibitors	50S Inhibitors
Penicillin Carbapenems Cephalosporins Beta-Lactamases	Glycopeptides Lipopeptides Polymyxins	Sulphonamides Trimethoprim	Rifampicin	Fluoroquinolones	Aminoglycosides Tetracyclines	Amphenicols Pleuromutilin Macrolides Ketolides Lincosamides Streptogramins

1.3.1 Quinolones

Quinolones are a class of broad-spectrum antibiotics (Pham et al. 2019) typically administered as a second-line treatment when risk-factors or multi-drug resistance contraindicate the usage of first-line treatments such as penicillin, macrolides or tetracyclines (Sánchez et al. 2004; Vázquez-Laslop and Mankin 2018b). Fluoroquinolones, a subset of quinolone antibiotics, are marked by their bicyclic core structures with an integrated 4-quinolone molecule (Pham et al. 2019).

Fluoroquinolones such as ciprofloxacin, levofloxacin, moxifloxacin, and ofloxacin are by far one of the most common types of antimicrobial treatment used in contemporary clinical treatment, with one of the key benefits of fluoroquinolones as a treatment being that they can be quickly absorbed by bone tissue without chelating it as tetracyclines do, providing a safer treatment for patients suffering osteomyelitis (Ezelarab et al. 2018). Another benefit is their broad effectiveness across both Gram-positive and Gram-negative pathogens, allowing them to function as a second-line treatment for a variety of infections that do not respond to first-line STI treatments such as doxycycline (Ezelarab et al. 2018) such as tetracycline resistant MG (Braam et al. 2018; van der Schalk et al. 2020). The usefulness of fluoroquinolones is tempered by the severe set of side-effects that can contraindicate their usage. Fluoroquinolones most notably can induce tendon pain and rupture (Godoy-Santos et al. 2018; Jupiter et al. 2018; Morales et al. 2019), along with arrythmia in extreme cases (Vivithanaporn et al. 2021), emphasizing the need to temper their prescription with appropriate monitoring of antibiotic resistance, to minimize the risk exposure to such harsh side-effects.

Fluoroquinolones target the type II topoisomerase genes of the bacterial cell, DNA gyrase and topoisomerase IV, genes vital to the proper function of cellular homeostasis. The two type II topoisomerases are heterotetrametric enzymes: DNA gyrase is composed of two *gyr*A and two *gyrB* subunits while topoisomerase IV is composed of two *par*C and two *par*E subunits, all approximately 70-97kDa in size. The *gyr*A/*par*C subunits form the active unit of the protein complex that mediates enzymatic activity, while the *gyr*B/*par*E subunits mediate ATPase-based reactions to produce energy for the protein complex.

DNA gyrase regulates DNA supercoiling, a necessary mechanism of prokaryotic regulation that ensures DNA is efficiently packaged in a cell. When not being replicated or transcribed, DNA is coiled in the direction of the double helix and then against the direction of the helix, producing the super-condensed supercoil or “negative supercoil”. When DNA replication is initiated, DNA gyrase binds to the replication fork produced during DNA replication and unwinds the dual helix by introducing negative superhelices that twist ahead of the replication fork, allowing the binding of DNA polymerases and preventing supercoiling from damaging the DNA structure. Topoisomerase IV acts during cell division to disentangle DNA after semi-conservative replication, cutting and repairing interlinked loops so that they can be segregated into daughter cells, a process called decatenation. Together, these topoisomerases mediate the segregation and condensation of chromosomal pairs, as well as altering the super-helicity of DNA (Berger et al. 1996), aiding the regulation of DNA repair and division. The type II topoisomerase genes are highly conserved genes observed across the bacterial kingdom (Talbert et al. 2019) and share a variety of similar homologs across the other biological kingdoms (Duprey and Groisman 2021). As such, they are a core part of even a simplistic genome and thus function as one of the few avenues of antibiotic treatment for mollicute-based pathogens. Within the genomes of the bacteria described in this work, they are comprised of the DNA gyrase genes *gyr*A, *gyr*B, and the topoisomerase IV genes *par*C and *par*E)

Fluoroquinolones function as a bactericidal treatment by disrupting the DNA replication of pathogens. This is achieved via the fluoroquinolone molecule binding non-competitively to type II topoisomerases as detailed in Figure 1 5, disrupting DNA ligation, and thus preventing ligation occurring in tandem with cell nucleases (Gutierrez et al. 2018). This leads to both single-stranded and double-stranded breaks in the bacterial DNA during the DNA replication process that in turn leads to cell death (Hall et al. 2019). In addition, fluoroquinolones have been identified as having a disrupting effect on the function of topoisomerase IV of segregating chromosomes during cell division, further contributing to its bactericidal properties (Gutierrez et al. 2018).

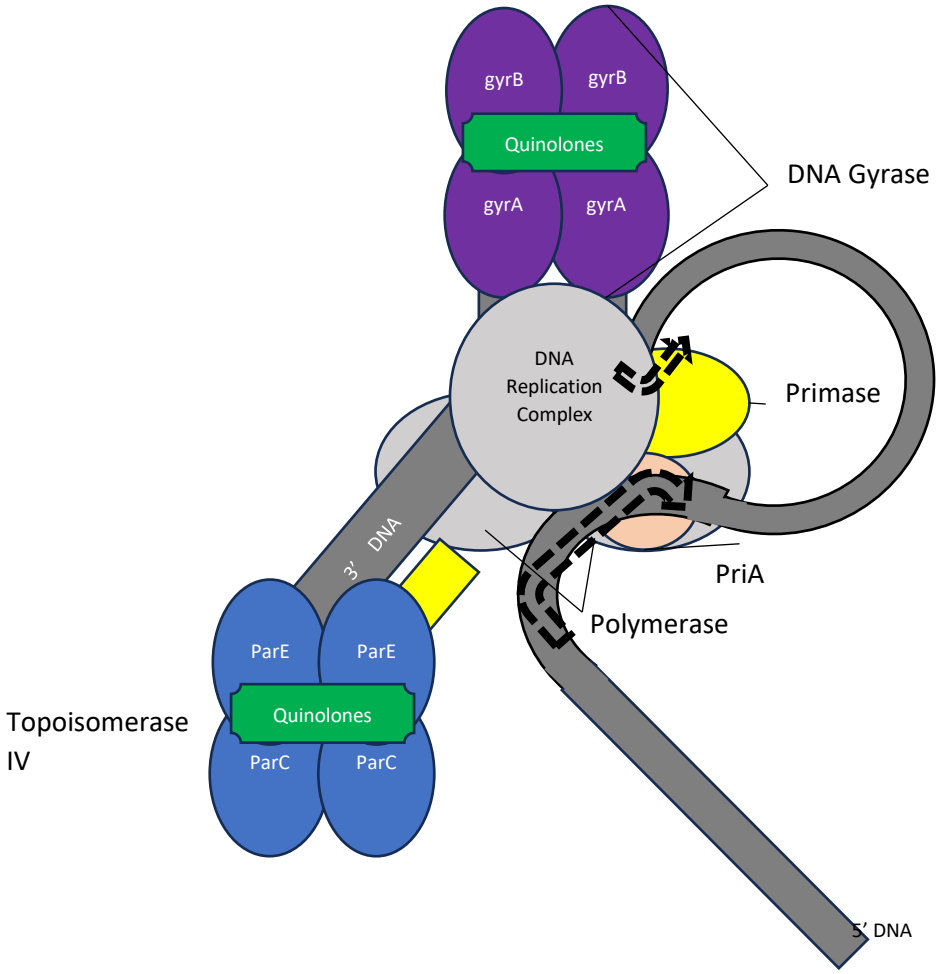

Figure 1-4 – Tertiary complex of type II topoisomerases, DNA, and quinolones. Quinolones disrupt the active sites of the *gyr*(A) and *par*(C) complexes, preventing the enzyme from cutting and releasing DNA after binding, causing double-stranded DNA and apoptosis. Primase, Polymerase and PriA make up the DNA Replication complex that is assisted by type II topoisomerases and is subsequently disrupted by quinolones. Adapted from Hooper (2002).

While less selective for equivalent human enzymes, interactions between fluoroquinolones and human genomic machinery is thought to be the most likely source of the severe side-effects that can arise from administering these drugs, with photosensitivity (Kim et al. 2018; Loupa et al. 2020) and damage to connective tissues (Godoy-Santos et al. 2018; Jupiter et al. 2018; Morales et al. 2019) being the most prominent side-effects. Such is the severity of these side-effects that their use is severely restricted in all but the most severe and/or resistant bacterial infections (EMA 2019).

Adding to these severe side-effects, the mechanism for fluoroquinolone resistance in *M. hominis* is well established as arising from Single Nucleotide Polymorphisms (SNPs) in the Quinolone Resistance Determining Region (QRDR) of the topoisomerase genes, as best characterised for *E. coli* (Van Der Putten et al. 2019) but mirrored in function across most other bacteria (Bebear et al. 1997; Hamasuna et al. 2018; Ostrer et al. 2019; Zhang et al. 2019; Esfahani et al. 2020). By altering the protein conformation of the resulting topoisomerases, they lack the relevant complementary sites and thus quinolones cannot bind to the bacterial ribosome, thus conferring immunity to the disruptive effect of fluoroquinolones.

Rates of quinolone resistance are incredibly high in certain pathogens, with 97% of countries reporting resistance rates to the World Health Organisation recording the presence of ciprofloxacin-resistant GC (Wi et al. 2017). Rates vary across different pathogens, but broadly are high enough that it is no longer recommended to treat GC with fluoroquinolones as a first-line treatment (Dalhoff 2012). As MH and U. *spp*. are not commonly surveyed or treated as pathogens, MG is the only *Mollicutes* with considerable clinical data, with moxifloxacin resistance rates existing at 4-10% (Deguchi et al. 2016; Le Roy et al. 2016; Li et al. 2017), varying across different geographies. In practice, older quinolones such as ciprofloxacin and ofloxacin have been established to have significantly poorer cure rates than moxifloxacin (Jernberg et al. 2008), which has a cure rate approaching 100% in susceptible strains (Jensen et al. 2016) and thus the recommended treatment for macrolide-resistant MG.

1.3.2 Macrolides

Macrolides are a class of broad-spectrum antibiotics, predominantly administered for Gram-positive bacteria for which penicillin would be ineffective, but also for a small variety of Gram-negative bacteria (Vázquez-Laslop and Mankin 2018b). Erythromycin was first discovered in 1952 as an equivalent treatment for penicillin-resistant illnesses and patients allergic to penicillin (Vázquez-Laslop and Mankin 2018b). One of the major side-effects of erythromycin is moderate gastrointestinal issues (Arsic et al. 2018), so early developments into the synthesis of variants such as azithromycin and clarithromycin were driven by the desire for less severe side-effects, although it should be noted that even modern variants of macrolides such as azithromycin can cause nausea and diarrhoea in patients. For the purposes of this work, focus will be on erythromycin, azithromycin, clarithromycin and josamycin as the four most relevant variants of macrolide antibiotics for this body of work.

Macrolides functions as a bacteriostatic by binding to the A2058 and/or A2059 residues of the 23S rRNA region of the 50S ribosome subunit, as well as the V region (Vázquez-Laslop and Mankin 2018a). Macrolides act by blocking the exit tunnel of the ribosome causing disengagement after only 7-8 peptides have been linked. The effects of this inhibition are reversible (bacteriostatic), but prolonged inhibition leads to cell death. Azithromycin is thought to be the most effective of the macrolides commonly used for the treatment for MG treatment due to its irregular 15-membered lactone ring, in comparison to the standard 14-membered ring of older macrolides such as erythromycin (Bradshaw et al. 2017). Josamycin is observed to be even more effective and has a 16-membered lactone ring but is not as commonly prescribed as azithromycin (Arsic et al. 2018), which remains a potential second-line treatment of susceptible MG strains if doxycycline is not a viable option for treatment. However, doxycycline is usually chosen as the first line treatment for MG over azithromycin, notwithstanding any reason to contraindicate the use of tetracyclines in a patient such as the patient being prepubescent or pregnant (Mello 1967; Tötterman and Saxén 1969).

Macrolides are generally ineffective in MH, with 14- and 15-ringed macrolides being unable to bind to the V region of the 50S ribosomal subunit due to a conserved G2057A polymorphism blocking binding (Pereyre et al. 2002; Krausse and Schubert 2010; Waites et al. 2012), modifying the active site of the macrolide binding region. This adaptation is not effective against larger ringed macrolides (Arsic et al. 2018), which means that of all the

commonly prescribed macrolides, only josamycin is effective against MH. GC also exhibits resistance to macrolides using a similar mechanism of modifying the V region of the 50S subunit but rather than a point mutation, resistant strains instead carry genes encoding rRNA methylase called *erm* genes to methylate the macrolide binding site of the V region (Svetlov et al. 2021), preventing macrolides from binding to the region and blocking ribosome subunit interactions. *erm* genes are regularly found on conjugative elements and can spread via conjugal transfer (Cousin et al. 2003; Derbie et al. 2020), with variants observed across a variety of gonococci pathogens. GC can also develop resistance to macrolides via similar mechanisms of rRNA mutations as observed in MG, with MICs increasing in line with the frequency of mutation across the four 23S rRNA alleles of GC (Hall et al. 2019). Methylation of the 23S rRNA region carries with it a fitness cost of inefficient protein synthesis relative to a wild-type variant, but this can be alleviated through macrolide-dependent mechanisms in the ribosome, whereby transcription of methyltransferases are heightened in the presence of intercellular macrolides (Vázquez-Laslop and Mankin 2018b).

Macrolide resistance is a growing feature of many infections, with resistance hovering between 2.5% to 5% in most countries reporting resistance (Unemo and Jensen 2017; Wi et al. 2017; Unemo et al. 2019). This is relatively consistent when compared to tetracycline and quinolone resistances, which tend to be much broader in range and normally much higher overall (Bradshaw et al. 2017; Godman et al. 2017; Unemo and Jensen 2017; Derbie et al. 2020). While low, the potential for anywhere between one in forty to one in twenty patients presenting as having macrolide-resistant GC makes resistance a growing concern in the treatment of a variety of other pathogens. These are varied but are predominantly pathogens already of interest due to their growing resistance rates. STIs such as GC (Hall et al. 2019; Ma et al. 2020) and MG (Machalek et al. 2019; Machalek et al. 2020) have been noted as potential superbugs due to the rising levels of macrolide resistance (Unemo and Jensen 2017; WHO 2019) and the lack of feasible alternative treatments. Macrolide resistance is an ongoing concern in other areas of clinical practice such as methicillin-resistant *Staphylococcus aureus* MRSA (Bishr et al. 2021) and necessary changes to treatments such as the prescription of macrolides for patients allergic to penicillin can increase the risk of developing this infection (Blumenthal et al. 2018). *Clostridium difficile* (Tilkorn et al. 2020) and *Klebsiella pneumoniae* (Zieliński et al. 2021) are also diseases with rising levels of resistance, as are GC and MG,

proving that macrolides are a vital tool that is becoming significantly less effective at treating serious pathogens both considered STIs or otherwise.

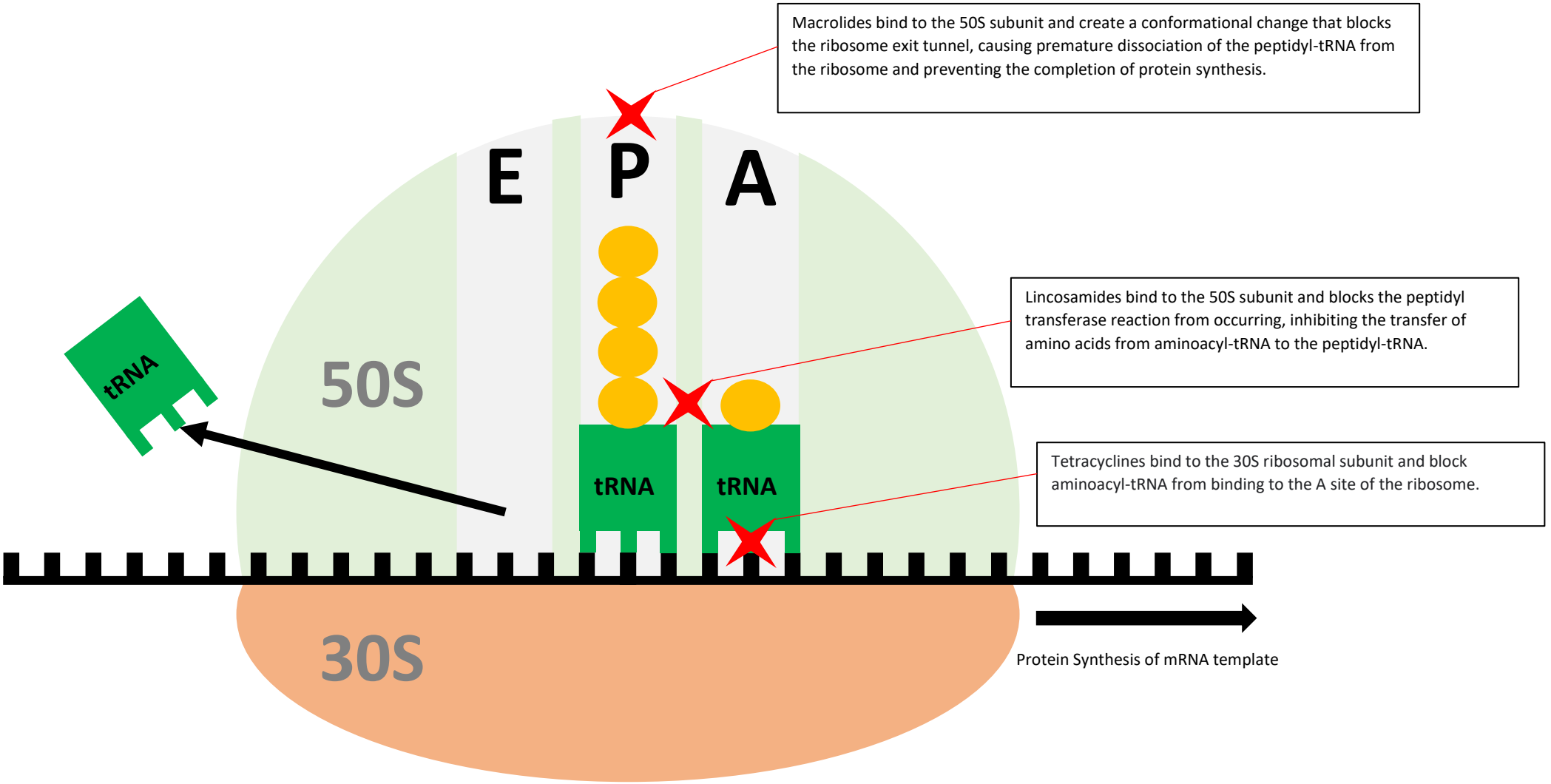

Figure 1-5 - Mechanism of function for antibiotics that inhibit protein synthesis. For all three classes of antibiotic illustrated here, the overarching mechanism of resistance is an alteration of one or more tRNA binding sites that in turn prevents the binding of the antibiotic to the relevant ribosomal site, blocking inhibition.

1.3.3 Tetracyclines

The tetracycline group of antibiotics includes range of broad-spectrum antibiotics, used for the treatment of a variety of infections. These include infections such as STIs, acne, cholera (di Cerbo et al. 2019), agricultural purposes (Koike et al. 2017) and even rarer diseases such as *Yersinia pestis* and *Bacillus anthracis* infections (Yang 2017; Brightman 2019). In addition, tetracyclines have a chelating property that makes them a useful biomarker of bone growth in rare cases (Macri-Pellizzeri et al. 2018; Martin and Bettencourt 2018). This class of drug is widely used and relied upon and as such is listed by the World Health Organization as one of the most critically important antimicrobials (WHO 2019).

Historically, the generations of tetracycline drugs describe their synthesis – 1st generation tetracyclines are biologically derived, those of the 2nd generation are modified after biological synthesis and 3rd generation tetracyclines are artificially synthesized in their entirety (Krueger et al. 2004). Further derivatives are either in development or starting to become licenced for more general use (Watkins and Deresinski 2019; Zhanel et al. 2019), but for the purposes of this thesis, the well-established derivatives of tetracycline listed here will be the main topic of discussion.

The tetracycline family of antibiotics functions as a bacteriostatic via the inhibition of protein synthesis. This is achieved by competitively inhibiting the attachment of charged aminoacyl-tRNA to the A-site of the ribosomal complex. Tetracyclines function via a broad mechanism of attack that can theoretically affect human cells as well (Chukwudi and Good 2019), but there are variances between cell toxicities that can be observed and are due to bacterial cells actively pumping tetracycline into their cytoplasm, where mammalian cells only absorb the drug via osmosis (Chukwudi 2016). This limits how much damage tetracyclines can do to a patient receiving treatment but does mean that some tetracycline-mediated damage of human cells will likely occur.

Furthermore, it has been observed that the metabolic by-products of tetracycline derivatives are themselves cytotoxic (Burgos et al. 2011; Tong et al. 2016), compounding potential side-effects. The developments in the R groups of tetracyclines has been driven by the need to reduce this toxicity, with newer drugs achieving an overall improvement in that regard (Borghi and Palma 2014). This has led to the replacement of tetracycline with doxycycline

in almost all cases (Heaton et al. 2007), but ultimately there is still some level of toxicity present in all tetracyclines (Burgos et al. 2011; Tong et al. 2016).

The most well-known side-effect is the chelating property of tetracyclines, which will discolour bone and teeth with continual usage (Sánchez et al. 2004; Judge et al. 2018). This is magnified in children (Sánchez et al. 2004), although evidence suggests a lack of staining from doxycycline (Gaillard et al. 2017; Pöyhönen et al. 2017) still is the effect on unborn foetuses, where tetracycline can more severely affect foetal development (Tötterman and Saxén 1969; Nahum et al. 2006; Muanda et al. 2017). As such, this family of drugs cannot be proscribed to pre-pubescent patients and is very rarely proscribed as a long-term form of treatment. These more general side-effects include phototoxicity (Kim et al. 2018) and aggravation of pre-existing liver impairment and/or diabetes (Buckler et al. 2017). all presents serious barriers to the prescription of these drugs, even before considering the development of AMR in pathogenic eukaryotes.

The potential for increasing resistance rates to tetracyclines via the acquisition of the *tet*M gene is of concern. This gene encodes for a ribosomal protection protein (RPP) that protects the carrier from the effects of tetracycline. The *tet*M protein binds to ribosomes conjugated with tetracycline and induces permanent conformational changes in the 16S RNA region (Dönhöfer et al. 2012), ejecting the tetracycline molecule from the ribosomal complex and preventing re-binding, thus conferring effective resistance to the drug. The gene is inherited primarily by horizontal gene transfer (HGT) via transposons and/or plasmids, with transposon *tn*916 most associated with its dissemination.

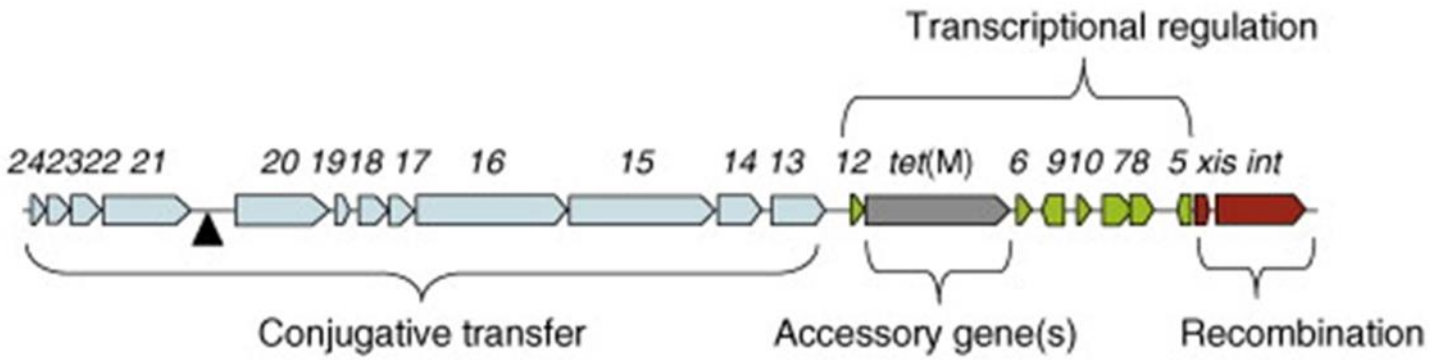

Figure 1-6 - The structure of the Tn916 transposon. Each of the four gene clusters mediating different functions of the transposon are marked in a different colour. Figure taken from Roberts and Mullany (2009).

The Tn*916* transposon is composed of four gene clusters that mediate the conjugative transfer, transcriptional regulation, and recombination of the transposon as well as the *tet*M accessory gene (Wright and Grossman 2016), as illustrated in Figure 1-6. Upon expression of its recombinase gene cluster, the transposon is excised from the genome on either end of the transposon and via further regulation of its topology is reformed into a circular mobile transposon (Rubio-Cosials et al. 2018), which is then transmitted via the usual mechanisms of plasmid transmission. Tn*916* normally has specific insertion targets formed of two coupling sequences, one of which is integrated into the excised plasmid to bond the two ends of the transposon together to form the circular structure. This cannibalization of one coupling sequence forms a motif that can be used to detect the presence and subsequent excision of a transposon in what would otherwise appear as a wild-type variant (Lunde et al. 2021). The mechanism of excision and insertion are applicable to a variety of different species, allowing for not only inter-species transmission but also intra-species transmission.

The absence of *tn*916 conjugation genes led to speculation as to whether the *tet*M element retained mobility or not (Calcutt and Foecking 2015). AMR of Tetracyclines is developing at a rapid rate across a variety of pathogens and *tet*M-mediated tetracycline resistance in pathogens typically confers resistance to all members of the drug family (Schmitz 2001; Beeton et al. 2016a; Meygret et al. 2018). In GC, tetracyclines are long-established to be an ineffective treatment (Unemo et al. 2019) with 75.2% of UK isolates tested by UKHSA found to be tetracycline-resistant (UKHSA 2022). Furthermore, only 30% of MG infections are successfully cleared by doxycycline (Pitt and Fifer 2022), with little or no understanding of the genotyping of the resistance strains.

Our understanding of tetracycline resistance in *U. spp.* and MH is even less detailed due to the lack of standardized routine surveillance, and so relies on aggregation of clinical studies. A meta-review by Ahmadi (2021) found that tetracycline resistance in both pathogens was

relatively high and increasing over time, although they also noted that the lack of standardization between commercial kits was a major issue for determining accurate resistance rates. Ahmadi noted that across 37 studies, the average rates of doxycycline resistance was 28.6% for *Ureaplasma spp*. and 9% for MH. This data is again noted to vary geographically, with rates ranging from 0-100% resistance for tetracycline and 9-18% resistance for doxycycline, as observed in other studies (Wang et al. 2016; Beeton and Spiller 2017; Meygret et al. 2018). The wide variance in rates of resistance can be primarily attributed to the varying rate of prescription of these drugs, with countries using more stringent guidance showing lower levels of tetracycline resistance (Godman et al. 2017). This is counterbalanced by the significantly higher cost of alternative treatments in low-income countries, forcing clinicians to proscribe tetracyclines and without antimicrobial resistance testing. In addition, the overuse of tetracyclines (and other drugs) in agriculture has been noted as a contributor to the generation of AMR, with livestock making up the bulk of antimicrobial consumption worldwide (He et al. 2019). These factors have contributed to an overall drop in the effectiveness of tetracyclines as a treatment for urogenital mycoplasmas, relative to other options such as fluoroquinolones or macrolides. Nonetheless, they are a key family of antibiotics worldwide and their resistance rates are an important component in the surveillance of antimicrobial resistance.

1.3.4 Lincosamides

Lincosamides are a relatively small class of broad-spectrum antibiotics, most used as an alternative treatment for patients who are allergic to penicillin (Armengol Álvarez et al. 2022). Lincomycin was first isolated as a secondary metabolite produced by *Streptomyces lincolnensis* and licenced for medical use in 1964 (Macleod et al. 1964), with current production still mediated by genetically modified cultures of *S. lincolnensis*. Clindamycin was quickly semi-synthesized using lincomycin as a template to increase the drug's activity spectrum and reduce the impact of its considerable side-effects, being licenced for clinical use in 1966 (Magerlein et al. 1967). Lincosamides are used to treat a variety of pathogens, showing good activity against staphylococci, streptococci and most anaerobic bacterium but typically lack efficacy against enterococcii, *Neisseria meningitidis* and aerobic gram-negative bacilli (Schwarz et al. 2016). Clinical use of clindamycin significantly outstrips the usage of lincomycin due to its broader range of susceptible organisms and higher overall efficacy as a clinical treatment (Spížek and Řezanka 2004). Furthermore, Pirlimycin is only approved by European regulators for the treatment of bovine mastitis (Birkenmeyer et al. 1984). As such, clindamycin is the only clinically relevant lincosamide for the treatment of urogenital infection and subsequent antimicrobial resistance.

Lincosamides primarily function as bacteriostatic protein synthesis inhibitors, targeting the 50S region of the bacterial ribosome subunit akin to macrolides. Where lincosamides differ is that instead of targeting the peptide exit tunnel like macrolides, they act earlier in the initiation of protein synthesis. Lincosamides bind to a 50S site that somewhat overlaps with the binding site of macrolides (Spížek and Řezanka 2004). By non-covalently binding at this site, lincosamides interact with both the A-site and P-site of the subunit (Tenson et al. 2003). This interferes with the positioning of both the peptidyl-tRNA and aminoacyl-tRNA, disrupting the peptidyl transferase-mediated reaction required to elongate the peptide chain. Tenson and colleagues also identified an inverse link between peptide chain length and lincosamide effectiveness, as the increased affinity of peptidyl-tRNAs with long peptide chains is sufficient to out-compete the lincosamide molecule.

As macrolides and lincosamides share similar binding sites, they also share mechanisms of resistance. As the binding sites of macrolides and lincosamides significantly overlap, erm-mediated methylation of the 50S binding sites confers resistance to both macrolides and

lincosamides, as well as group B streptogramins and azalides (Lewis and Jorgensen 2005). Constitutively resistant bacteria uniformly express an altered 50S site that confers resistance, while inducible resistance occurs when macrolides bind to upstream translational attenuator sequences that in turn induce the expression of erm methylases to alter the 50S binding site (Weisblum 1995). In addition to conformational changes, lincosamide-specific resistance can also occur. This is typically mediated via enzymatic inhibition (Schaenzer and Wright 2020) or drug efflux (Schwarz et al. 2016) and is typically a less common form of lincosamide resistance in pathogens. A key aspect of lincosamide usage in sexual health is the mechanism of application. There are no NICE guidelines for the use of clindamycin in male sexual health patients, and guidelines for treatment of pelvic and female genital infections are to be treated via intravenous infusion. In practice, an IV (and thus a longer-term hospital visit) would be considered a last resort, sharply limiting the applications of clindamycin for the treatment of vaginitis and PID. Furthermore, it is well-established that oral or parenteral applications of clindamycin carry a breadth of side-effects such as skin irritation, diarrhoea, and potentially life-threatening antibiotic-associated colitis.

Within the context of treating urogenital mycoplasmas, the application of clindamycin is limited. Because erm-mediated resistance to lincosamides can also confer resistance to macrolides, erythromycin and azithromycin are preferred over clindamycin due to their higher sensitivity (Taylor-Robinson and Jensen 2011) and there are no official recommendations for the use of clindamycin to treat MG infections. Furthermore, *Ureaplasma* spp. demonstrate a uniform resistance to lincosamides (Redelinghuys et al. 2014), the mechanisms of which are not fully understood. On the other hand, MH is inherently immune to 14- and 15-membered macrolides due to mutations in 23S rRNA (Pereyre et al. 2002) but is sensitive to clindamycin (Chang et al. 2022) and so is recommended as a second-line treatment for MH infections. The limited spectrum of lincosamides in the treatment of mycoplasma infections means that they did not form a significant component of this study.

1.4 Aims and Objectives

The overarching hypothesis of this work is that that antimicrobial resistance and its genomic mechanisms can be rapidly and effectively identified using modern molecular methods through the link between mycoplasmas and symptoms of disease, with both established links with mycoplasma infections and symptoms not yet associated clinically with mycoplasma infections such as *Mycoplasma hominis*. The means by which I will explore and demonstrate this hypothesis are:

- Determining mechanisms of tetracycline and quinolone resistance in *M. hominis*, looking at both mobile genetic elements transmitted inter- and intra-species.
- Determining the mechanisms of transposon-mediated resistance in *Ureaplasma spp*.
- Examining if there are significant benefits for the clinical usage of molecular diagnostic methods over traditional culture-based methods for GC and M. hominis.
- Examining rates of antimicrobial resistance pre- and peri-COVID pandemic for GC, *M. hominis*, *M. genitalium* and *Ureaplasma spp*.
- Investigating if incidence rates of Ureaplasma spp. between sexes can be related to variations in growth rates in the presence of sex hormones.

While relatively understudied in the context of a pathogen, the molecular mechanisms of resistance in *M. hominis* are well-established enough to be explored in more detail. Relating back to *M. genitalium* and referencing work already conducted on antimicrobial resistance in *M. hominis*, the main areas of clinical concern are increasing levels of resistance to tetracycline- and quinolone-family antibiotics observed in clinical trials. As such, it was deemed relevant to explore the underlying mechanisms behind these adaptations.

Likewise, while understudied as a sexual pathogen *Ureaplasma spp*. is regarded as a pathogen of concern in fertility and obstetrics, and as such there is a great deal of benefit to understanding the mechanisms of resistance in these organisms and how they contribute to the overall burden of antibiotic resistance. In addition, analysis of these resistance mechanisms and how they relate to the resistance mechanisms present in *M. hominis* offers a chance to further explore the molecular regulation and adaptations of mollicutes as a species and how the mechanisms present in each species interrelate.

The point of interest that underlies the study of these organisms is the growing levels of antimicrobial resistance in each of these pathogens, and how the mechanisms of resistance are not fully understood and thus offer opportunities to improve treatment outcomes for patients in a clinical setting. As such, the core question of my work is to take these observations and interrogations of antimicrobial resistance mechanisms and attempt to combine this knowledge with the practical analysis of whether the molecular detection of antibiotic resistance can be integrated into clinical analysis in a cost- and time-efficient manner. To do this, this work will investigate at a variety of factors relating to the incidence rates and AMR rates of GC, *M. hominis*, *M. genitalium* and *Ureaplasma spp.* It will examine methods for the purpose of their molecular detection and use the information generated along with linked demographic data to observe if there are correlations between any demographic variance and any of the chosen bacterial targets. Considering the Covid-19 pandemic and its associated challenges, this will also include an analysis of the effects of the pandemic on rates of antibiotic resistance present in the target pathogens of this study.

2 Materials and Methods

2.1 *M. hominis* culture

Mycoplasma hominis strains investigated were derived from clinical samples or isolates referred to Public Health England (PHE) reference laboratory for diagnostic investigation or antimicrobial susceptibility testing (from 21st February 2005 to 9th October 2015). Isolated strains kindly supplied by PHE were archived at -80°C as agar cubes in mycoplasma liquid media and/or mycoplasma liquid cultures (with or without beads) until investigated for this work. Samples submitted were derived from a range of clinical specimens including neonatal, obstetric, and sexual health as well as invasive infections (blood culture, CSF, heart valve, ascitic fluid and pleural fluid). Four vaginal reference strains from Australia were also kindly provided to me by Dr Matthew Payne, University of Western Australia, for comparative analysis (3 from the same patient (AH3) collected at 20, 28 and 36 weeks gestation, as well as a single isolate from a separate patient (AH58) (Payne et al. 2016). Apart from the *M. hominis* strains isolated in Havana, Cuba, the remaining strains were archived from previously published studies (Beeton et al. 2009b; Morris et al. 2020; Boostrom et al. 2021). Recovery of frozen archived isolates was performed via resuspension in Mycoplasma Experience Limited (Reigate, UK) selective media. Plates sealed with clear adhesive film were incubated in a humidified chamber at 37 °C for up to 5 days. Cultures and plates were checked daily, with their growth recorded. Growth in broth culture was visualized as a yellow to red colour change in the absence of turbidity.

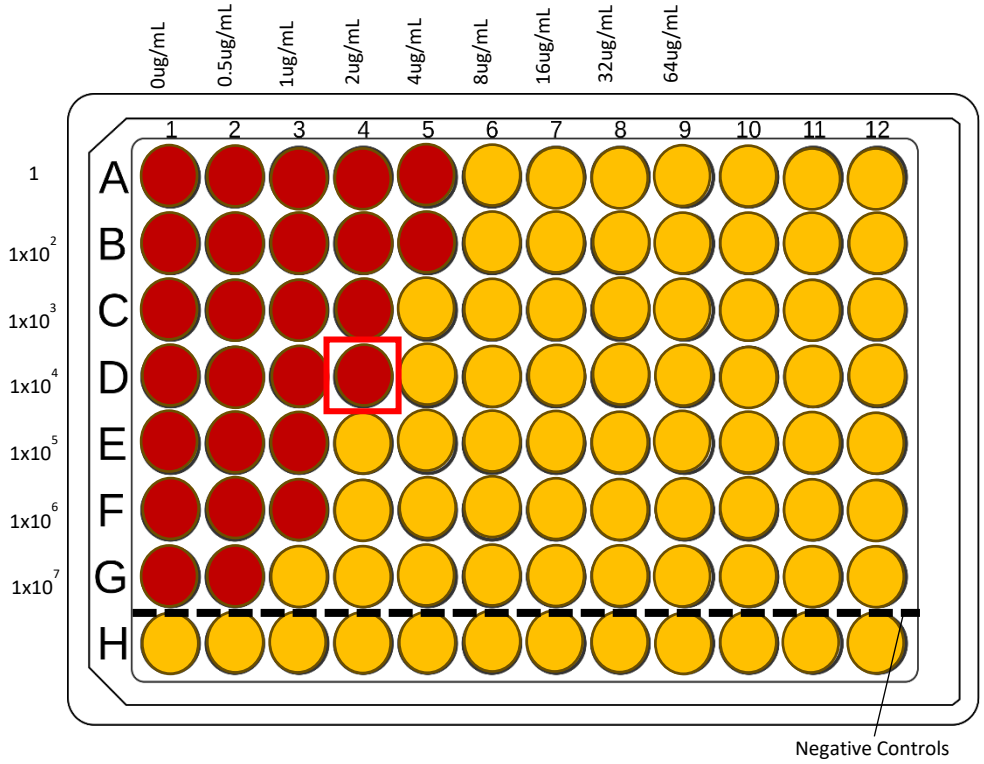

Figure 2-1 - 96-well Plate Determination of Minimum Inhibitory Concentration. This plate shows the result of a hypothetical tetracycline-resistant *M. hominis* isolate. Dark red wells indicate growth-mediated colour change, while orange-yellow wells indicate a lack of growth sufficient to induce colour change. A Minimum Inhibitory Concentration was determined by the highest concentration of antibiotic to produce colour change at $1x10^4$ or higher Colour Change Units.

2.1.1 Antimicrobial Resistance Screening

For the determination for antimicrobial resistance in *M. hominis*, mycoplasma selective agar (Mycoplasma Experience Limited) with inclusion of specific concentrations of antimicrobials for threshold resistance screening was used. For minimum inhibitory concentration (MIC) determination, microbroth dilution methods utilised Mycoplasma selective medium prepared by CPM SAS (Rome, Italy), optimised for colour change indicative of *M. hominis* growth. Agar and microbroth dilution methods were performed as outlined by CLSI guidelines (CLSI 2011) including use of defined resistance thresholds: 2 $\mu g \cdot mL^{-1}$ levofloxacin, 0.5 $\mu g \cdot mL^{-1}$ moxifloxacin, 0.5 $\mu g \cdot mL^{-1}$ clindamycin, and 8 $\mu g \cdot mL^{-1}$ tetracycline. For agar plates, four 10-fold dilutions of each strain were inoculated per 90mm plate, and only the dilution that yielded 30-300 colonies on the antimicrobial-free control plate were used for analysis of antimicrobial-containing agar plates. Microbroth dilution methods to determine MIC for tetracycline, levofloxacin and moxifloxacin was undertaken according to CLSI guidelines (CLSI 2011) examining $1x10^4$ colour-changing units in comparison to the antimicrobial-free growth control medium, as established previously by Beeton et al. (2009b). Briefly, colour-changing units relate to a well of unknown bacterial concentration (set at 1 CCU) being diluted tenfold across a broth microdilution plate, creating a ten-fold gradient of growth for the determination of growth in the presence of a parallel gradient of antibiotic concentrations.

While no resistance thresholds have yet been determined for josamycin and tigecycline, MICs were determined under the same parameters. 120 *M. hominis* isolates from 81 patients were analysed, with initial screening for *tet*M presence was performed by traditional PCR (AppTaq RedMix (Appleton Woods, UK), 40 cycles, Tm=56^oC, extension 30 sec) visualising the expected 419 bp product by transilluminated ethidium bromide containing 1% agarose gel electrophoresis using PCR primers (designed in this study) tetM1338F 5'-TATCTGTATCACCGCTTCCG-3' and tetM1758R 5'-AATACACCGAGCAGGGATTT-3'.

Induction of fluoroquinolone resistance was performed similarly to the agar-based, single-step induction method described by Bebear et al. (1999), except that selection occurred in microbroth dilution conditions. CLSI guidelines require determination of susceptibility at a strict 24–48 h incubation point, but by allowing incubation of the MIC plates a further 24–72h beyond the initial MIC determination, spontaneous colour change was observed for wells of 4 mg/L for levofloxacin. Sub-culture of these isolates then found that they now had an MIC of 8 mg/L. However, this phenomenon was only observed in isolates that already had pre-existing gyrA QRDR mutations. For the purposes of this work, bacterial referred to as a bacterial strain is an identified and characterised subgroup of a given bacterial species, while a bacterial isolate is merely a single bacterial organism been identified in each patient sample but has not yet been fully purified and characterised.

2.1.2 Ethical Considerations

Work undertaken for the analysis of *M. hominis* genomes was classified as service evaluation and clinical investigation, and so was exempted from NHS Research Ethics Committee Review. Original ethical approval covering the reference strains included from Australia were approved by the Human Research Ethics Committee of Western Australian Department of Health, Women and Newborn Health Service (2056/EW).

The evaluation of UK and French Ureaplasma bacterial isolates for antimicrobial resistance was exempt from NHS Research Ethics Committee review, due to the investigation's role in clinical service evaluation. Cuban isolates were acquired in a study approved by the 'Pedro Kourí' Tropical Medicine Institute Ethical Board (approval CEI-IPK 33-12).

2.2 *Ureaplasma spp.* culture

Archived *Ureaplasma* strains investigated for tetracycline resistance were derived from clinical samples or isolates referred to Public Health England (PHE) reference laboratory for diagnostic investigation or antimicrobial susceptibility testing from 2003 to 2013 (Beeton et al. 2009a; Beeton et al. 2016a). Samples submitted were derived from a range of clinical specimens including neonatal, obstetric, and sexual health as well as invasive infections. Two were isolated from Havana, Cuba (Preval et al. 2022), two from France (Meygret et al. 2018); eight from the United Kingdom as well as the American Type Culture Collection (ATCC) serovar 9 prototype reference strain from Vancouver. Apart from the *Ureaplasma spp.* strains isolated in Havana, Cuba and Bordeaux, France, the remaining strains were archived from previously published studies (Meygret et al. 2018; Preval et al. 2022), as well as from an unpublished UROGEN study (IRAS 251053; Spiller O.B. and Jones L.C.). Recovery of frozen archived isolates was performed via resuspension in Ureaplasma selective media purchased from Mycoplasma Experience Limited (Reigate, UK).

Recovery from frozen archives was performed by resuspension in Ureaplasma selective medium purchased from Mycoplasma Experience Limited (Reigate, UK). Plates were sealed with clear adhesive film in a humidified chamber and incubated at 37^{o}C ovenight. Cultures and plates were checked daily, and growth recorded and confirmed utilizing qPCR methods detailed in previous studies (Beeton et al. 2016a; Preval et al. 2022). MIC analysis of isolates was conducted in accordance with CLSI guidelines (CLSI 2011) using the methodology outlined by Beeton et al. (2009b) previously in this section, with isolates meeting the tetracycline MIC threshold values of 2ug/mL or higher being chosen for sequencing.

Induction of tetracycline resistance via repeated challenge with sub-lethal concentration of tetracycline was induced as per the well-established methods of Evans and Taylor-Robinson (1978), with a concentrations of ranging from 0.125-1 mg/L tetracycline. Briefly, cultures of *Ureaplasma spp*. were grown as per normal MIC methodology, with strains growing at any concentration of tetracycline sub-cultured after 24 hours to a fresh MIC plate and repeatedly exposed to scaling levels of tetracycline until full resistance was observed to have developed.

For the subset of strains to be examined by whole genome sequencing, individual colonies were grown in 500mL Ureaplasma selective medium, pelleted at 13,000xg for 3h, resuspended in 400 µL of sterile distilled water as the first step of DNA extraction using the Qiagen EZ1 Advanced XL automated extractor utilizing the EZ1 DSP Virus Kit as per manufacturer's instructions. DNA yields were between 1-8 ng/µL DNA (Qubit 4.0, Life Technologies).

2.2.1 Clinical Samples

Ureaplasma strains investigated were derived from clinical samples or isolates referred to Public Health England (PHE) reference laboratory for diagnostic investigation or antimicrobial susceptibility testing from 2003 to 2013 (Beeton et al. 2009a; Beeton et al. 2016a). Samples submitted were derived from a range of clinical specimens including neonatal, obstetric, and sexual health as well as invasive infections. Two were isolated from Havana, Cuba (Preval et al. 2022), two from France (Meygret et al. 2018); eight from the United Kingdom as well as the serovar 9 prototype reference strain from Vancouver. Apart from the *Ureaplasma spp.* strains isolated in Havana, Cuba and Bordeaux, France, the remaining strains were archived from previously published studies (Meygret et al. 2018; Preval et al. 2022), as well as from the UROGEN study (IRAS 251053; Spiller O.B. and Jones L.C. unpublished). Recovery of frozen archived isolates was performed via resuspension in Ureaplasma Experience Limited (Reigate, United Kingdom) selective media.

2.2.2 Ethics Considerations

The evaluation of UK and French bacterial isolates for antimicrobial resistance was exempt from NHS Research Ethics Committee review, due to the investigation's role in clinical service evaluation, apart from those arising from the UROGEN study (IRAS 251053; Spiller O.B. and Jones L.C.). Cuban isolates were acquired in a study approved by the Pedro Kourí Tropical Medicine Institute Ethical Board (approval CEI-IPK 33-12).

2.2.3 Whole genome sequencing and bioinformatic analysis.

For the subset of strains to be examined by whole genome sequencing, individual colonies were grown in 500mL Ureaplasma selective medium pelleted at 13,000xg for 3h, resuspended in 400 µL of sterile distilled water as the first step of DNA extraction using the Qiagen EZ1 Advanced XL automated extractor utilizing the EZ1 DSP Virus Kit as per manufacturer's instructions. DNA yields were between 1-8 ng/µL DNA (Qubit 4.0, Life Technologies).

Full genomic sequencing of all extracted isolate DNA was kindly undertaken by Edward Portal, Kirsty Sands and Jordan Mattias as I was not able to access the sequencing laboratory at UHW during the Covid-19 lockdown. This was undertaken using a Nextera XT library preparation with V3 chemistry on an Illumina MiSeq platform.

Bioinformatics analysis of genomes was comprised of 3 main processes:

- QC pipeline to validate & trim the raw sequence reads using FastQC and Trimgalore (Andrews 2010)
- a whole genome assembly and mapping utlizing Flash, SPAdes, BWA, pilon and quast; (Li and Durbin 2009; Magoč and Salzberg 2011; Bankevich et al. 2012; Gurevich et al. 2013; Walker et al. 2014)
- Whole genome annotation and profiling of genetic determinants using a combination of available software (using both fastq and de novo assembled reads): prokka, NCBI BLAST, kmerfinder, CARD, srst2, ARG-ANNOT and VFDB.

Assembled contigs were further analysed utilising Geneious sequence analysis software (BioMatters ltd. New Zealand) and aligned and assessed against a reference *tet*M sequence from *Enterococcus faecalis* (GenBank no. EFU09422) for the identification of genetic elements, point mutations and gene rearrangements via the Snippy and Roary programs (Page et al. 2015). Phylogenetic trees were generated using FastTree (Price et al. 2010) both via the Geneious Prime UI and via Linux-based command line.

2.3 Interventional Clinical Study

2.3.1 Samples and methods relating to the "Antibiotic Guardian Study".

Figure 6 shows indicates the sample collection, processing, and reporting pathways for the Antibiotic Guardian Study. The collection and testing of patient samples was conducted as part of an interventional clinical study, and so Health Research Authority (HRA) and Health and Care Research Wales (HCRW) ethical approval was obtained before the commencement of the study under IRAS project ID 269508. Patient samples were collected from Dewi Sant Hospital and Keir Hardie University Health Park in the Rhondda Cynon Taf and Merthyr Tydfil counties of South Wales, part of Cwm Taf Morgannwg University health board. Participants were symptomatic patients attending a sexual health clinic at either the Department of Integrated Sexual Health at Dewi Sant or Keir Hardy. Patients attending were asked to complete a self-triage form by sexual health staff detailing whether they had symptoms. A member of the clinical research team then reviewed a self-triage form and determined if the patient was symptomatic from their answers. Patients were not eligible to participate in the study if they did not meet any of the exclusion criteria (less than 16 years of age, were in attendance following sexual assault or were deemed to lack capacity to consent) and presented with any of the following symptoms:

- Dysuria (Painful urination)
- Urethral/Vaginal discharge (itchy/sore sensation, unusual colour, or smell)
- Rectal discharge (Mucous and/or purulent)
- Dyspareunia (painful intercourse)
- Pelvic pain (both acute and chronic)
- Epididymitis (Inflammation of epididymis/testicular tube)
- Post-coital bleeding
- Acute Vulval/Glans/Perianal pain.

Patients were given an opportunity to ask questions about the proposed study and were provided with clarification as necessary. If patients wished to participate in the study, written informed consent was gained by a member of the clinical research team trained in Good Clinical Practice (signed consent form). Although this did not occur, participants were able to withdraw from the study at any time – all data derived from their participation would have been removed in this event. Participants were then seen by a doctor specialising in Sexual Health and HIV, who took a full medical history and performed appropriate examination of patients as required. All participants underwent additional STI testing for Chlamydia, HIV, Syphilis, Hepatitis B, and Hepatitis following examination in addition to the testing undertaken as part of the clinical study. All participants with positive results were reviewed three weeks after treatment as per routine practice and were offered the opportunity to undergo a 'test of cure' via standard NHS testing – in practice, patients did not take up offers of 'test of cure' due to the pandemic restrictions and post-pandemic this continued.

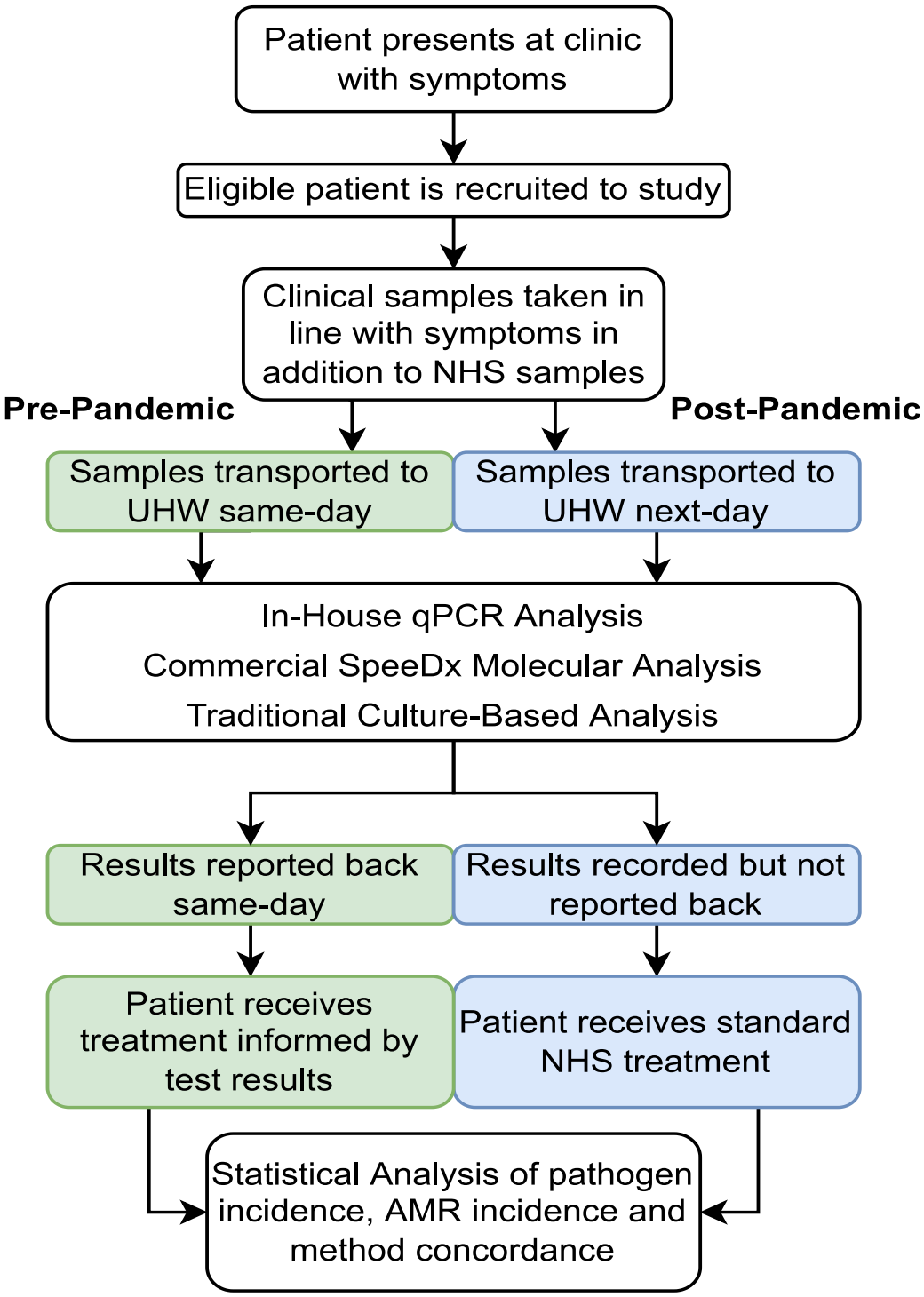

Figure 2-2 – Summary of clinical testing regime undertaken for the interventional clinical study. Only symptomatic patients were part of the clinical study. The standard NHS treatment regime takes approximately two weeks, with immediate treatment given empirically and further informed by laboratory results as appropriate.

The samples used for this study were taken in line with symptoms and could be any number of the following:

- Heterosexual male symptomatic patients.
    - Leftover “waste” first-catch urine
    - Penile and/or urethral swab sample from routine testing
- Men having sex with Men (MSM) symptomatic patients.
    - Leftover “waste” urine sample from routine screening
    - Oral swab
    - Rectal swab from routine testing
- Female symptomatic patients.
    - Leftover “waste” urine
    - Vulvo-vaginal swab sample from routine testing
    - Pharyngeal swab

Swabs containing residual participant material were pseudo-anonymised by the direct clinical care team in clinic with a unique research number before being transferred for transport to the Cardiff University at UHW (University Hospital of Wales) for processing. Additional participant residual samples consisted of up to 3 swabs for each participant (endocervical or urethral, oral, and rectal – sample site identified by the extension S, F and R, added to the common unique research number for that participant, respectively) and/or waste urine (identified by the extension U) following standard NHS clinical sample provision. The transport of patient samples to UHW for processing was conducted same-day pre-Covid and next-day post-Covid, using UN3373 compliant transport containers to prevent the risk of sample contamination or exposure to any biohazards.

2.3.2 Sample Processing

Swab samples were suspended in 3ml of 0.85% saline solution and then separated into two 400µl archive samples and a 200µl sample for extraction. Urine samples were spun down and pelleted at 10,000rpm for 5 minutes before being resuspended in 3ml of 0.85% saline solution and separated into two 400ml archive samples and a 200ml sample for extraction. For the purposes of clinical validation agar plates and a microdilution broth culture all selective for mycoplasma and Ureaplasma were set up to act as secondary tests for both *Mycoplasma spp*. and *Ureaplasma spp.* as per previously established methodologies (Morris et al. 2020) and Mycoplasma MST test kits were inoculated as per manufacturer’s instructions to act as a secondary validation for molecular detection of antimicrobial resistance. Sterile 1-microlitre bacterial loops were used to inoculate 1 x Mycoplasma selective agar (A7, BioMerieux) plate and 1 x Gonococcus selective agar (Oxoid, Basingstoke) plate, which were then transferred to the 5% CO_2 incubator to recover viable bacteria from resuspended swabs and/or urine. The detection of *Neisseria gonorrhoea* was also validated using agar-based culture. For each

sample determined by qPCR to be GC positive, sterile 1-microlitre bacterial loops were used to inoculate 1 x Gonococcus selective agar (Oxoid, Basingstoke) plates with a lawn of bacteria. MICs were then determined by the application of antibiotic-impregnated disks (MAST Group, Bootle) as per manufacturer's instructions. Disks were used to test for Ceftriaxone susceptibility (8mg/L) and two concentrations of Ciprofloxacin (1mg/L and 5ml/L) were used to test for fluoroquinolone susceptibility, all as per manufacturer's instructions and in line with current CDC guidelines (CDC 2023).

The molecular targets of this clinical study were *Mycoplasma hominis*, *Mycoplasma genitalium*, *Ureaplasma parvum*, *Ureaplasma urealyticum* and *Neisseria gonorrhoeae* (GC). DNA extraction was undertaken using 400µl of swab suspension with the Qiagen EZ1 Advanced XL automated extractor utilizing the EZ1 DSP virus kit as per the manufacturer's instructions to produce a 40µl DNA extraction for each sample. For each DNA extraction of a patient sample, BioRad Laboratories CFX96 Touch Real-Time PCR thermocycler and BioRad Maestro software were used to run the assays, along with SsoAdvanced™ Universal Probes Supermix (Bio-Rad Laboratories) for hydrolysis probes and plated using 96-well plates and optical clear adhesive seals. The molecular targets of this analysis, their primers and probes are detailed in Table 2-2.

Table 2-1 - The panel of targets of both assays run on each sample.

Probe	**ROX**	**HEX**	**FAM**	**CY5**	**C5-5**
GC Panel	*M. hominis*	*GC opa*	*GC por*(A)	*M. genitalium*	*E. coli (Extraction control)*
***Ureaplasma spp*. Panel**	*M. hominis*	*U. parvum*	*U. urealyticum*	*M. genitalium*	*tet*M

SpeeDx assays were undertaken in 96-well amplification plates (including the inclusion of a no template control, wild type control and mutant control), as per manufacturer's guidelines. This included addition of a non-viable *E. coli* source to act as a measure of successfµl DNA extraction – for the purposes of this assay, the proprietary SpeeDx *E. coli* DNA was used. As at time of analysis no commercial assay for *M. hominis* existed for purchase or use, analysis of extracted samples for the presence of *M. hominis* was undertaken by utilizing a previously published *yid*(C) Taqman hydrolysis probe assay (Férandon et al. 2011). Furthermore, the use of these hydrolysis probes for all five targets (*M. hominis*, *M. genitalium*, *U. parvum*, *U. urealyticum* and GC) in a multiplex assay had been clinically validated in previous studies (Morris et al. 2020) before their use here. For each assay, genomic copy equivalents were determined against a 7-point standard curve of micro-diluted primer and probe targets as per manufacturer's instructions.

The total volume of each reaction was 20µL, comprised of the following:

- 5µL of clinical sample suspended in 0.85% saline solution.
- 10µL SsoAdvanced™ Universal Probes Supermix

- 0.25pmol/µL forward primer, 0.25 pmol/µL reverse primer, and 0.01 pmol/µL probes for each of the molecular targets of the assay panel.
    - For the GC Panel, this was composed of *M. hominis*, GC *opa*, GC *por*(A), *M. genitalium* and *E. coli* (extraction control).
    - For the *Ureaplasma spp*. panel, this was composed of *M. hominis*, *U. parvum*, *U. urealyticum*, *M. genitalium* and *tet*M
- Remainder volume was made up with molecular grade water.

Table 2-2 – Primers and probes used in qPCR multiplex assay.

Target	Name	Sequence (5'-3')	Length (bp)	G+C (%)	Tm (°C)	Amplicon size (bp)
U. parvum	UUP_FP	AAGGTCAAGGTATGGAAGATCCAA	24	41.7	59.3	90
	UUP_RP	TTCCTGTTGCCCCTCAGTCT	20	55.0	59.4	
	UP_HP	(FAM)-TCCACAAGCTCCAGCAGCAATTTG-(BHQ1)	24	50.0	62.7	
U. urealyticum	UUP_FP	AAGGTCAAGGTATGGAAGATCCAA	24	41.7	59.3	90
	UUP_RP	TTCCTGTTGCCCCTCAGTCT	20	55.0	59.4	
	UU_HP	(HEX)-ACCACAAGCACCTGCTACGATTTGTTC-(BHQ1)	27	48.1	65	
M. hominis	MH_FP	TCACTAAACCGGGTATTTTCTAACAA	26	34.6	58.5	94
	MH_RP	TTGGCATATATTGCGATAGTGCTT	24	37.5	57.6	
	MH_HP	(ROX)-CTACCAATAATTTTAATATCTGTCGGTATG-(BHQ2)	30	30.0	59.9	
M. genitalium	mgpB_BnB_FP	GAAGGTATGATAACAACGGTAGAGCTT	27	40.7	61.9	88
	mgpB_BnB_RP	CCATTTAAACCCCTTTGCACCGT	23	47.8	60.6	

	mgpB_BnB_HP	(CY5)-ACTTAGCAAAAATGGAAAACCCCTC-(BHQ3)	25	40.0	59.7	
tetM	MH-tetM_FP	CCTGTTCCCTGTTTATCACGG	21	52.4	58.6	137
	MH-tetM_RP	ACTCAATTTTGAAAACTTTTCCGCA	25	36.5	59.1	
	MH-tetM_HP	(CY5.5)-TTTATTCATCAACACATCGAGGTCAG-(BHQ3)	26	38.5	59.7	
N. gonorrhoea (*por*(A))	PorA-FP	CCGGAACTGGTTTCATCTGATTAC	24	48.8	61	102
	PorA-RP	GTTTCAGCGGCAGCATTCA	19	52.6	56.7	
	PorA-HP	(HEX)-TTCCAGCGTGAAAGTAGCAGG-(BHQ1)	21	52.4	59.8	
N. gonorrhoea (opa)	GC opa-FP	TTGAAACACCGCCCGGAA	18	55.6	59.8	68
	GC opa-RP	TTTCGGCTCCTTATTCGGTTTAA	23	39.1	58.4	
	GC opa-HP	(HEX)-CCGCCCGGCAACATCAGTGA-(BHQ2)	20	55.0	60.3	

Bio-Rad CFX96 cycling conditions were as follows: initial activation and denaturation 95 °C for 5 min, next 45 cycles of 95 °C for 15 s and 60 °C for 15s. Readings were acquired in between cycles on channels FAM, HEX, ROX, CY5 and CY5.5, with data produced analysed using Bio-Rad CFX Maestro software. For samples that were found to contain *M. genitalium* by the SpeeDx ResistancePlus® MG, five microliters of that sample's extract were subsequently tested using the SpeeDx MG+parC assay to determine the presence of fluoroquinolone resistance-mediating mutations. Likewise, if *N. gonorrhoeae* was detected by initial analysis, 5µl of sample extract was tested for the presence of fluoroquinolone resistance-mediating mutations utilizing the SpeeDx ResistancePlus® GC as per the manufacturer's instructions. After all samples were fully analysed for pathogenic targets and their associated antimicrobial resistances, all detected pathogens and any corresponding antimicrobial resistances were recorded. On proceeding days, follow-up observations of the well plates and/or agar plates

were made, recorded and if bacterial growth was observed, samples were taken for archival purposes and tested in the same manner as swab suspensions (DNA extraction and qPCR analysis) for further validation of initial molecular diagnosis.

In line with ethical approval, there was no interaction between patients and researchers, with clinicians undertaking all aspects of patient interaction and sample collection. Patients were assigned unique research numbers as part of their clinic notes with their research proforma and consent form, by the senior clinician responsible for their care. These notes were not shared by the clinical team to me or any other researcher, and instead a sanitised and anonymous set of demographic information was recorded during the interview and shared with researchers approved by the ethics board to have access to this information.

This information was composed of:

- Gender
- Age
- Sample types taken.

After laboratory analysis, each research number was also assigned:

- Detection of pathogens. Each sample from an individual patient was recorded separately. In addition, as the detection of each target bacterium was conducted in parallel, this provided information on potential co-infections. Furthermore, as many of these pathogens can be commensal, a binary presence/absence determination was made using a gene copy number threshold ($>1x10^{-2}$) generated from (Morris et al. 2020). Bacterial load was used as the threshold unit due to European treatment guidelines (Horner et al. 2018) acknowledging that controversial bacteria such as U. urealyticum should be treated if present at sufficiently high bacterial load. The target panel was:
    - *Mycoplasma genitalium*
        - Presence or absence of MG Azithromycin resistance
    - *Mycoplasma hominis*
    - *Neisseria gonorrhoeae*
        - Dual target detection of *opa* and *por*(A) was required for a positive detection.
        - Presence or absence of GC ciprofloxacin resistance
    - *Ureaplasma urealyticum*
    - *Ureaplasma parvum*
    - Non-specific presence of *tet*M

- If patient samples produced growth on appropriate agar culture plate for confirmation purposes
- Mycoplasma/Ureaplasma Broth Microdilution Titration Value (CCU)

2.3.3 Alterations to Clinical Study and Reporting

Due to the Coronavirus pandemic and subsequent restrictions on my ability to conduct this study, several alterations to the original research plan were made. The initial design of the study included the results of the rapid diagnostic test of GC, MG and MH being fed back to the clinical team on the same day as sample collection and reported back the next day. As it was no longer feasible for results to be reported back to patients due to the pandemic, results of the rapid diagnostic tests for GC, MG and MH were not made available to participants and were only fed back to the clinical research team in the pseudo-anonymised form of a unique study ID for data analysis. This change was incorporated into the original ethical permission as a major amendment. These results were not recorded in the patients notes but in a study database on secure, password protected encrypted NHS computers. Instead of the original plan of informing patients of their test results the following day, patients were informed by telephone call of any NHS standard positive results as per routine care by a qualified sexual health nurse and advised that they needed to attend for treatment. Appointments were then made for patients with positive NHS laboratory tests to be treated as per guidelines (Soni et al. 2019; Fifer et al. 2020) and routine practice.

Due to alterations made to the overall study in response to the pandemic, variations occurred in the dataset of this study between the data collected pre- and post-pandemic. To add to the statistical power of the study, data comparable between both between pre- and post-lockdown was analysed via a two-sample t-test when possible. Data analysed separately was then compared to detect any observable differences in treatment outcomes before and after the pandemic.

2.3.4 Statistical Analysis of Clinical Study Primary Outcomes

The primary outcome measures of this study that were statistically analysed were:

- Incidence rates of the five target pathogens (*N. gonorrhoea*, *M. genitalium*, *M. hominis*, *U. ureaplasma* and *U. parvum*).
- Incidence rates of tetracycline resistance observed in *M. hominis*, ciprofloxacin resistance observed in *N. gonorrhoea* samples, and azithromycin resistance observed in *M. genitalium* samples.
- Disparities in pathogen incidence rates between genders
- Disparities in pathogen incidence rates between pre- and post-Covid time periods.

An analysis of the tetracycline resistance rates of *Ureaplasma spp.* was initially planned but could not be undertaken due to intractable issues with the non-specificity of the Ureaplasma *tet*M primers, as outlined later in this work. To assess the reliability of our study and the likelihood of detecting true effects, I initially conducted a power analysis using SPSS with a threshold power of 0.8 or higher. This calculation was repeated post-hoc when the sample

size was reduced due to pandemic restrictions. Statistical analysis was undertaken using either the Chi-square or Fisher's exact test for the comparison of categorical variables between demographic groups, with datasets tested in advance using the Kolmogorov–Smirnov test to assess parametric or non-parametric distribution for the most suitable test, and an independent samples t-test or Mann–Whitney U-test was used for comparing continuous variables with normal and non-normal distribution between demographic groups respectively. All statistical calculations were generated via use of SPSS (v29).

2.3.5 Growth of Isolates in Presence of Sex Hormones

As there is an observed link between the prevalence and bacterial load of *U. parvum* in female patients, as compared to *U. parvum* in men or *U. urealyticum* in women, I attempted to investigate potential mechanisms behind this variation in prevalence. The usage of sex hormones was based on the established relationship between Ureaplasma culture and sex hormone injections observed in rat models by Furr and Taylor-Robinson (Furr and Taylor-Robinson 1989; Taylor-Robinson and Furr 1990).

To investigate this phenomenon, three *U. parvum* strains (HPA5, ATCC-27815 and F2) and three *U. urealyticum* strains (HPA71, Vancouver and UUg1) were chosen to be analysed due to the availability of their fully sequenced genomes. Previous trial runs of this experiment suffered issues as ureaplasma strains grown overnight from frozen produced wildly disparate but uniformly low growth rates, so to standardize growth rates pre-experiment, the strains were grown for two days before the experiment. Each day, a sub-culture taken was taken from the highest positive culture dilution well (provided this was $\geq 1x10^{-4}$ CCU) that demonstrated colour change in line with bacterial growth (from yellow to red), to prevent the recent thawing of the strains influencing bacterial growth during the 12 hours of monitored growth. Each of the six isolates were grown in ten sterile 1.5ml Eppendorf tubes each containing 1ml of Mycoplasma Experience Ureaplasma media (Reigate, UK), with each containing one of the ten concentrations of sex hormone (six concentrations of testosterone and four concentrations of beta-oestradiol) chosen for testing, for a total of sixty cultures. In addition to these test cultures, a 1ml vial of culture of each isolate was grown with no additional hormones as a negative control. There is (currently) no known growth-enhancing reagent for Ureaplasma and growth-restricting reagents would likely kill the culture mid- or pre-experiment, so it was not possible to add a positive control to the experiment.

Both testosterone and beta-oestradiol were purchased from Sigma-Aldrich and prepared as per manufacturer's instructions. The beta-oestradiol used was in the form of water-soluble cyclodextrin-encapsulated 17β-oestradiol, while the testosterone used was at 1.0 mg/mL concentration in acetonitrile. Both reagents were dissolved in molecular grade water to produce the appropriate concentrations of stock solutions. Stock solutions were diluted in Mycoplasma Experience Ureaplasma media to achieve the necessary concentrations. This formulation of Ureaplasma selective media invariably contained hormones found in bovine serum extract (Naskar et al. 2016) as a necessary growth metabolite, but the same formulation of media was used to ensure minimal bias between the two cohorts of varying beta-oestradiol and testosterone concentrations.

Hormone concentrations were chosen to approximate detectable levels of beta-oestradiol and testosterone in blood across the different stages of the menstrual cycle, which is established to have effects on the pH, presence of hormone receptors and overall cytology of the urogenital tract (Robinson et al. 2013; Malik Aubead 2021). These values were based on reference ranges of both beta-oestradiol and testosterone in turn taken from recent clinical studies as appropriate for male and female patients (Tourgeman et al. 1999; Anckaert et al. 2021). For beta-oestradiol, a concentrations of 0pg/ml were chosen as a negative control value, with further concentrations of 62.5pg/ml chosen to approximate patients on combined oral contraceptives and general oestradiol levels in males, 300pg/ml chosen to approximate the follicular phase of the menstrual cycle, and 1000pg/ml was chosen to approximate both the luteal and ovulatory phases due to significant overlap in the hormone levels of these two phases. For testosterone levels, concentration ranges are far broader and relate more to individual physiological variations, so a range of concentrations of 0, 0.5, 1, 2, 8 and 30 nmol/L were chosen to cover the total possible range of clinically relevant concentrations in men and women. There was no known reagent that was established to promote Ureaplasma growth at the time of the experiment, and as such it was not possible to use a positive control for growth. As such, optimal conditions established as conducive to good growth were used instead.

For each of the specified sex hormone concentrations, 1 ml of Ureaplasma broth media with the requisite hormone concentration was inoculated with 30µl of the prepared Ureaplasma culture. The mixture was briefly agitated to ensure a uniform distribution of bacterial cells across the 1 ml. Subsequently, 50µl samples were extracted at inoculation (0 hours) and every 2 hours post-inoculation for testing and archival purposes across 12 hours. From each of these samples, 5µl was then analysed using the qPCR assay detailed in section 2.3.2 to determine the initial quantity of bacterial cells. These values were in turn statistically analysed using GraphPad Prism software version 7 to assess changes in growth of statistical significance. Mann Whitney U-tests were conducted on each growth curve generated from each hormone concentration to ascertain if variations in the concentrations of hormone significantly altered growth curves from the negative control. In addition, Tukey Fence calculations were undertaken to confirm the appropriate usage of U-tests for the values generated. The generation time of isolates was calculated using the bacterial cell count at 0 hours and the final recorded value at 12 hours, input into the following equation: Doubling time = [Duration·ln(2)]/[ln(Initial concentration/Final concentration)]. The relationship between doubling time and hormone concentration was interrogated via linear regression, with an R^2 value of >=0.2 used as an initial threshold for a significant relationship between the two variables.

3 Antimicrobial Guardian Study – An Interventional Clinical Study

3.1 Introduction

The management and treatment of sexually transmitted infections is of increasing importance to health care practitioners and providers, due to the growing rates of infections that are resistant to available treatment options, as well as the short- and long-term effects these pathogens can have on wider health. This can include chronic, painful conditions such as non-gonococcal urethritis in men (Beeton et al. 2019; Henkel 2021) and pelvic inflammatory disease in women (Darville 2021), infertility (Boujemaa et al. 2018) and even increased prevalence of urogenital cancers (Fiori et al. 2013; Garbas et al. 2021). According to NHS Wales, rates of *Neisseria gonorrhoeae* have increased by 14% between 2018 and 2019 (PHW 2019) in both heterosexual men and MSM (Men having Sex with Men). Rates of *Mycoplasma genitalium* were not diagnosed until much more recently, and due to the pressures of the Covid-19 pandemic, Public Health Wales has not been able to publish data since 2019 at time of writing. As of 2019, BASHH guidelines estimated the prevalence of *M. genitalium* at 1-2% of the total population and between 4-38% of STI clinic attendees (Soni et al. 2019), demonstrating both a relatively high prevalence but also a lack of precision in our ability to monitor the prevalence of this pathogen.

STIs such as *N. gonorrhoea* and *M. genitalium* not only affect the short-term quality-of-life of patients but also the long-term outcomes in closely related areas such as fertility and obstetrics. *M. genitalium* has been noted by researchers to significantly increase the risk of pre-term birth and female infertility (Jensen et al. 2016; Ma et al. 2021), long-term cell damage that can lead to urogenital cancers (Tantengco et al. 2021a) as well as neonatal infections such as ophthalmia neonatorum (Vallely et al. 2018). STIs specifically have been identified internationally as pathogens of concern due to their rising rates of resistance to the remaining antibiotics available to treat them (WHO 2018,2019) with *N. gonorrhoea* and *M. genitalium* rapidly developing high rates of antimicrobial resistances. The *Mycoplasma genitalium* Antimicrobial Resistance Surveillance (MARS) pilot studies conducted from March 2019 to March 2020 (Fifer et al. 2021) showed a macrolide resistance rate of 69% across both studies, a rise in fluoroquinolone resistance from 9% to 11% and a rise in dual resistance from 5% to 10%. The current gonococcal resistance to antimicrobials surveillance programme (GRASP) report (UKHSA 2022) notes that ciprofloxacin resistance has increased from 44.3% to 46.9% from 2020 to 2021 and that penicillin resistance has increased from 9.6% to 14.1% across the same period. Furthermore, azithromycin has shown similar increases from 8.7% to 15.2%, and tetracycline resistance is so high in GC (75.2%) that it is not considered as a treatment option. Ceftriaxone resistance is incredibly low (0.07%), but there has been a substantial increase in the number of ceftriaxone-resistant cases referred to UKHSA, with 11 cases in 2022 as compared to none in 2020 and only 3 in 2021. Relating to the rise of antimicrobial resistance in *Mollicutes* pathogens, a key component of this study is the inclusion of *M. hominis* and *Ureaplasma* spp., pathogens which have generated concern as their proposed screening has been posited to contribute to the excessive treatment of STIs with unnecessary antibiotics, most prominently in commercially available tests with limited clinical oversight (Horner et al. 2018).

Another key factor in the treatment of STIs are the various barriers to treatment that can delay prompt and effective care. While treatment failures due to antibiotic resistance are the main topic of this work, there are other important factors that impact treatment outcomes such as potential reinfection from untreated partners and a lack of capacity for test of cure procedures (Ovens et al. 2020), which can create difficulties in the integration of AMR surveillance into pre-existing sexual health care services. Another aspect of AMR surveillance that presents issues is the ability to provide healthcare in a relevant timescale. The current time taken for an STI diagnosis provided by NHS Wales from initial consultation to the results being reported back to the patient can take up to two weeks (Blomquist et al. 2018). As a result, patients who present at a sexual health clinic could be left with painful and distressing symptoms (Raffe and Soni 2022), which in turn leads to clinicians regularly prescribing antibiotics using an initial empirical diagnosis until a laboratory diagnosis can be obtained. This can in turn act as a selective pressure for AMR in these pathogens, further reducing the effectiveness of these antibiotics (Guschin et al. 2015; Bottery et al. 2017; Boujemaa et al. 2018; Porse et al. 2020). Therefore, one objective of this chapter is to demonstrate the prevalence of these organisms as clinically relevant pathogens that can be observed in symptomatic patients, which can then be rapidly assessed for antimicrobial susceptibility in tandem with their detection.

Previously outlined reservations on the usage of these rapid assays and their contribution to the development of AMR (Jensen et al. 2016) are valid and considered in this work, this objective is significantly informed by the realities of sexual healthcare. Rapid diagnostic assays have already been widely proliferated across both sexual healthcare (Karellis et al. 2022). While pre-existing issues with their formulation and resistance thresholds drive a great deal of this aspect of my work (Beeton and Spiller 2017), ultimately it is my opinion that these types of diagnostic kits are an established tool that cannot be put back in Pandora's box, but must instead be improved and refined to mitigate and ultimately eliminate their substantial downsides. A second objective of this chapter is to analyse the accuracy, precision and specificity of these assays used and their subsequent suitability for clinical purposes. This part of my work is the most extensively altered from its original design due to the Covid pandemic impacting the clinical study component of the chapter. As such, it also includes an analysis on the variations between infection rates and AMR prevalence pre- and post-pandemic.

In summary, the primary questions this chapter sought to address were:

- What are the incidence rates of *M. hominis*, *U. ureaplasma* and *U. parvum* in the specified sexual health patient cohort, and how do these compare to the prevalence rate of two well-established sexual health pathogens *N. gonorrhoea* and *M. genitalium*?
- What is the frequency of antimicrobial resistance present in these target pathogens? This question focuses on those resistances most clinically relevant to my target pathogens - tetracycline resistance in *M. hominis*, ciprofloxacin resistance in *N. gonorrhoea* and azithromycin resistance in *M. genitalium*.
- Can we observe disparities in pathogen incidence rates between genders, and between pre- and post-Covid time periods?
- Can we observe any changes in *Ureaplasma* growth when cultured in varied concentrations of sex hormones (beta-oestradiol and testosterone) that correspond to levels as they vary across hormonal cycles?

3.2 Results

3.2.1 Patient Demographics

In total 160 patients were successfully recruited to the study, with 79 patients recruited pre-pandemic and 81 patients recruited post-pandemic, Of the 160 patients there was a clear gender bias present in the cohort with 112 female patients and 48 male patients consenting for participation in the study. More specifically, 20 male patients and 61 female patients were recruited pre-pandemic, and 28 male patients and 51 female patients were recruited post-pandemic. While this means there is an overall gender bias in the study cohort between male and female participants, the distribution of male and female patients was not significantly different before and after the pandemic, suggesting that this is a consistent gender disparity in clinic attendance. In total, *U. parvum* was present in 22.5% of patients, *M. hominis* was present in 11.9% of patients, *U. urealyticum* was present in 8.1% of patients, *N. gonorrhoea* was present in 4.4% of patients and *M. genitalium* was present in 2.5% of patients, with 41.25% of patients being found to carry any one or more of the study's target pathogens.

3.2.2 Analysis of Pathogen Incidence

Due to the pandemic requiring the study to reduce the sample size from 1000 patients to 160, the study was limited in the statistical power that was initially expected when this experiment was first designed. Nonetheless, Fisher's exact analysis found that both *U. parvum* and *M. hominis* were significantly more prevalent in female patients ($p > 0.05$). *U. parvum* was the most common pathogen detected in all cohorts (male, female, before pandemic and after pandemic) and was present in almost a quarter of all female patients tested in the study and had over twice the detection rate (as a percentage) in symptomatic female patients as the pathogen did in symptomatic male patients, suggesting a gender bias in *U. parvum* pathogenicity. A similar gender disparity was detected in *M. hominis*, which as the second most prevalent pathogen detected in female patients was three times more prevalent (12% versus 4%) in female patients than in male patients. In comparison, the other target pathogens (*M. genitalium*, *U. urealyticum* and *N. gonorrhoea*) were not significantly more prevalent in either gender, although only *U. urealyticum* had a lower prevalence in female patients over male patients, with other target pathogens being equal or higher in prevalence. This was also in line with female patients being found by t-test to be significantly more likely to carry one or more of the study's target pathogens ($p > 0.05$).

In comparison, it was surprising to find that there was no statistically significant difference in pathogen incidence before and after the pandemic overall, considering the legal restrictions on movement that were in place during the pandemic. One potential hypothesis for these observations could be that in both cohorts, transmission predominantly occurred between co-habiting sexual partners (which precludes a degree of promiscuity), and another might relate to dysbiosis caused by Covid leading to immunologically mediated symptoms. In their case, quantifying these theories would require access to detailed patient records unavailable as per ethical approval. In any event, the level rates pre- and post-pandemic are unusual considering the legal restrictions on meeting people outside of one's "bubble", but this does reply on the assumption that patients universally adhered to these restrictions, which cannot be assumed.

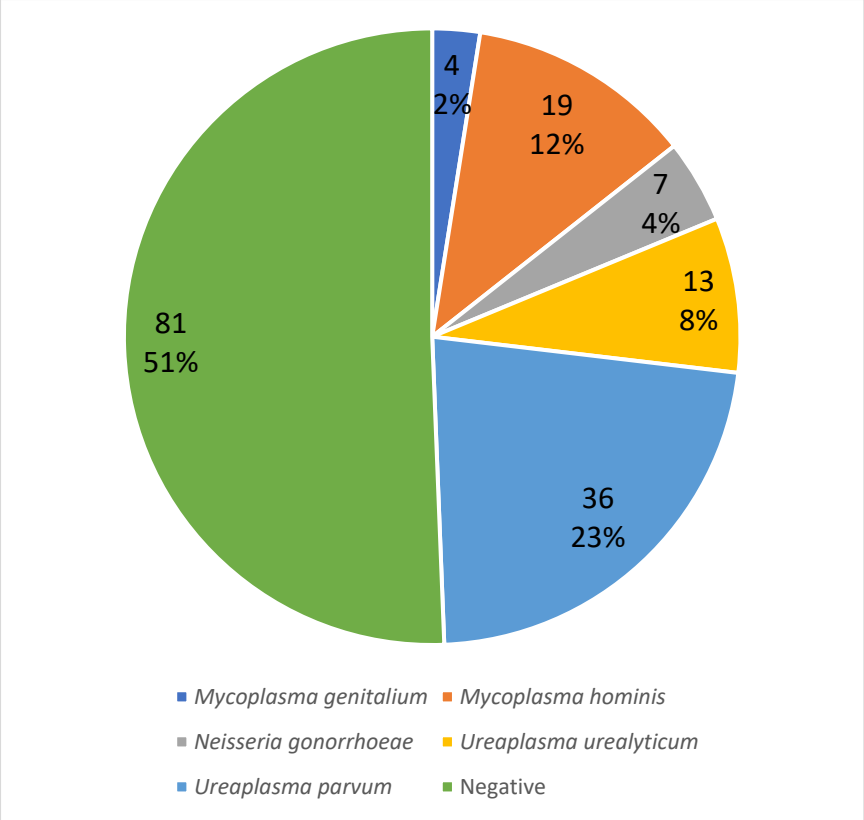

Figure 3-1 - The distribution of pathogens detected in 160 patients tested as part of the Antibiotic Guardian Study. Patients may have been tested via multiple sample types based on symptoms, with one or more positive detection leading to treatment.

As seen in Table 3-1, vaginal samples were determined by chi-squared test to be significantly more common in *U. parvum*, M. hominis, and negative samples. *M. genitalium* was not detected frequently enough to draw any inferences, but the significant prevalence of positive vaginal samples in *U. parvum* and *M. hominis* as compared to *N. gonorrhoeae* and *U. urealyticum* does make sense when considered in the context of acknowledged gender disparities in Ureaplasma infections (Horner et al. 2018). Nonetheless, this variance must also be considered in the context of the study's sampling bias, with a 2:1 female-to-male ratio. Another finding is that the initial bacterial count threshold of $1x10^2$ was likely overly conservative. *M. genitalium* was detected at around the $1x10^2$ threshold, but all other pathogens targeted had an average bacterial count of $>1x10^4$. Once again, the small sample size of *M. genitalium* makes it difficult to infer aspects such as bacterial count, but when considering the other pathogenic targets in this study, it may be possible to adjust the threshold of positivity. This would have to be backed by analysis of asymptomatic patients, which would require further work. Both the in-house qPCR methodology outlined in this work and the commercial SpeeDx kits used for the detection of pathogens demonstrated a 100% concordance of results, with all samples tested for *N. gonorrhoea* and *M. genitalium* producing the same detection results with both assays used. Furthermore, while only 50% of samples that tested positive for GC via PCR subsequently produced viable agar cultures, 100% of all cultures successfully grown correlated with the initial qPCR results.

Table 3-1 - Clinical data by sample type collected during AGS clinical study. Multiple sample types could be collected from patients in line with presenting symptoms. Bacterial count was calculated by gene copy count generated as part of RT-qPCR analysis.

Target	***M. genitalium***					***M. hominis***					***N. gonorrhoeae***					***U. urealyticum***					***U. parvum***					**Negative**				
Sample Type	**Oral**	**Rectal**	**Urethral**	**Vaginal**	**Urine**	**Oral**	**Rectal**	**Urethral**	**Vaginal**	**Urine**	**Oral**	**Rectal**	**Urethral**	**Vaginal**	**Urine**	**Oral**	**Rectal**	**Urethral**	**Vaginal**	**Urine**	**Oral**	**Rectal**	**Urethral**	**Vaginal**	**Urine**	**Oral**	**Rectal**	**Urethral**	**Vaginal**	**Urine**
Samples Collected	0	0	0	3	1	0	0	0	12	2	2	0	0	4	1	0	1	0	8	4	4	0	0	27	5	10	2	0	63	33
Total Samples Collected	4					14					7					13					36					108				
Concordance (%)	-	-	-	100	100	-	-	-	100	100	50	-	-	50	0	-	100	-	100	100	100	-	-	100	100	-	-	-	-	-
Bacterial Count by Sample (Av.)	-	-	-	$1x10^2$	$1x10^2$	-	-	-	$8.42.x10^4$	$4.5x10^4$	$1.96x10^4$	-	-	$3.40x10^5$	$2.79x10^4$	-	$1.0x10^2$	-	$1.26x10^5$	-	$1x10^2$	-	-	$1.43x10^5$	$3.33x10^5$	-	-	-	-	-
Bacterial Copy by Species (Av.)	$1x10^2$					$6.46x10^4$					$1.29x10^5$					$6.3x10^4$					$1.59x10^5$					-				

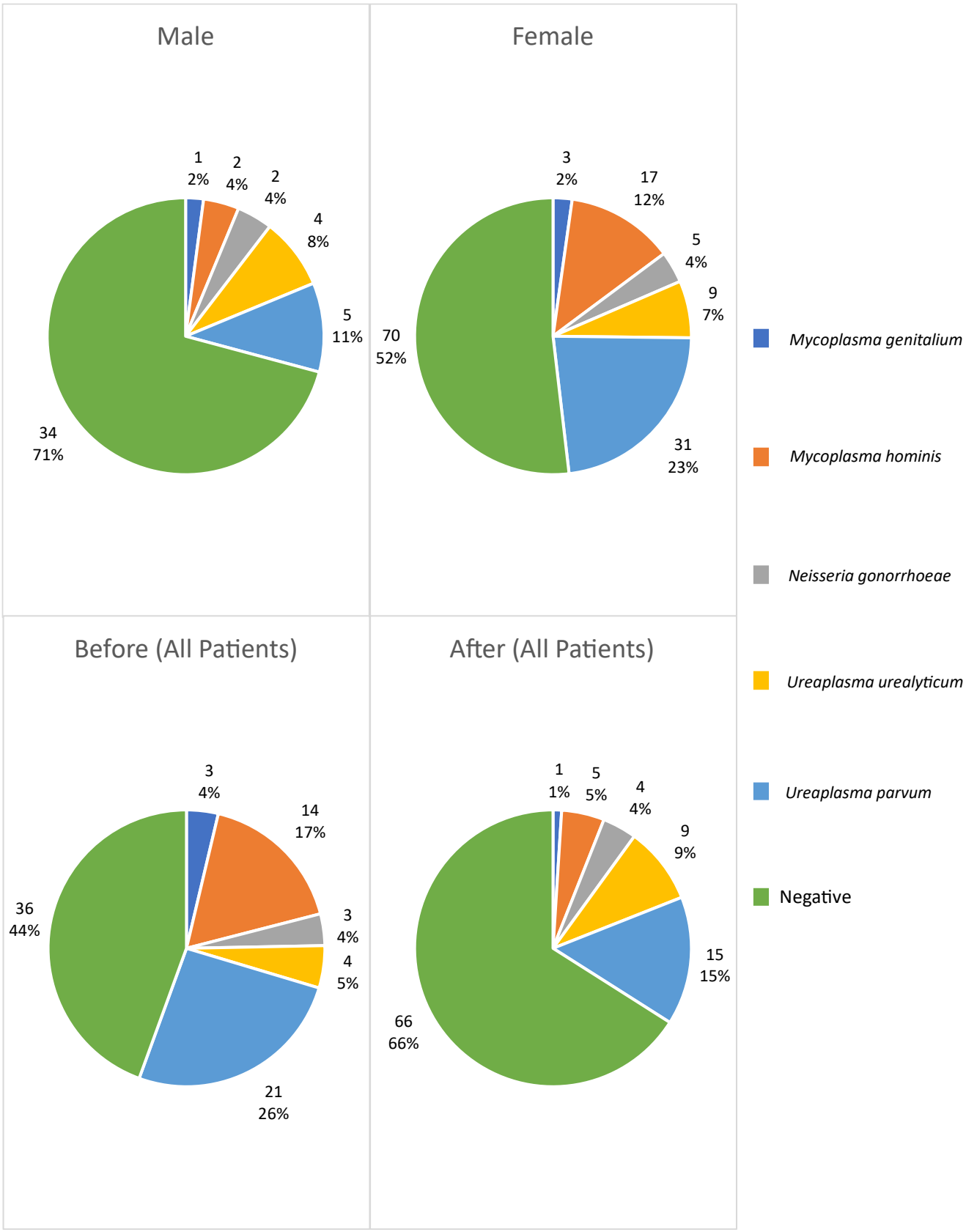

Figure 3-2 – The distribution of pathogens detected in male and female patients, as well as before and after pandemic restrictions. All four categories show that *U. parvum* is the most common bacteria present. *M. hominis* is the second most prevalent pathogen overall and in women specifically, while *U. urealyticum* is the second most prevalent pathogen in male patients.

3.2.3 Detection of Resistance in Clinical Samples

Antimicrobial resistance in samples was relatively high, with three out of four (75%) *M. genitalium* samples testing positive for 23S-mediated azithromycin resistance and 42.9% of all *N. gonorrhoea* samples testing positive for S91F-mediated ciprofloxacin resistance. Tetracycline resistance in *M. hominis* was unable to be accurately quantified from initial qPCR analysis as of the 37 samples that tested positive for the presence of *tet*M, 14 had no target pathogens detected and thus carried *tet*M due to the presence of other tetracycline-resistant pathogens. I ultimately determined that tetracycline resistance in *M. hominis* specifically could not be accurately detected by initial testing of patient samples with the in-house assay, as the *tet*M primers and probes I used were not specific enough to both accurately detect the presence of *tet*M in *M. hominis* while also excluding the presence of *tet*M homologs contained in other commonly occurring endogenous bacterium in the same sample. Furthermore, due to the truncation of this aspect of my research due to the pandemic, there was no scope for a re-design of my Ureaplasma. *tet*M primer and probe set. However, after culture enrichment of Mycoplasma and Ureaplasma in selective medium, qPCR presence of *tet*M was specific only to tetracycline-resistant isolates and the background *tet*M signal was removed. Tetracycline resistance was detected in 75% of *M. hominis* isolates. Figure 3-4 shows an alignment of *tet*M genes from reference strains corresponding to each of the target pathogens of the clinical study, demonstrating the high level of homology between each gene, well above 90% for each pathogen. Furthermore, when antimicrobial susceptibility testing was conducted via culture-based methods, 100% of *M. hominis, U. parvum and U. urealyticum* shared concordance of antimicrobial susceptibility results, but only 50% of *N. gonorrhoea* was able to be cultured to verify the phenotypic ciprofloxacin resistance relative to the molecular detection methods.

3.2.4 Genomic Analysis of Fluoroquinolone-Resistant GC

In total, four patients were diagnosed with a GC infection with the in-house kit and confirmed by supplementary culture-based testing. An additional six patient samples tested with the in-house kit were found to test positive for one diagnostic probe (Opa OR *por*(A)) but could not be considered a positive result for GC due to the lack of concordance between the two gene targets. Furthermore, supplementary same-day analysis with SpeeDx confirmed these findings as negative results. Post-pandemic, a concordance of results between the in-house and SpeeDx kits was used to detect GC in patient samples as results were not reported back to clinicians for next-day treatment (which was part of the pre-pandemic study design), and all three samples positive for GC were identified concurrently with the in-house assay and the commercial SpeeDx assay. All three ciprofloxacin-resistant *N. gonorrhoea* samples being identified post-pandemic was considered notable in a clinical context, but likely due to the small sample size no statistically significant variances in resistance before and after the pandemic could be detected. The genomes of all three ciprofloxacin-resistant isolates detected jointly by the in-house assay and the SpeeDx commercial kit (AGS151, AGS 138, and AGS152) were analysed via disk diffusion using CDC thresholds of resistance and it was found that the Oxoid GC specific agar plate used was contaminated with *Kingella dentrificans*, a closely related bacterium that under initial observation appeared as *N. gonorrhoea*. Subsequent testing showed that the *K. dentrificans* genome sequenced contained neither of the *opa* or *por*(A) targets utilized by either

molecular assay which means it therefore could not have been detected by either assay. Ciprofloxacin-resistant *N. gonorrhoea* sampled from patient AGS138 was found to contain the expected S19F point mutation that was both detected by the assay and is established to confer clindamycin resistance in *N. gonorrhoea*, as shown in Figure 3-3.

Figure 3-3 – An alignment of the variation between *gyr*(A) QRDR regions present in two *N. gonorrhoea* genomes. Pictures A and B show both bacteria sampled from patients AGS138, AGS151 and AGS152 on Oxoid agar plates, with antibiotic diffusion disks containing ciprofloxacin. AGS138 demonstrates a 20mm diameter zone of inhibition surrounding a Cip 5 disk, while AGS152 demonstrates a 55mm diameter zone with the same Cip 5 disk, demonstrating a significantly lower resistance to ciprofloxacin. AGS151 contains a ciprofloxacin-susceptible bacterium (zone diameter <20mm) now known to be *K. dentrificans*.

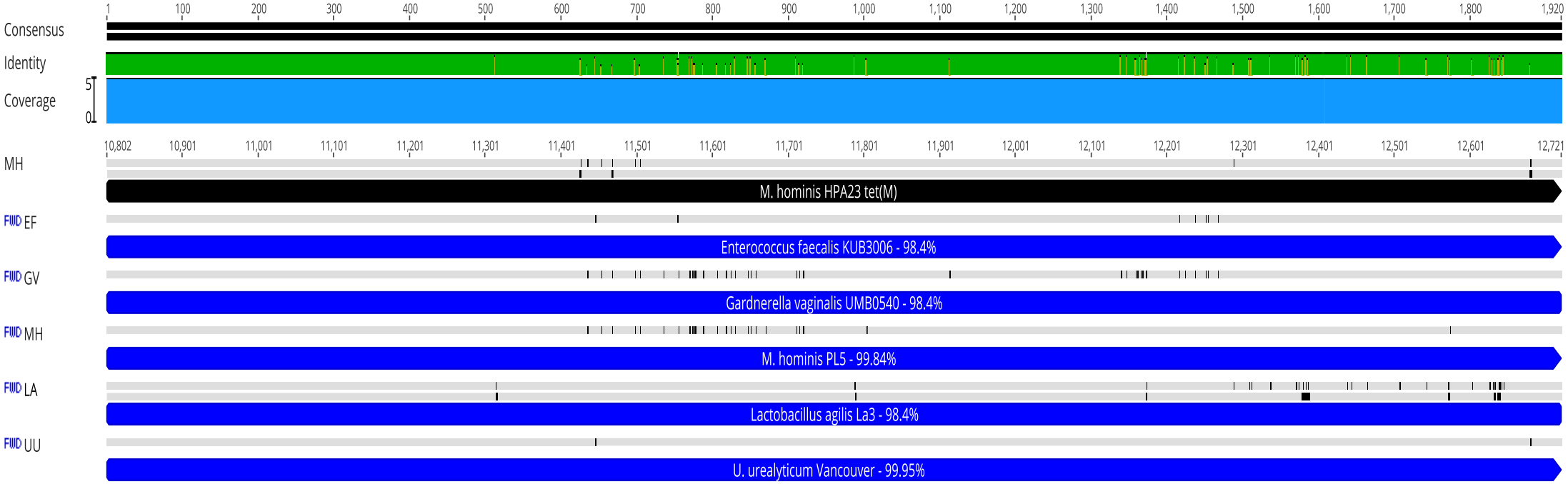

Figure 3-4 – An alignment of tetM genes from common urogenital microflora and target pathogens of the study. Species, reference strain designation, and percentage similarity is indicated in the blue labels, relative to the HPA23 strain of *M. hominis* labelled in black. Point mutations are indicated in yellow lines present in the green identity bar and as black dashes relative to each gene in the alignment.

3.2.5 Growth of *Ureaplasma spp*. in the Presence of Sex Hormones

Given that *U. parvum* has been observed to have a much greater propensity to be isolated from female patients (Redelinghuys et al. 2014) and that previous mouse model experiments indicate that female mice can only be colonised with Ureaplasma strains following pre-treatment with oestrogen (Furr and Taylor-Robinson 1989), experiments were designed to see if there was a direct effect of oestrogen or testosterone on the growth of *Ureaplasma parvum* and/or *Ureaplasma urealyticum*. growth. Full growth curve data can be found at Appendix Table 8-8 and Table 8-9, and is summarised and discussed here.

As cultures were sub-cultured each day for 48 hours in advance of the experiment (as per the methods described in section 2.2.1), bacterial growth was consistently observed to be exponential for the entire 12 hours of growth of both *U. parvum* and *U. urealyticum*, as shown by Appendix Figures 8-2 through 8-5. Final bacterial growth values of *U. parvum* were consistently higher than that of *U. urealyticum* when grown in the presence of beta-oestradiol, in line both with previous culture of these strains and the wider literature (Furr and Taylor-Robinson 1989; Taylor-Robinson and Furr 1990). In comparison, *U. urealyticum* cultured in testosterone consistently grew to final bacterial counts an order of magnitude higher than comparable cultures grown in beta-oestradiol. There was no statistical significance found in the variance of testosterone levels, but the broad observation of testosterone as a growth agent for *U. urealyticum* may relate to its acknowledged role in non-gonococcal urethritis (Horner et al. 2018).

Variation in growth rates was further investigated via Mann-Whitney U-Test, comparing each hormone concentration to its relevant negative control to detect any statistically significant increase and/or decrease in growth rate. Statistical analysis found no statistically significant variation in any of the hormone concentrations from the negative control cultures lacking in sex hormone. Furthermore, the p-values of these growth curves were incredibly high, with only two data points demonstrating a p-value <0.10. It should be noted that both "near-fails" were for the *U. parvum* isolate F2 when grown in the presence of testosterone, although which may indicate an avenue for further investigation. Nonetheless it is difficult to determine a causal link due my inability to repeat the experiment again due to pandemic-related time constraints.

When the relationship between doubling time and hormone concentration was examined via linear regression, *U. parvum* isolate F2 once again demonstrated interesting data, with an R^2 value of 0.273, signifying hormone concentration contributed to 27.3% of the variance in doubling time. As these concentrations relate to specific points of the menstrual cycle, it is notable that the doubling times of both these cultures was significantly lower in the concentrations relating to the luteal and ovulatory phase, which form the "latter half" of the menstrual cycle. *U. urealyticum* isolate UUg1 demonstrated an even stronger regression R^2 value of 0.796, but this is tempered by poor growth of the isolate overall, suggesting the results observed may likely be a statistical outlier I was unable to repeat due to pandemic-related time constraints.

Ultimately, there was no discernible variation of growth that correlated with any of the specific concentrations of either beta-oestradiol or testosterone that were tested, but broad

trends relating to hormone/pathogen concentrations were observed. *U. parvum* grew to consistently higher final bacterial counts in the presence of beta-oestradiol than *U. urealyticum*, and *U. urealyticum* grew to consistently higher final bacterial counts in the presence of testosterone. The lack of statistical significance between hormone concentrations makes it difficult to ascribe a mechanism for this variation, but nonetheless provides a starting point for further work. Likewise, the relatively high relationship between hormone levels and doubling time in some *U. parvum* isolates cultured in beta-oestradiol provides some evidence for a link between U. parvum growth and variations ion hormone levels during the menstrual cycle that could form the basis further investigation.

Table 3-2 – Doubling times for *Ureaplasma parvum* and *Ureaplasma urealyticum* isolates cultured in sex hormone. Datapoints found to be significantly correlate with hormone concentration are written in **bold**. Datapoints that are suspected to be outliers are written in *italics*.

(a) Concentration	Doubling Time (mins)					
	U. parvum			*U. urealyticum*		
	HPA5	ATCC-27815	F2	HPA71	Vancouver	UUg1
0	110.6	176.4	189.0	269.7	176.4	330.0
62.5pg/ml	115.6	129.7	79.2	79.1	129.7	373.0
300pg/ml	136.2	97.3	109.6	220.4	97.3	172.5
1000pg/ml	121.3	129.3	82.0	164.5	128.3	95.2
R^2 value	0.092	0.110	**0.273**	0.016	0.120	***0.796***

(b) Concentration	Doubling Time (mins)					
	U. parvum			*U. urealyticum*		
	HPA5	ATCC-27815	F2	HPA71	Vancouver	UUg1
0	56.5	100.2	114.2	56.5	100.2	114.2
0.5nmol/L	110.3	97.7	229.0	110.3	97.7	229.0
1nmol/L	111.2	191.6	77.6	111.2	191.6	77.6
2nmol/L	69.5	139.1	88.3	69.5	139.1	88.3
8nmol/L	106.8	134.4	103.5	106.8	134.4	103.5
30nmol/L	76.8	87.0	172.4	76.8	87.0	172.4
R^2 value	0.022	0.188	0.075	0.022	0.188	0.075

3.3 Discussion

U. parvum was the most prevalent urogenital bacterium detected, present in both 11% of males and 23% of females, followed by *M. hominis* being detected in 8% of males and 7% of females. 86.1% and 89.5% of samples of *U. parvum* and *M. hominis* respectively were found in female patients, showing a clear bias towards being isolated in female patients and in tandem with the selection criteria of symptomatic patients further demonstrates a potential link between these bacteria and pathogenicity. This is further emphasized by the higher incidence rate of these pathogens than *N. gonorrhoea* or *M. genitalium*, while presenting with the same spectrum of symptoms. However, this must be considered in the context of the cohort, as 112 female patients and 48 male patients creates an almost 3-to-1 ratio between female and male patients and a clear bias in the sampling. This was not entirely unexpected, as there is a known bias towards female attendance of STI clinics (Tanton et al. 2018), although the severity seen in this dataset was surprising.

A key finding was the strong bias of *U. parvum* present in female patients, over twice as often as in male patients. *U. parvum* has long been established as a pathogen in neonatal medicine, with studies demonstrating its link to chorioamnionitis (Knox et al. 2010; Sweeney et al. 2017a; Sprong et al. 2020), premature birth (Motomura et al. 2020) and spontaneous abortion (Oliveira et al. 2020), yet studies assessing its role in symptomatic women that have fed into official guidelines have cited the lack of evidence of its pathogenicity as a reason why it should not be screened as a sexual pathogen (Horner et al. 2018). This is counterbalanced by the fact that the official guidance stating the "non-pathogen" status of urogenital ureaplasma is tempered by the possibility that it could be the causal bacterium if at high enough levels in non-gonococcal urethritis patients (Jensen et al. 2016), complicating matters.

The work described in this chapter seeks to answer the call for more data and evidence, with a focus on patients presenting with specific symptoms, as a major source of doubt as to the pathogenicity of *U. parvum* has been its ubiquity as a commensal in the urogenital tract of asymptomatic patients (Ikonomidis et al. 2016; Beeton and Spiller 2017; Viscardi and Waites 2017). This has meant that meta-analysis has identified previous studies as suffering from an inability to control for the presence of other infections and/or conditions (Jonduo et al. 2022) such as the common pathogenic causes of bacterial vaginosis (Frølund et al. 2019; Rittenschober-Böhm et al. 2019). Therefore, a key focus of this work was the observation of abnormally high levels of urogenital mycoplasmas in symptomatic patients while being able to exclude confounding conditions such as BV and *Candida*. Likewise, *M. hominis* had a far higher incidence in female patients (12%) than male patients (4%), once again suggesting a sex-based component to its pathogenicity. *M. hominis* has also been implicated in adverse neonatal outcomes (Ansari et al. 2021), as well as being extensively investigated for its role in infertility (Huang et al. 2016; Boujemaa et al. 2018; Wang et al. 2019; Paira et al. 2021), so an area of further work might be investigating the reasons for this disparity.

Several demographic factors relating to the observed disparity in incidence across the sexes have been implicated as contributing factors towards the incidence of these diseases (Siles-Guerrero et al. 2020), but the factors that were available for study were limited by ethical and practical considerations to age and biological sex. One major component that I was not able

to examine was sexual partners, as the limitations on patient data made this infeasible to track and confirm in practice. Understanding the relationship between STI pathogenicity and the influence of sexual dynamics is a major component in not only the obvious contributions to the spread of an STI (Wihlfahrt et al. 2023), but also a key component in understanding the duration of infectiousness and overall infection timespans (Cina et al. 2019)

These factors could be able to provide the body of evidence required to firmly establish the pathogenicity of mollicutes such as *M. hominis* and *Ureaplasma spp*. Hormonal levels are a well-known factor in the development of pathogenic STIs, with the first works describing the link between hormonal injections and the growth of Ureaplasma in rat models dating back over twenty years (Furr and Taylor-Robinson 1989; Taylor-Robinson and Furr 1990). While there is a body of evidence on the links between hormonal levels and the promotion of bacteria-cell adhesion in infection of the urinary tract and reproductive tract in animal models (Kemp et al. 2014; Pavlidis et al. 2020; Tantengco et al. 2022), there is far less work that describes the direct effect of hormonal levels on the growth of Ureaplasma. While it is hard to theorise on the reasons why research was not carried out, it is likely due to both the disparate ranges of hormonal concentrations that can occur in the human body, along with the difficult nature of culturing Ureaplasma in what could easily be non-optimal conditions caused by the presence of these hormones.

The wide range of concentrations that were tested to ascertain a primary effect were further exacerbated by the relatively slow growth of Ureaplasma, with *U. parvum* and *U. urealyticum* demonstrating 60-90 minute doubling times under laboratory conditions (Stemler et al. 1987), making the detection of growth in real-time unfeasible in many circumstances. Other aspects of Ureaplasma culture such as its fastidious culture requirements have been described earlier in this work, and only added to the difficulty in testing any hormone-related hypothesis. As such, a key part of analysing the primary effects of hormone concentration on *Ureaplasma* growth was to develop a methodology that was able to accurately and repeatably test whether such a primary effect occurred in vitro. If such an effect could be observed, it would have helped explain the variable nature of *Ureaplasma spp*. pathogenicity that has made widescale healthcare surveillance difficult to implement. Due to time constraints (relating once again to pandemic restrictions and the subsequent limitation on *in vitro* work), the methodology used in this study was not able to be refined and developed based on the initial findings outlined here. Nonetheless, the broad trend for U. parvum to grow more readily in the presence of beta-oestradiol and the likewise trend for U. urealyticum to grow more readily in the presence of testosterone provides some information that might form the basis of further investigations into the underlying mechanisms of these trends.

As an avenue of research, secondary mechanisms for the pathogenicity of *Ureaplasma spp.* have been investigated in previous works, with meta-analyses (Sweeney et al. 2017a) describing the current understanding of *Ureaplasma* spp. pathogenicity and how the main virulence factors considered currently relate to the avoidance of host immune systems. Multiple banded antigens (MBA) are well-established to aid in the avoidance of immune system recognition (Aboklaish et al. 2016; Sweeney et al. 2017b; Yang et al. 2020a) and it was established as early as 1984 that Ureaplasma was capable of synthesizing Immunoglobulin A

(IgA) Protease as a defence mechanism (Kilian et al. 1984). Less work has been done on the links between sex hormones and *Ureaplasma* infections, with works investigating the effects of *Ureaplasma* spp. on female patients strongly focusing on the impact the species can have on pregnancy and neonatal outcomes (Knox et al. 2010; Payne et al. 2016; Beeton and Spiller 2017; Sweeney et al. 2017b). As such, broader comparisons of sex bias are not as available in the literature, demonstrating a gap of knowledge in our understanding. Cytokines are a family of signalling proteins that form a key component of a healthy immune system by helping regulate the cell signalling systems of the immune system (Kany et al. 2019), and are in turn regulated by the secretion of oestradiol and progesterone (Bereshchenko et al. 2018). The levels of these cytokines have been observed to vary across pre-menopausal female patients with *U. urealyticum* and *M. genitalium* infections when compared to control groups (Garza et al. 2021), in turn effecting the composition of *Lactobacillus* colonies in the patient microbiome. The confounding presence of microbiomes is regularly cited as a confounding factor in the analysis of urogenital *Mollicutes* pathogenicity, with the observed links between these bacterium and bacterial vaginosis (Frølund et al. 2019; Rittenschober-Böhm et al. 2019; Vodstrcil et al. 2021) and/or *Trichomonas vaginalis* (Fürnkranz et al. 2018; Dessì et al. 2019) being two such factors that complicate efforts to narrow down the precise mechanisms that cause the variable pathogenicity of these bacterium.

Mollicutes such as *M. genitalium*, *M. hominis* and *U. spp*. are still extremely difficult to culture within an NHS diagnostics lab (Soni et al. 2019) and require specialized training that many laboratories may not be able to access. Therefore, while molecular tests for STIs such as *M. genitalium* are recommended for all patients who present with relevant symptoms, such tests are conducted by special request only (Soni et al. 2019) and so in practice many STIs can be missed or simply unviable to consistently achieve in sexual health practice. Modern molecular testing kits such as the SpeeDx kits used in this work are available, but even clinically validated kits usable in a healthcare setting can present significant financial problems. A study by Huntington et al. (2018) demonstrated that multiplex qPCR testing provided many benefits to patient outcomes that could contribute to lowering the overall cost of NHS STI care provision but was counterbalanced by significant increases in the up-front cost of implementing and providing these services. While it is acknowledged that the implementation of such molecular assays carries benefits, an aversion to funding the significant costs in implementation is by far the most common reason for the lack of provision currently available (Wi et al. 2019; Footman et al. 2021). Nonetheless, current gold standards in culture-based diagnosis are known to be too slow, insufficiently comprehensive, and prone to overly subjective interpretations (Trotter et al. 2019) and NAATs such as multiplex qPCR-based analysis, while expensive, can overcome these persistent difficulties (Raffe and Soni 2022). Therefore, it is acknowledged by official bodies that more evidence is needed (Horner et al. 2018), to better define the long-term benefits of NAATs as a diagnostic tool for sexual health pathogens of growing importance and the substantial financial costs of implementing these assays in healthcare settings.

Of the two molecular methods used for the detection of target pathogens, the in-house molecular method demonstrated a 100% concordance with both the commercial SpeeDx ResistancePlus® MG and SpeeDx MG+parC assays and the traditional culture-based methods

commonly used in a laboratory setting, demonstrating the reliability of these assays to produce trustworthy results. The culture-based confirmation of ciprofloxacin resistance in *N. gonorrhoea* could only be tested on 50% of the patient isolates with the commercial SpeeDx ResistancePlus® GC, because of the variable nature of growing *N. gonorrhoea* from patient samples. Additionally, though the percentage of *N. gonorrhoea* found to be clindamycin resistant was high (42.86%), the sample size of four out of seven instances of *N. gonorrhoea* makes it impossible to make inferences of statistical significance.

The necessity to grow *M. hominis* in selective media to ensure the accuracy of the detection of tetracycline resistance was an additional burden that could add an additional 12-24 hours to a diagnostic turnaround in the context of rapid diagnostics, but a two-day turnaround is still far shorter than the two-week turnaround that is expected of current NHS services. Tetracycline resistance mediating genes such as *tet*M share high levels of homology not just within species but also between species, as shown in Figure 3-4. A high level of homology can be accounted for in molecular assays such as the SpeeDx MG+parC assays that detect resistance-mediating point mutations, but this would require assay optimisation beyond the scope of this work's timescale.

In conclusion, I have demonstrated the both the reliability and speed of the molecular detection of the four urogenital mollicute pathogens chosen as the focus of this work, along with determining the incidence rates of relevant resistances to tetracycline, ciprofloxacin, and azithromycin. Furthermore, Figure 3-3 demonstrates the reliability of these methods for the detection and resistance profiling of *N. gonorrhoea,* as compared to traditional culture-based methods. Resistance rates were high for all relevant pathogens surveyed. Furthermore, a high degree of accuracy was demonstrated by the molecular methods when compared to traditional culture-based methods. This was apparent when these methods were used to detect both antimicrobial resistances and more broadly to ascertain the presence of these pathogens. This confirms the need for further surveillance to achieve optimal treatment outcomes, and to help control the development of antimicrobial resistances in these bacteria. Furthermore, the presence of these pathogens in symptomatic patients further suggests that while they are present only as commensals in much of the adult population, there are factors that lead to their pathogenicity in symptomatic patients that are worthy of further investigation.

4 Polymorphism Analysis of Quinolone Resistance Determining Mechanism-Mediated Resistance of Fluroquinolones in *Mycoplasma hominis*

4.1 Introduction

M. hominis as a species is naturally resistant to 14- and 15-membered ring macrolides (Krausse and Schubert 2010), due to a conserved point mutation in its 23S rRNA region (Pereyre et al. 2002). Furthermore, as a mollicute, *M. hominis* is structurally resistant to beta-lactams and cephalosporins. The remaining therapeutics for treatment are 16-membered ring macrolides, tetracyclines, and fluoroquinolones, the last of which is the family to which ciprofloxacin, levofloxacin, and moxifloxacin belong. Fluoroquinolones target the type II topoisomerases that facilitate alterations in chromosomal supercoiling necessary for transcription and DNA replication. By binding to these topoisomerases, they render the topoisomerases unable to disassociate from the DNA molecule so it cannot reform, creating wide-scale nucleotide breaks and ultimately cell death. While effective therapeutics for *M. hominis*, they can be associated with a variety of adverse side effects. These include tenonitis, tendon rupture, potential prolonged QT intervals, and, rarely, cardiac arrhythmia (Thai et al. 2021). This means that application of this treatment needs to be highly targeted and account for issues such as drug resistance. The mechanism for fluoroquinolone resistance in *M. hominis* has been established as arising from Single Nucleotide Polymorphisms (SNPs) in the Quinolone Resistance Determining Region (QRDR) of the topoisomerase genes, as best characterized for *E. coli* but also reported for *M. hominis* (Bebear et al. 1997; Bébéar et al. 1998; Van Der Putten et al. 2019). By altering the protein conformation of the resulting topoisomerases, they become immune to the disruptive effect of fluoroquinolones. As this mutation can arise with a single-point mutation, it can occur spontaneously in clinical isolates regardless of lineage, and therefore I analysed the entire *gyrA*, *gyrB*, *parC*, and *parE* genes (including the QRDR) of 72 different sequenced strains to determine the genomic mechanisms that conferred phenotypic antibiotic resistance to fluoroquinolones in these strains. I further examined fluoroquinolone-susceptible strains carrying QRDR mutations in the *gyrA* gene (but no concurrent *parC* gene) to determine if they were able to spontaneously gain a *parC* mutation after prolonged incubation with higher concentrations of levofloxacin. I also

investigated the prevalence of non-resistance polymorphisms in these key genes for *M. hominis*, to ensure they did not obfuscate my genomics-based analysis.

The main questions this chapter seeks to address are:

- What are the levels of genomic variation between *M. hominis* isolates, considering both total frequencies of SNPs and non-synonymous SNPs in particular?
- What are the levels of fluoroquinolone resistance in this cohort of *M. hominis* isolates, and what is the level of genomic variation we can observe in the genes that mediate fluoroquinolone resistance, as compared to total levels of genomic variation?
- When fluoroquinolone resistance is induced in *M. hominis* via exposure to sub-lethal levels of fluoroquinolone antibiotics, how does the genomic architecture of the mediating topoisomerase IV genes change, and can this be linked to resistance phenotypes?
- Does the development of resistance occur spontaneously, or is it in some way linked to geographic and/or genomic clades?

4.2 Results

4.2.1 Genomic Analysis

One limitation of this aspect of my work is that not all the genomes analysed here were able to be matched with a viable bacterial isolate. As such, any bacterial genome that could not have its genotype matched to a corresponding phenotype (via MIC) was excluded. Nonetheless, a total of 72 *M. hominis* isolate genomes were analysed across a wide range of geographic locations. 12 were isolated from Havana, Cuba; seven from Pančevo, Serbia; 31 from various locations in England, five from Perth, Australia; and 14 from Pontypridd, Wales. These were analysed as well as the ATCC prototype reference strains from France and the USA. Reference strain ATCC 33131 (Sprott strain) was also examined as a prototype strain. Table 4-1 lists the variations in SNP frequency between all isolates, both for all SNPs present and for just functional/non-synonymous SNPs. Of the four genes analysed, *gyr*A contained the highest number of SNPs of any of the four genes, with between 25 to 97 SNPs present in any one isolate relative to ATCC 23114. When non-synonymous SNPs were examined, *par*C contained the highest levels of variation with five to 10 amino acid polymorphisms in any one isolate relative to ATCC 23114.

4.2.2 Antimicrobial Resistance Testing

Of the 72 isolates that were initially analysed, three demonstrated phenotypic resistance to fluroquinolones (levofloxacin MIC > 2 mg/L and moxifloxacin MIC > 0.5 mg/L): U006, MH10-09, and MH15-03. All other isolates demonstrated conventional susceptibility to fluoroquinolones, and these three isolates were found to have non-synonymous mutations in both the *gyr*A and *par*C genes (Figure 4-2). However, full gene analysis also identified two isolates, DF28 and S019M, that carried the same *gyr*A mutation observed in U006 and MH15-3 (S153L; E. coli numbering S83L). They presented as susceptible to levofloxacin with MICs of 0.5 mg/L, although they both had an intermediate moxifloxacin MIC of 0.25 mg/L.

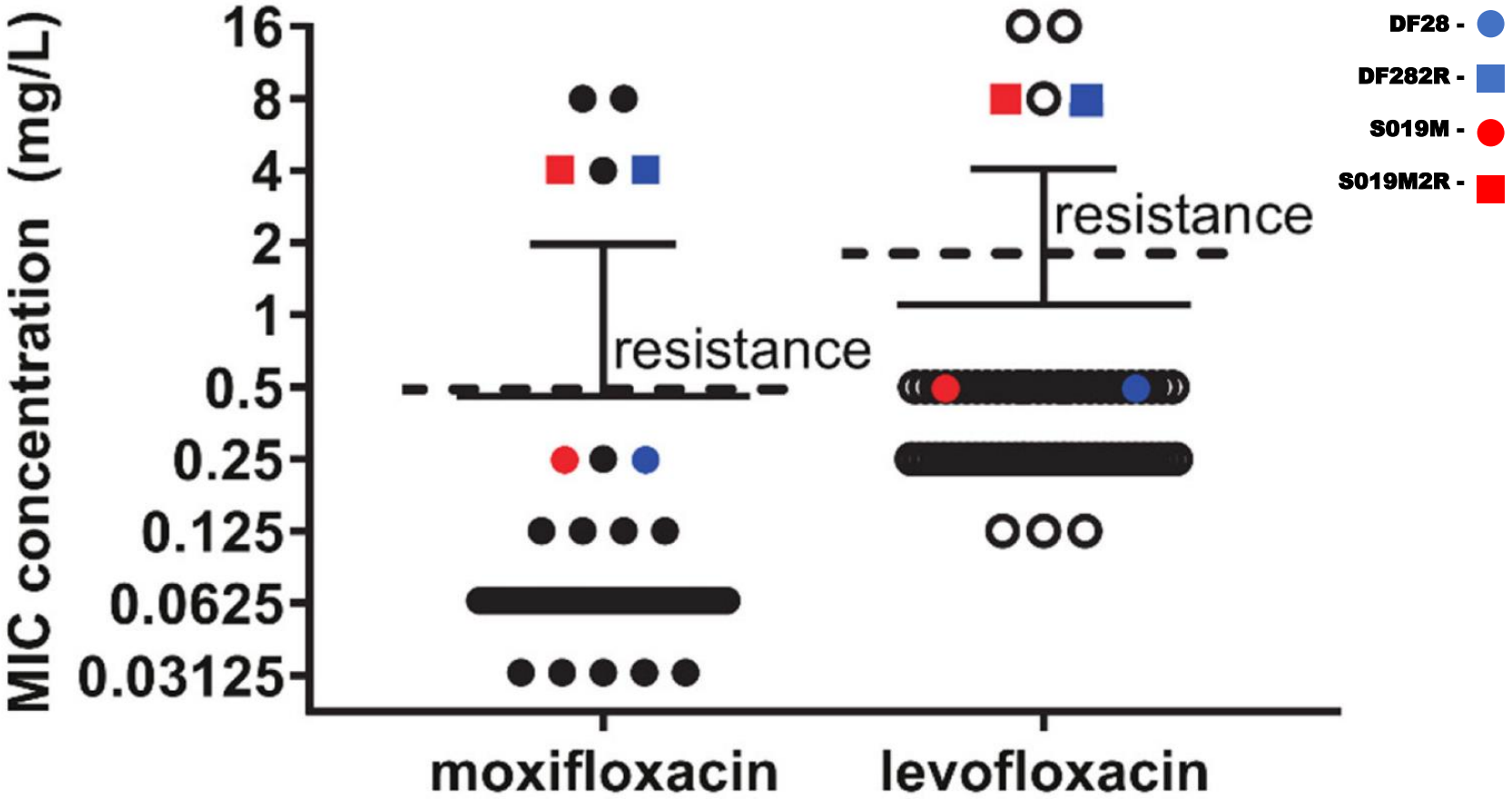

Figure 4-1 - Minimum inhibitory concentration for M. hominis isolates for levofloxacin and moxifloxacin. Internationally agreed thresholds for resistance are shown as dotted lines and isolates above the line are resistant to the respective fluoroquinolones. Isolates with QRDR *gyr*A-only mutations are shown (S019M as red circles and DF28 as blue circles), and MICs for these isolates following one step induction of resistance, resulting in an additional QRDR *par*C mutation, are shown as coloured squares (S019M2R as red squares and DF282R as blue squares).

4.2.3 Spontaneous Induction of Antimicrobial Resistance

Prolonged incubation of DF28 and S019M with levofloxacin resulted in spontaneous induction of resistance (MIC 4 or 8 mg/L for moxifloxacin and levofloxacin, respectively; Figure 2) within 24–48 h later, which was not observed for other isolates. Resequencing of these induced resistant isolates (named DF282R and S019M2R respectively) found induction of the same *par*C mutation as observed for MH10-9 S91I (S81I *E. coli* numbering; Figure 4-2). While the *gyr*A SNP of MH10-09 was observed to be shifted 12 bases downstream relative to the QRDR mutations present in other isolates, no significant difference in MICs were detected between these three isolates. It is interesting to note that while S019M and DF28 shared the observed *gyr*A mutation with MH15-3 and U006, the induced *par*C mutation aligned with that observed for MH10-9. Furthermore, U006 additionally contained an K144R mutation proposed as a source of fluoroquinolone resistance in a previous report (Zhang et al. 2019), but this polymorphism can be observed in 28 other isolates without elevated MICs in the genome cohort and therefore is clearly a non-resistance polymorphism. There can be a risk of incorrectly correlating observed genotypes with observed phenotypes, either by using an improper reference strain or automatically assuming a previously observed phenotype must automatically correlate with the genotype observed with the original observation. Regarding *par*C QRDR mutations, this appears to be the dominant determinant for levofloxacin and moxifloxacin resistance as only those strains carrying either S91I (*E. coli* numbering S81I) or E95K or V (E. coli numbering E85K or V) were phenotypically resistant. The *gyr*) mutation S153L is only capable of mediating an intermediate moxifloxacin MIC = 0.25 mg/L when present alone (Figure 4-1). A full breakdown of point mutations can be found at Appendices A1-A4.

4.2.4 Phylogenetic Variation of Resistant and Susceptible Isolates

As demonstrated by Figure 4-3, phylogenetic variance between resistant isolates varied significantly. DF28 and DF282R did not cluster together with S019M and S019M2R even though both pairs of isolates were a product of spontaneous resistance induction. The naturally occurring resistant isolates (MH10-09, MH15-03 and U006) were spread throughout the phylogenetic tree, indicating a low chance of resistance being shared along a subgroup or to represent clonal expansion. A full MLST-based phylogenetic analysis was not possible as the sequence quality of some of the 74 isolates was not sufficient, but an incomplete MLST-based phylogenetic analysis was undertaken and the resultant phylogenetic tree can be found at Appendix Figure 8-1. A more detailed list of (Non-)Synonymous SNPs can be found at Appendix Table 8-7.

Table 4-1 - A summary of the ranges of SNP frequencies observed across the topoisomerase genes of each of the seventy-four isolates analysed.

Gene	Total SNPs	Non-Synonymous SNPs
gyrA	25-98	2-6
gyrA QRDR	0-4	0-1
gyrB	1-34	0-3
gyrB QRDR	0-2	0
parC	3-63	5-10
parC QRDR	0-4	0-1
parE	14-62	1-6
parE QRDR	0-4	0

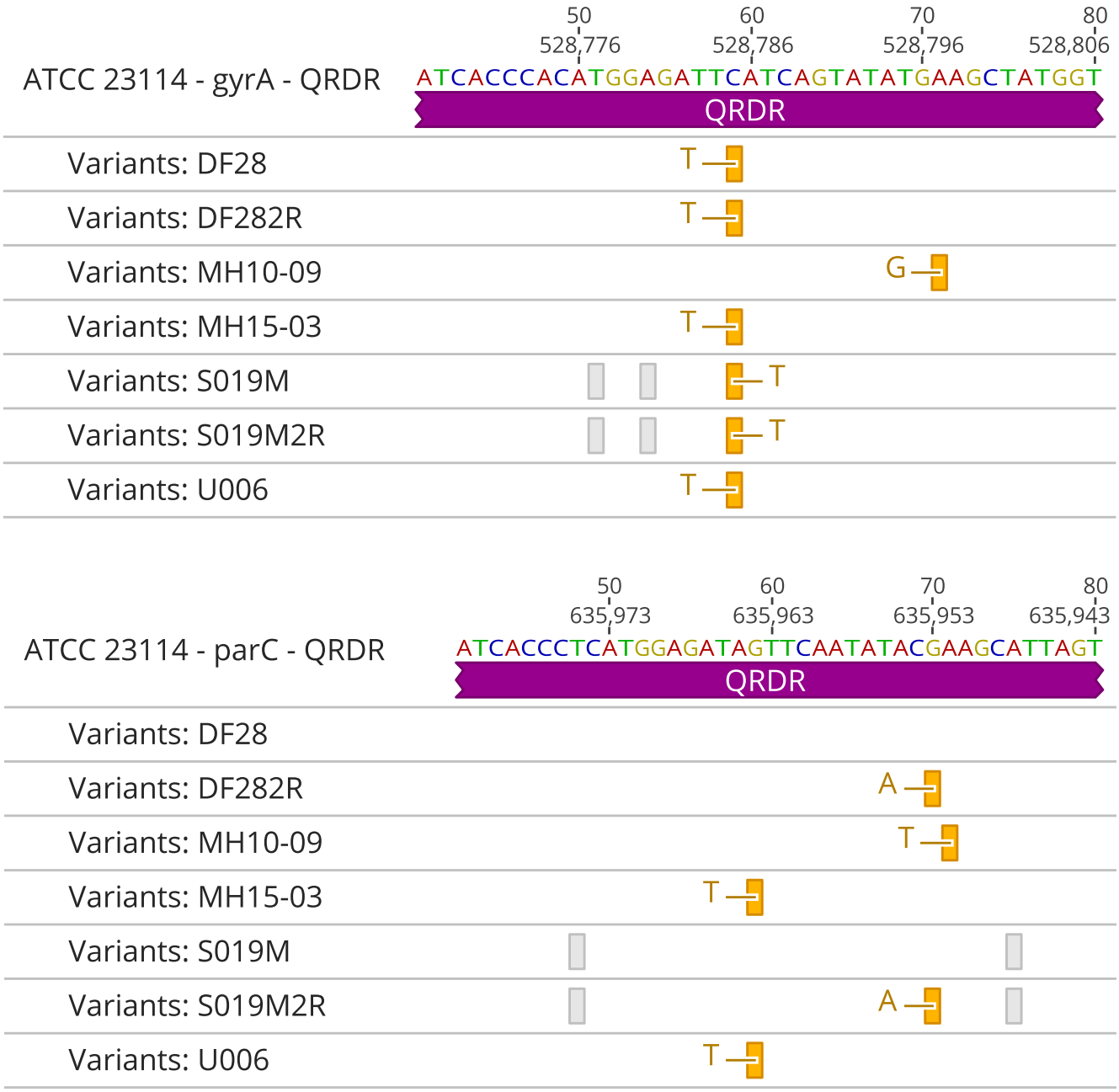

Figure 4-2 - The single nucleotide polymorphisms (SNPs) of *gyr*A and *par*C genes found in the QRDR regions of all strains analysed. Orange lines represent functional or non-synonymous mutations. No functional mutations were found in the QRDR regions of *gyr*B or *par*E.

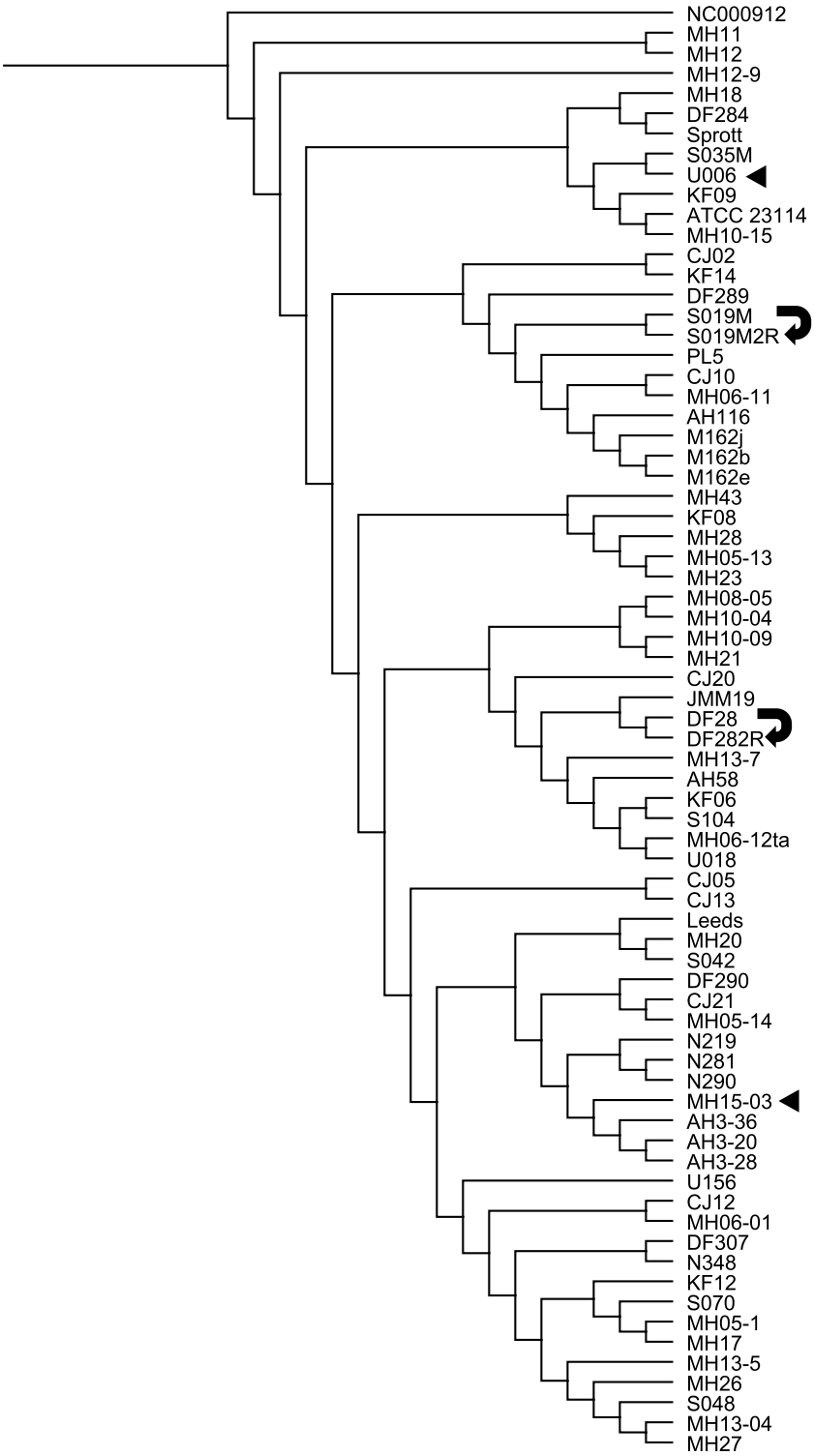

Figure 4-3 - A phylogenetic tree of 72 isolates analysed, with two induced resistant strains linked by arrows. Phylogenetic tree was constructed via concatenations of all four topoisomerase genes (*gyr*A, *gyr*B, *par*C and *par*E. The outgroup used was *Mycoplasma pneumoniae* M129, listed on the tree under its accession number (NC000912) and marked with a red circle. Naturally occurring resistant strains are identified by arrowheads. Induced mutant resistant strains are identified by curved arrows from parent to daughter strain.

4.3 Discussion

The most prominent finding of this work was that while a single mutation in the *gyr*A QRDR was found associated with susceptible fluoroquinolone MICs, resistance was found to require an additional *par*C QRDR mutation. This is not a definitive claim however, as no isolates with an isolated *par*C QRDR mutation were identified. As such, I could only observe isolates with the pre-existing *gyr*A QRDR mutation spontaneously developed induced resistance in my work. Resistance against levofloxacin and moxifloxacin was examined, despite the fact these are rarely used to treat sexually transmitted infections (ciprofloxacin or ofloxacin being more frequently prescribed). This is as the only available internationally agreed thresholds for fluoroquinolone resistance determination (CLSI 2011) are using levofloxacin and moxifloxacin. However, MICs for ciprofloxacin are usually the same or slightly higher than those observed for levofloxacin. In our study, of the 72 isolates that underwent AMR testing, only 4% of isolates were identified as being resistant to fluoroquinolones. While this appears much lower than the >90% observed in Chinese studies that specifically investigated fluoroquinolone resistance rates (Zhang et al. 2019), this is consistent with those observed in a recent multi-national study on patients undergoing infertility investigation and symptomatic sexual health patients in the UK, France, and Serbia in 2019 that found resistance in only 2 out of 85 *M. hominis* isolates (Boostrom et al. 2021). Furthermore, a study examining 1000 sexual health patients in Wales identified 100 *M. hominis* isolates with no fluoroquinolone resistance (Morris et al. 2020). This cohort contained samples from a variety of geographical regions, which included all previously available, archived fluoroquinolone-resistant isolates, but were chosen because they had associated whole genome sequences, and so is not representative of a complete clinically relevant data set.

Of the three resistant isolates and two inducible isolates, there was a consistent modification of S153L of the QRDR section of *gyr*A, with MH10-09 being the exception having a E157G mutation. While not entirely uniform, the narrow band of mutation positions provides a starting point for further analysis regarding the functional effects of a mutation in this region. Mutations in the *par*C genes of these isolates were more varied, with S91I mutations observed in U006 and MH15-03, but a E95V mutation observed in MH10-09. This was further supported by the observation of variable resistance observed in S019M and DF28, which contained the exact same set of SNPs in the QRDR section of its *gyr*A region but had an E95K mutation, rather than an E95V mutation, in its *par*C gene. The earliest investigation of induced

fluoroquinolone resistance in *M. hominis* through multi-step repeated challenge of isolates with ciprofloxacin, norfloxacin, pefloxacin, and ofloxacin, by Bebear et al., only reported *gyr*A QRDR mutations (Bebear et al. 1999). However, study did not have the ability to additionally amplify and examine the *par*C gene for the same resistant isolates to see if corresponding mutations were present for that gene as well. These historical studies relied on Sanger sequencing of amplicons using primers designed against conserved regions from other bacteria, while a modern analysis such as the one I have conducted has the benefit of access to long sequence contigs from high-depth next generation whole genome sequencing.

Similar examples of co-mutations contributing to a variance of antimicrobial resistances have been observed in previous studies of *M. hominis*. Observations specifically regarding the gyrase genes in other human mollicutes have been made before (Beeton et al. 2009a; Guo et al. 2019; Yang et al. 2020b), but the variance of resistance and its corresponding regulation is, to our knowledge, a novel observation made here. More broadly, other mutations have been implicated as the source for fluoroquinolone resistance such as *par*C K144R mutation (Zhang et al. 2019), which was found in resistant strain U006, but also in 28 susceptible strains. Zhang and colleagues also reported A154T *par*C mutation as a potential resistance marker; I did not find any A154T mutations, in either the susceptible or resistant strains, which does not preclude its lack of function but determine that it is not a sole determinant of fluoroquinolone resistance. Many other polymorphisms were identified but the putative *par*E mutation A463S was not found in any susceptible or resistant isolate.

One observation that can be made is the significant heterogeneity in the reference genomes that are conventionally used for *Mycoplasma hominis*. Of the 72 isolates used, two reference strains were included, ATCC 23114 and Sprott. Although reference strains, both exhibited incredibly high levels of heterogeneity relative to each other, which further exemplifies the unusually high polymorphism prevalence of this species. Nonetheless, ATCC 23114 is an established reference genome and therefore a touchstone for comparisons between works. More broadly, QRDR-mediated resistance is a common occurrence in a variety of similar pathogens and similar amino acid changes are conserved across multiple species (which is the basis for determining the E. coli numbering equivalents). QRDR-mediated mutations have been observed in *Streptococcus spp.* (Varon et al. 1999) and *Enterococcus faecalis* (Esfahani et al. 2020), both of which has been suggested as a putative source of the original

gyrase/topoisomerase complex via non-specific horizontal gene transfer (Oliveira et al. 2017; Citti et al. 2018). The seemingly non-specific nature of horizontal gene transfer implies there may be a relationship between antimicrobial resistance development and co-infections. However, this mechanism was not supported by our study despite the high inter-isolate variability, as the topoisomerase and gyrase genes are still much more closely related to each other than to out-groups by phylogeny analysis.

As demonstrated in Figure 4-3, the genetic diversity of *M. hominis* is incredibly high. Multi-locus sequence typing schemes developed for *M. hominis* have previously reported that identical sequence types are only found when isolated from the same patients at multiple timepoints (Boujemaa et al. 2018). The highest degree of variation found was present in the *gyr*A gene (the lowest having 25 and the highest having 97 SNPs relative to ATCC 23144), What is notable from a phylogenetic standpoint is the distinct lack of interrelation between the resistant isolates, beyond the interrelatedness of induced resistance-linked isolates. This not only reiterates the highly variable nature of *M. hominis* as an organism, but also the variable nature of the resistance-determining polymorphisms I observed in resistant isolates. Antimicrobial resistance in *M. hominis* conferred via *in vitro* selection pressure is well documented (Bebear et al. 1999). However, the finding that pleiotropy observed between *gyr*A and *par*C does not seem to correlate with any sort of genomic interrelatedness means that the ability of *M. hominis* to develop fluoroquinolone resistance so rapidly could itself be considered a mechanism of resistance in tandem with the QRDR polymorphisms themselves. The ability for previously susceptible variants of *M. hominis* to develop clinically relevant levels of resistance to fluoroquinolones demonstrates the need to expand our surveillance methodologies to include the capacity for identifying these potentially resistant variants before treatment is undertaken, to prevent resistance developing against one of the few therapeutics that clinicians have left to combat this pathogen.

5 Conjugative Element Analysis of *tet*M in *Mycoplasma hominis*

5.1 Introduction

Treatment options for *Mycoplasma hominis* are limited as the lack of a typical bacterial cell wall renders drugs such as beta-lactams (e.g. penicillin) ineffective, and the nucleotide scavenging of *M. hominis* excludes antifolates and trimethoprim (Waites et al. 2005). *M. hominis* is inherently resistant to rifampin and macrolides due to amino acid substitutions in the beta-subunit of their RNA polymerase complexes and sequence polymorphisms in their 23S rRNA gene, respectively (Pereyre et al. 2002). The remaining effective therapeutics include lincosamides, ketolides, tetracyclines and fluoroquinolones, which inhibit the protein synthesis mechanisms or DNA replication of the bacteria (Nguyen et al. 2014). Resistance to tetracycline-based antibiotics is mediated in *M. hominis* via *tet*M, which encodes for Tetracycline resistance proteins that produce a non-covalent modification of the host ribosome (Connell et al. 2003), rendering tetracyclines ineffective as a treatment. The Tn*916* transposon structure that carries *tet*M has been reported by Roberts and Kenny (1987) to be able to be conveyed by horizontal gene transfer from *Enterococcus faecalis*, a phenomenon not exclusive to *M. hominis* (Poyart et al. 1995). While these studies underpin our understanding of the mobility of the Tn*916* transposon, they pre-date the modern molecular techniques now available to study this mechanism of resistance, and as such require further investigation to better understand the underlying mechanisms of transposon-mediated resistance in this pathogen.

To identify possible mechanisms of *tet*M horizontal gene transfer, I undertook an investigation of archived *M. hominis* strains submitted to Public Health England (PHE) between 2005 and 2015 which were kindly made available to me by PHE. As part of this work, antibiotic susceptibility testing (AST) was performed to assess the antibiotic resistance rates amongst these isolates, the genome sequencing of these isolates and the genomic interrogation of subsequent data was also undertaken to analyse the underlying mechanisms of resistance observed and bioinformatic interrogation, to fully characterise the variability in the *tetM*-containing ICE.

In summary, the primary questions this chapter sought to address were:

- What are the incidence rates of samples submitted to PHE, and specifically what are the levels of tetracycline resistance in these strains of *Mycoplasma hominis*?
- What is the genomic mechanism of tetracycline resistance within these *M. hominis* isolates, and does any genomic variation in the Tn*916* transposon lead to a linked variation of tetracycline resistance?
- What is the mechanism for the development of tetracycline resistance in *M. hominis*? Does the Tn*916* transposon retain mobility between bacteria, or is the genetic element non-mobile and instead transmitted through clonal inheritance?
- Does *tet*M-mediated tetracycline resistance in *M. hominis* extend to novel variations of the tetracycline-family antibiotics such as tigecycline? Would the usage of such a variant still be effective as a treatment for these strains?

5.2 Results

5.2.1 Reference culture and PCR review

From 2011 to 2015, 4.7-6.7% of samples submitted to PHE for investigation of *M. hominis* infection were found to be positive for MH. The type of clinical specimens submitted for these archived positive strains are shown in Table 5-1.

Table 5-1 - Sample numbers per year, detailing detection of *M. hominis* by PCR and culture and associated antimicrobial resistance. Prior to July 2010 culture positive samples were recorded and post-July 2010 samples submitted for M. hominis investigation tested by molecular diagnostics were included. The one resistant isolate found in 2015 was a dual-resistant isolate.

Year	Culture Positive (Including referred isolates)	PCR Positive, Culture Negative	PCR Negative	PCR Positive	Total Samples	Total MH Detected (% total)	Tetracycline Resistant Isolates	Moxifloxacin Resistant Samples
2005	9	N/A	N/A	N/A	N/A	9	1	0
2006	11	N/A	N/A	N/A	N/A	11	3	0
2007	3	N/A	N/A	N/A	N/A	3	0	0
2008	8	N/A	N/A	N/A	N/A	8	0	0
2009	15	N/A	N/A	N/A	N/A	15	0	0
2010	16	N/A	94	0	94	16	1	1
2011	8	1	174	3	177	9 (5.0%)	0	0
2012	9	1	200	10	210	10 (4.7%)	1	0
2013	15	0	233	15	248	15 (6.0%)	3	0
2014	7	3	185	10	195	10 (5.1%)	0	0
2015	12	2	196	14	210	14 (6.7%)	1 tet^R/$moxi^R$	
Total	113	7	1082	52	1134	**120**	0	0

5.2.2 Antibiotic susceptibility testing evaluation on recovered viable isolates

A total of 96 of 120 archived isolates originating from 81 separate patients were successfully revived from frozen archival isolates. These were investigated in parallel for growth on inoculated plates containing a final concentration of either 2 mg/L Levofloxacin, 0.5 mg/L Moxifloxacin, 0.5 mg/L Clindamycin or 8 mg/L Tetracycline, representing the CLSI resistance breakpoints (CLSI 2011). No isolates were resistant to clindamycin. One isolate (MH10-9) from 2010 showed resistance to two separate fluoroquinolones (MIC= 8 mg/L for moxifloxacin and 16 mg/L for levofloxacin), and an additional isolate (MH15-3) from 2015 showed multi-drug resistance to both fluoroquinolones tested (MIC= 16 mg/L for moxifloxacin and 32 mg/L for levofloxacin) as well as tetracycline (MIC=16 mg/L). In total, 12/81 (14.8%) showed resistance to tetracycline distributed across the 10-year period (Table 5-1).

5.2.3 Mechanisms of antimicrobial resistance

PCR screening of all isolates identified the presence of the *tet*M resistance gene only in the 12 tetracycline-resistant isolates that grew in the presence of 8 mg/L of tetracycline, later confirmed by whole genome sequence analysis. Figure 5-1 outlines the gene composition of the ICE regions within these isolates. All ICE regions uniformly showed insertion at the 3' end of the *rum*(A) gene and ended at the hypothetical protein (relative to the reference strain Sprott, accession number NZ_CP011538). Five groups (Group I to V) were observed, characterised by genetic composition between all ICE regions.

Based on observation, five groups were devised for the isolates based on their *tn*916 transposon homology. Group I contained the six largest ICE regions (PL5, Sprott, MH13-7, MH10-4, MH06-11, MH12-9, and MH10-15) with only PL5 containing a full set of uninterrupted genes and thus the full-length ICE. Other isolates in Group I had mutations resulting in premature stop codons that truncated ORF16 (Sprott and MH06-11), ORF15 (MH10-15) or the serine recombinase gene (MH12-9 or MH06-11), precluding the mobility functions of the ICE.

Group II Integrated Conjugative Elements (ICE) had lost 4 ORFs preceding the serine recombinase and a further truncation of the multidrug ABC transporter ATP-binding protein in isolates MH06-12 and MH08-5 and truncation of ORF17 and ORF15 in MH06-12. This commonality in Tn916 truncation can be observed as a branch on the phylogenetic tree in Figure 4-3.

Group III contained the three reference isolates with different isolation dates originally collected from a single Australian patient (AH3) (Payne et al. 2016), as well as MH06-1, all of which had lost 7 ORFs from the 3' end including the serine recombinase relative to group I. Due to premature ORF termination by mutation, all three AH3 isolates also had a disrupted Tn*916* integrase, while MH06-1 retained a full integrase ORF but also a premature stop codon in the multidrug ABC transporter ATP-binding protein.

Group IV had lost all non- Tn*916*ORFs from Group I, apart from the serine recombinase, but the Tn*916*excisase and integrase were lost, thus displaying an 80% heterology of ORF7 genomic content relative to the other groups.

Group V consisted solely of MH05-14, which had lost all ORFs between the *tet*M gene and the insertion point at ORF MHOMSp_RS02665, as well as having truncating point mutations in both ORF15 and ORF16, resulting in the loss of all genes that could facilitate transfer to another genome. All *tet*M-positive strains analysed retained resistance to tetracycline irrespective of the composition of the ICE.

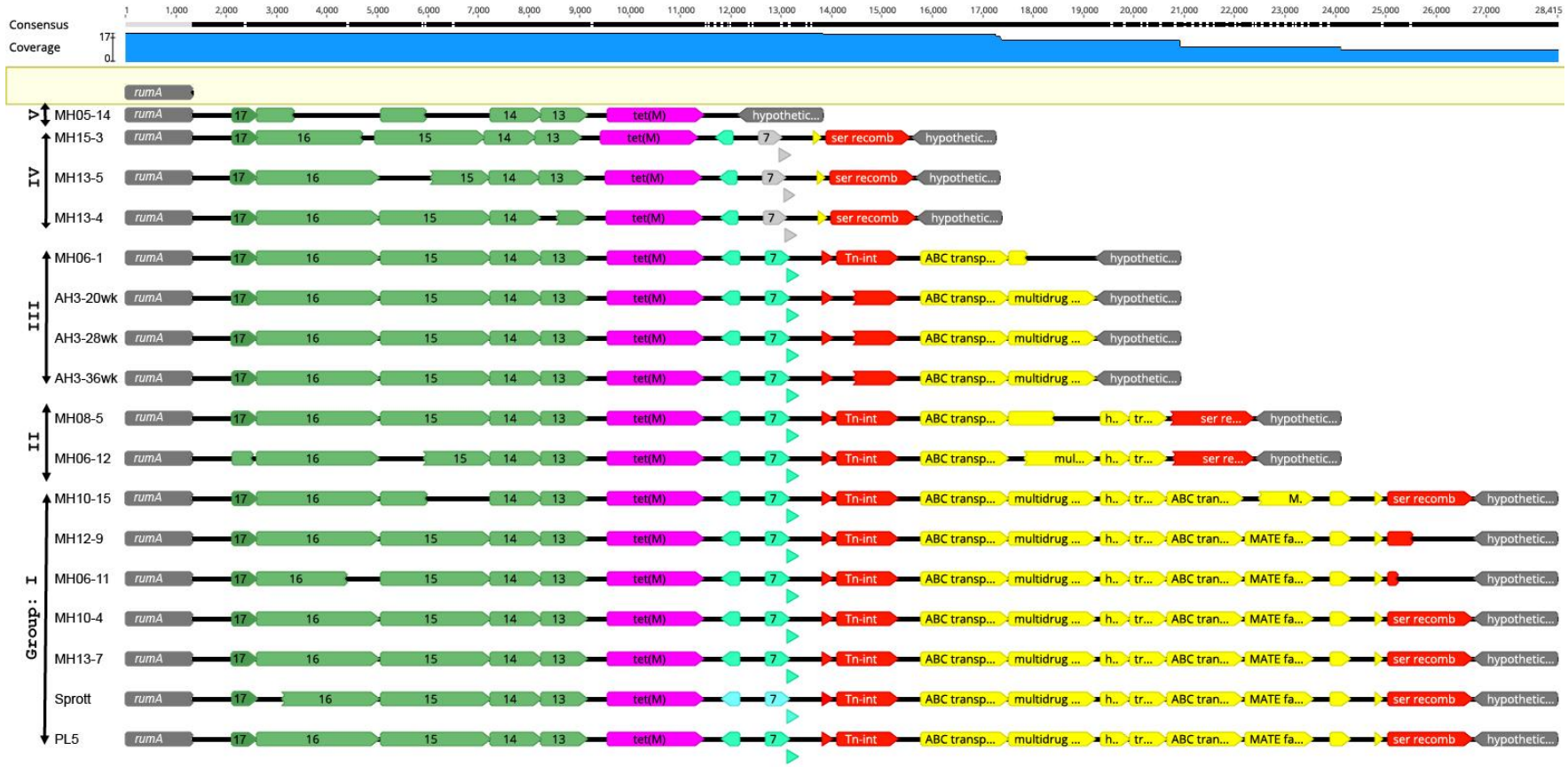

Figure 5-1 - Alignment of ICE elements carrying the *tet*M gene. Genes were aligned relative to their insertion at the 3' end of the *rum*(A) gene. Open reading frames for PL5 reference gene consist of *rum*(A); *tn*916 conjugation genes (green) ORF17, ORF16, ORF15, ORF14, ORF13; *tet*M resistance gene (pink); Tn*916*regulation genes (blue, or grey at 80% homology) ORF9, ORF7; Tn*916*excisase and integrase genes (red); accessory transporter genes from ICESpy2905 (accession number FR691055; yellow) which also includes the serine recombinase (red) at the end of the mobile genetic element. MH06-1 was absent from previous diagrams as it did not have phenotypic fluoroquinolone resistance.

5.2.4 Sequence veracity and genetic drift

The ICE analysis demonstrated a high frequency of truncation in the ORFs of ICE element genes mediated by SNPs, and as such the veracity and repeatability of my genomic sequencing methodology was investigated to ensure these stop codons were stable and not a result of aggregated sequencing errors. The resistant isolate MH06-12 was independently cultured in triplicate and the three sequences generated from each of these three separate cultures showed no sequence variation within the ICE, and only 2 SNPs external to the ICE on the contig were found (a G to T transition synonymous mutation located 21.9 kb 5' and an additional T added to a poly-T intergenic stretch 22 kb 3' of the ICE element), demonstrating high reproducibility of the SNPs identified within the ICE between isolates.

The sequence variation from Australian antenatal screening isolates (AH-3) taken at 20-, 28- and 36-weeks' gestation from the same patient were additionally analysed to further assess sequence veracity but also determine the level of genetic drift in this region of the genome. No SNPs were observed within the ICE and 5 SNPs were observed within the entire 114,794 bp contig containing *tet*M: A31,843G altering Ile642 in the exodeoxyribonuclease V subunit alpha (20 week isolate) to Val642 (28 and 36 week isolates); C53,551A truncates a hypothetical open reading frame position 181 of 284aa hypothetical protein (36 week isolate); variation in an intergenic poly-T region from 19 T and 21 T (28 week), 20 T (36 week); and T114,552C resulting in synonymous codon polymorphism for hypothetical protein MHOMSp_RS02740. This indicates that the rate of genetic drift for these isolates over 16 weeks was very low and negligible with regards to the ICE region of the contig.

5.2.5 Anecdotal evidence for ICE Mobility

The earliest description of the *tet*M-carrying ICE in *M. hominis* (Calcutt and Foecking 2015) suggested the absence of the Tn*916* conjugation ORFs 18-24 (which include the Ftsk translocase, ArdA superfamily protein and Cro/Cl family initiation replicator ORF) potentially resulted in lack of essential elements and therefore *tet*M gene mobility. This suggestion was supported by the lack of homology between the ICE found in *M. hominis* and any other bacteria in the genomic database, barring a single group B Streptococcus isolate (GB00555, accession number NZ_ALTN01000021) that retained ORFs 18-24. In order to explore the veracity of this suggestion, a genomic analysis of the major surface protein (variable adherence antigen or VAA) type for all *tet*M carrying ICE and other UK and Australian *M.*

hominis isolates was undertaken in line with a methodology used in an earlier study (Brown et al. 2014), to determine if *tet*M was restricted to a single VAA type (Table 5-2; Listed as coloured circles in 5). A more intensive examination of ICE(+) strain clustering compared to ICE(-) strains was performed by neighbour-joining tree constructions of concatenated multi-locus sequence typing loci (Figure 5-3) using previously defined gene targets (Jironkin et al. 2016).

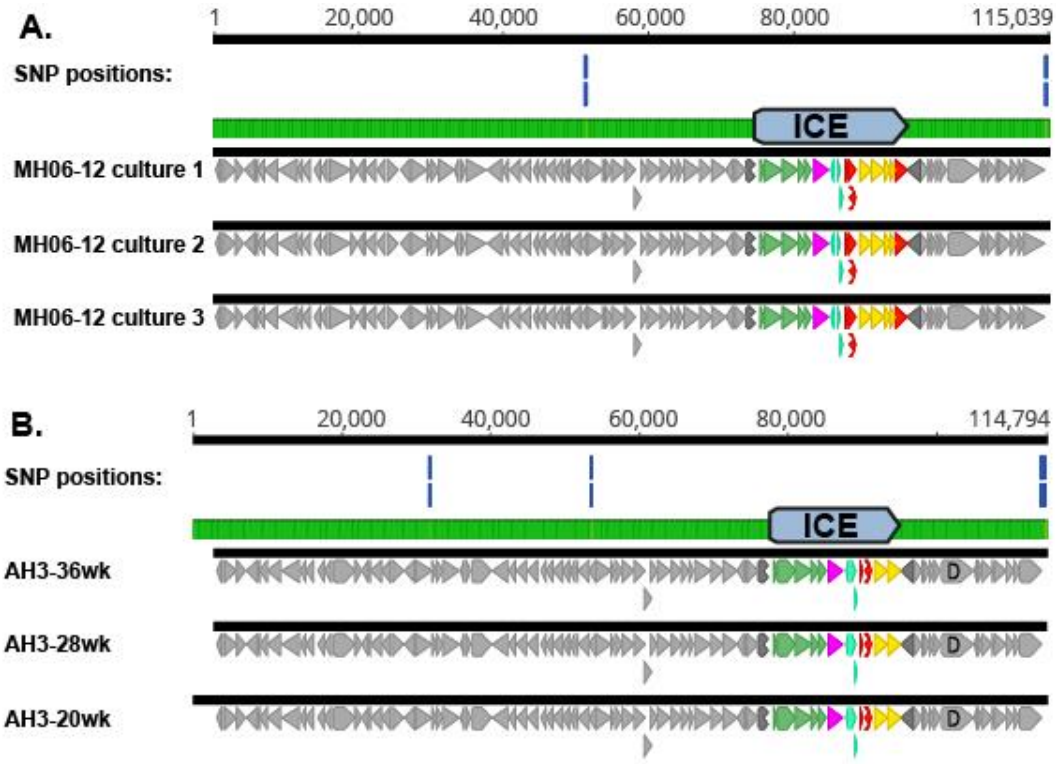

Figure 5-2 - Nucleotide alignments for the contigs containing the *tet*M gene showing SNP locations. These SNPs were identified when sequencing the same strain (MH06-12) three independent times (A) and three independent isolations (AH3-20wk, AH3-28wk and AH3-36wk) of *M. hominis* from the same patient at 20-, 28- and 36-weeks' gestation (B).

Table 5-2 - Variable Adherence-associated Antigen (VAA) typing for given strains. Strains detailed in **bold** contain *tetM*.

VAA Category	Total No. of Strains	No. of *tet*M positive strains	No. of *tet*M negative strains	Strain Identifiers
1	11	5	6	**MH13-7, MH05-14, AH3-20, AH3-28, AH3-36**, MH05-1, MH05-2, MH08-14, MH09-14, MH12-6, MH15-9
2	12	11	1	**MH06-1, MH06-12, MH12-9, MH15-3, MH06-11, MH10-4, MH10-15, MH13-4, MH08-5, PL5, Sprott**, AH58-bl
4a	1	0	1	MH05-13
4b	2	1	1	**MH13-5, MH**13-14
Novel	1	0	1	MH10-9 (modules I, II, II', IV, V, VI)
Total	27	17	10	-

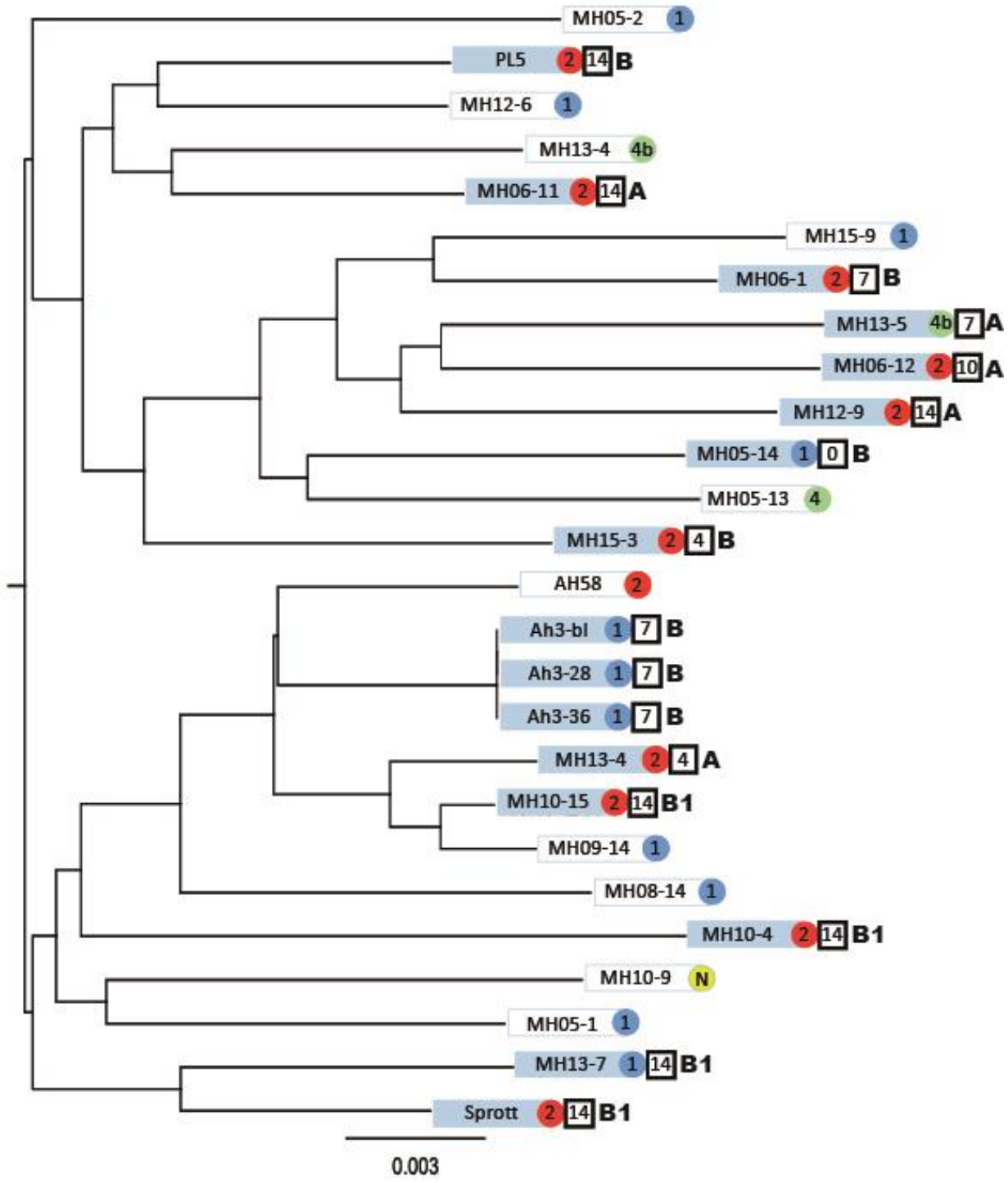

Figure 5-3 - Phylogenetic analysis of MLST genes. Phylogenetic analysis was conducted for strains with *tet*M (light blue box) relative to strains without (white box). Listed are the typing of major surface antigen (VAA) is shown next to each isolate with the VAA type (blue circle = type 1, red circle = type 2, green circle = type 4 or 4b (due to 1 or 2 copies of module III, respectively), yellow circle = novel VAA type), the total number of ICE genes (excluding *tet*M) and then the conserved SNP variation as per previous identifications in the literature (Mardassi et al. 2012), indicated as the last entry per line for type "A", "B" and sub-variant "B2".

ICE-carrying isolates spanned three separate VAA types, suggesting potential to move between lineages or loss from specific lineages due to selection pressures. Most notably the three strains lacking the serine recombinase were present in two different VAA types (MH05-14 and AH3, VAA-1; MH06-1, VAA-2). Group IV (retaining serine recombinase, but missing Tn*916*excisase and integrase) were also present in two different VAA types (MH15-3 and MH13-4, VAA-2; MH13-5, VAA-4b). While most ICE (+) isolates coincided with VAA-2, there was enough distribution across the different VAA types to suggest genomic mobility or sufficient selection pressures to select against the ICE: the ICE element distributed across the entire tree co-locating to each other rather than to ICE (-) VAA-2 isolates or to ICE (+) isolates of alternative VAA types. Early branching of fluoroquinolones and tetracycline resistant MH15-3 was noted suggesting dual resistance may have arisen early in the strain's evolution.

Mardassi *et al.* (2012) noted the existence of *tet*M sequence types based on conserved SNPs within the resistance gene at nt positions 593, G789A, T807C, C819A, G825A and G831A. 6/13 of the *tetM* genes analysed here match the proposed sequence type (type A *tet*M) by these authors (Figure 5-4) and while phylogenetic analysis of the isolated *tet*M gene confirmed that they clustered separately from type B *tet*M, they did not aggregate to a single VAA type; however all but one (MH13-5) were found in the top half of the MLST phylogenetic tree (Figure 5-5). Within the *tet*M genes lacking these conserved SNPs (type B *tet*M), a subtype with the conserved single SNP at nt position C839T (type B *tet*M) all clustered together on the gene phylogenetic tree. All Type A tetM containing strains co-located to the top half of the MLST phylogenetic tree (Figure 5-5), while all type B2 *tet*M containing strains collocated to the bottom half of the MLST phylogenetic tree, with type B *tet*M distributed equally across the tree. Identical grouping of strains from the same patient (i.e., AH3-20, 28 and 36) was noted. Overall, no defined lineage or common ancestry was observed between the isolates analysed here, accounting for the homology of *tet*M-positive strains relative to the *tet*M-negative strains.

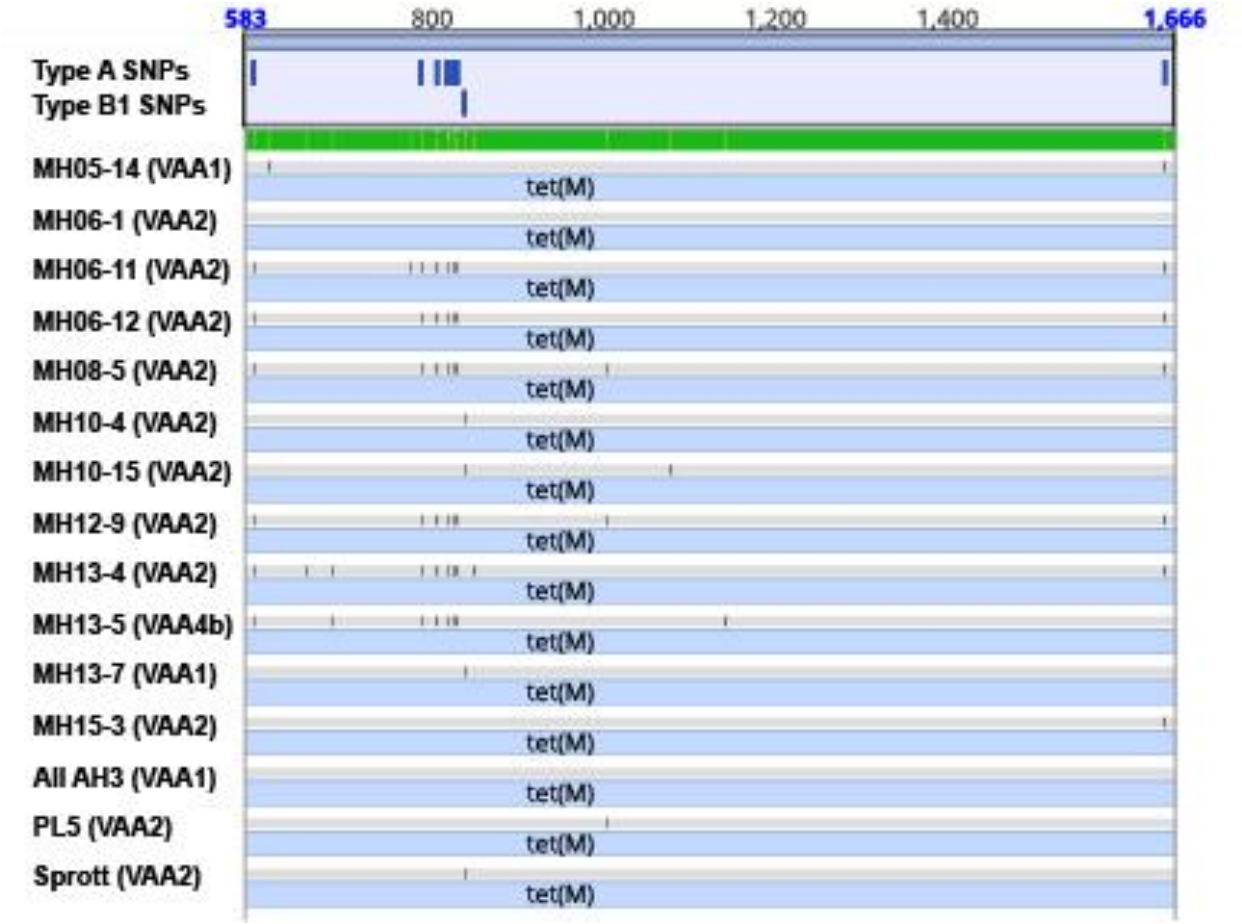

Figure 5-4 - Alignment of *tet*M genes showing conserved SNP variants. A threshold of >0.2 prevalence was used for *tet*M-type A (06-11, 06-12, 08-5, 12-9, 13-4 and 13-5) relative to the remainder that represent *tet*M-type B. Grey dashes indicate non-synonymous single nucleotide polymorphisms – tetM function was retained across all isolates.

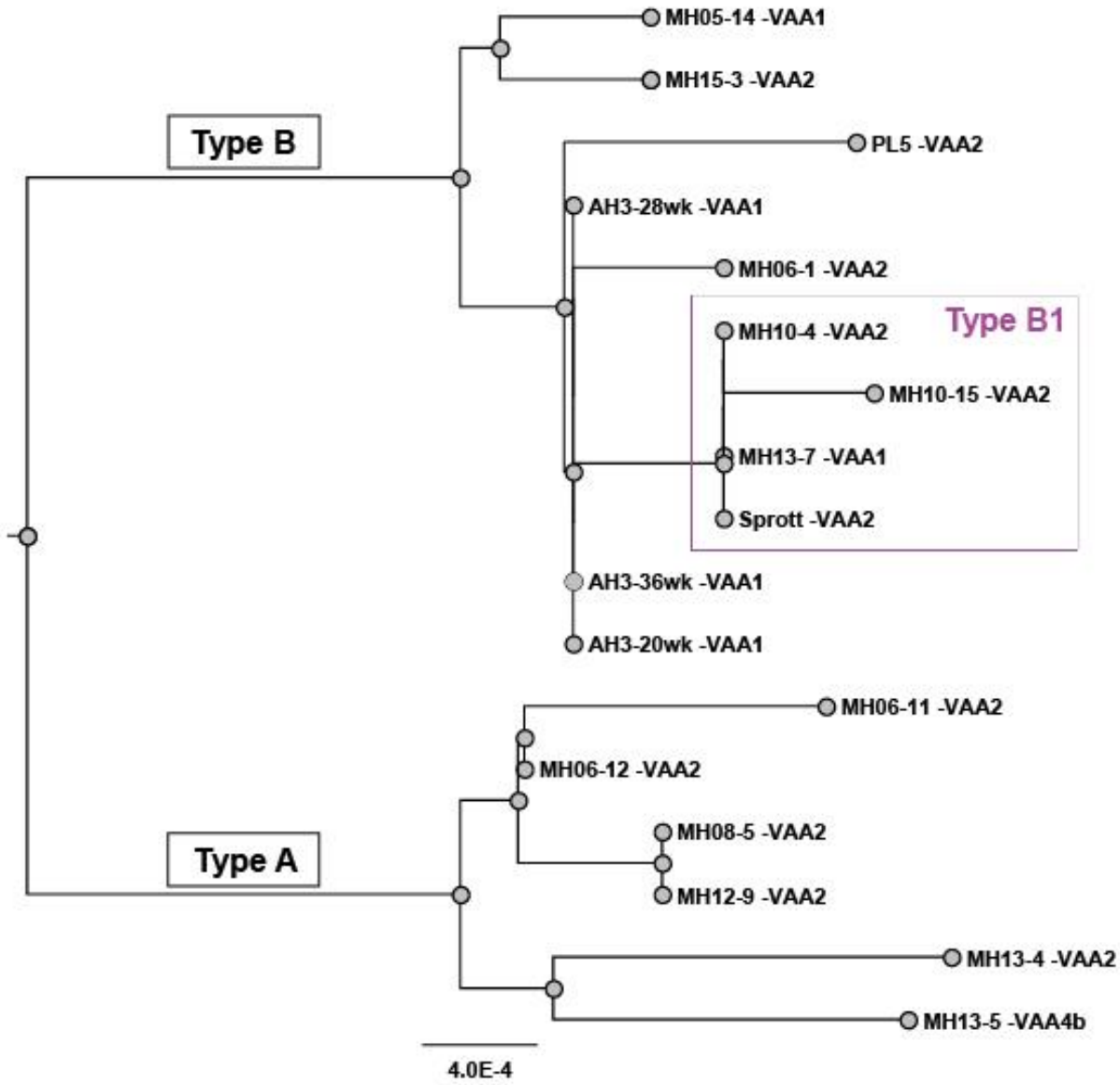

Figure 5-5 - Neighbour-joining phylogenetic analysis of the entire *tet*M gene between strains. Those with conserved SNPs distinguishing between *tet*M-type A and *tet*M-type B cluster separately, but no co-clustering of VAA type was seen.

5.2.6 Alternative therapies for tetracycline and multi-resistant strains

Agar-based resistance screening identified 12 tetracycline-resistant strains and 2 fluoroquinolone-resistant strains (one strain had combined resistance). Antimicrobial resistant testing on these 13 strains and 17 susceptible isolates was performed and their susceptibility was separated by *tet*M carriage as per Figure 5-6. The CLSI resistance breakpoints are shown as dotted lines, and the fluoroquinolone isolates (MH10-9 and MH15-3) are indicated on the levofloxacin and moxifloxacin graphs. Isolate MH15-3 was only susceptible to clindamycin. To examine alternative therapeutics, MIC values were also determined for these isolates against tigecycline, a third generation (glycyl)tetracycline to determine if *tet*M mediated an elevated MIC for this antimicrobial. No difference in MIC was observed for isolates with or without *tet*M. Furthermore, despite *M. hominis* inherent resistance to 14- and 15-membered macrolides, susceptibility to josamycin (a 16-membered ring macrolide commonly used to treat infections in France, Italy, Spain, and Russia) was also examined. The MIC for josamycin was 0.25 +/- 0.14 mg/L irrespective of *tet*M presence (as anticipated), demonstrating that the therapeutic options beyond clindamycin are available (i.e., josamycin and tigecycline) for multi-resistant *M. hominis* strains such as MH15-3.

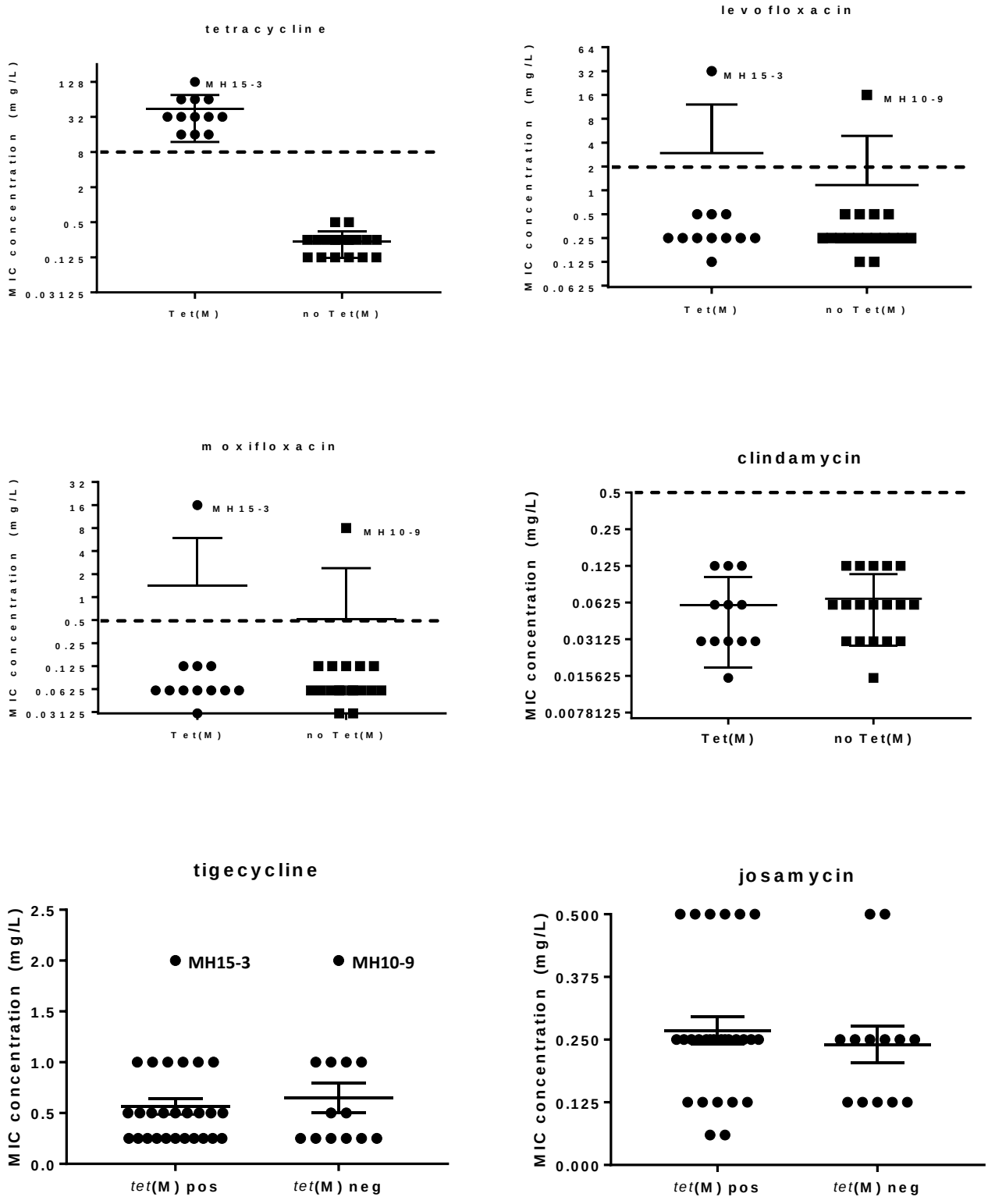

Figure 5-6 - Antimicrobial susceptibility testing for 40 isolates. Data is of 13 *tet*M-carrying and 27 randomly selected susceptible controls) for antibiotics with CLSI-defined resistance thresholds, along with for glycyl-tetracycline tigecycline and macrolide josamycin. No breakpoints have been assigned for these latter two antimicrobials at time of writing, so a more detailed range of breakpoints were tested instead of the usual two-fold dilutions seen in the preceding four antibiotics.

5.3 Discussion

Lincosamide, tetracycline and fluoroquinolone susceptibility testing of recovered isolates from 2005-2015 (67% of the total received) showed tetracycline resistance was the most common (12/81; 14.8%) followed by fluoroquinolone (2/81; 2.4%). One isolate present in the cohort (MH15-3) was resistant to both tetracycline and fluoroquinolones, leaving only clindamycin as the hypothetical therapeutic option for this strain. It is difficult to compare resistance rates across countries as CLSI international guidelines were only published in 2011 (CLSI 2011) and few contemporary studies were completed prior to this. Reports of tetracycline resistance in the international literature range from 0-58%, fluoroquinolone resistance from 0-94% and clindamycin resistance from 0-30%, ultimately making a geographical comparison impossible to make with any statistical weight. A greater and more prescient difficulty arises from the use of the range of commercial assays available, the majority of which do not conform to the CLSI thresholds for resistance breakpoints or utilise antimicrobials (e.g., ciprofloxacin and ofloxacin) with no current breakpoint determined. This is a problem that has previously been highlighted for susceptibility testing in *Ureaplasma* spp., (Beeton and Spiller 2017) and while these lyophilised commercial assays are convenient for screening, very few reports confirm resistance by determining the underlying mechanisms of resistance and as such are of limited relevancy to a clinically-focused analysis of antimicrobial resistance and its detection. It was found that all tetracycline resistance was mediated by the resistance gene *tet*M, and did not find any isolates with *tet*M that were tetracycline-susceptible as has been reported for rare *M. hominis* strains elsewhere (Meygret et al. 2018).

When MLST schemes for *M. hominis* were being first developed, inter-strain diversity was unusually high – to the point where each strain was presumed to have a unique ST unless it was isolated from the same patient (Jironkin et al. 2016). It was based on this observation that the source of failed lung transplants caused by *M. hominis* infection in a clinical cohort were able to be traced back to the original asymptomatic donor (Sampath et al. 2017) whom had right and left lobes transplanted into different recipients – both of which failed due to *M. hominis* infection.

More recent MLST schemes have extended to including surface antigens vaa, p120', p60, lmp1 and lmp3 to segregate isolates isolated from individuals with infertility from isolates from patients with gynaecological infections (Boujemaa et al. 2018) but these enhanced

MLSTs still show wide varieties of individual ST assignments. One would therefore expect SNP acquisition rate to be high, but when the rate of change was calculated in an isolate that was grown, sequenced, and analysed in triplicate, comparison of the 115kb ICE contig showed only two SNPs – one variation of a single T in an intergenic poly-T region and one synonymous mutation in an open reading frame. Therefore, short differences in passage history *in vitro* do not seem to result in significant sequence variation. Moreover, the changes in sequence from three isolates each obtained 16 weeks apart from another via an antenatal screening also only showed five SNPs over 112kb, two in intergenic mono-polynucleotide stretches and three additional SNPs. Comparison of the five longest contigs between the AH3 isolates collected at 20 weeks' and 36 weeks' gestation covered 537,152nt and showed 18 genomic variations accounting for approximately 76 SNPs per 700 kb genome per year, which would not account for the wide diversity in MLST profiles observed between individual isolates. Therefore, as it is more likely that the high diversity observed in these isolates has been produced by sporadic high genetic alteration caused by antimicrobial exposure akin to how fluoroquinolones are known to induce SOS-mediated rapid genomic mutation (Escudero et al. 2015), immune pressure or adaptation during the initial infection of a patient following transmission (Bottery et al. 2017).

The first observation I was able to make when undertaking an analysis of ICE regions of *tet*M positive strains was the degree of gene content disparity between isolates. Isolates ranged from having ICE regions consistent with the reference Sprott strain to having highly truncated regions, with almost all the isolates (except 13-7) containing SNP-mediated truncation of at least one ORF. Surprisingly, the reference strain Sprott was observed to contain a SNP in ORF16 leading to a truncation. PL5, the only other reference strain available did not have any truncations and had the same ICE gene contingent as originally reported for Sprott. The overall lack of homology between *tet*M positive and negative strains indicates a non-clonal method of *tet*M acquisition, with horizontal gene transfer (HGT) being the most likely avenue. Meygret et al. reported the presence of other mycoplasma-specific ICEs (MICE) in *M. hominis* (Meygret et al. 2019) although the essential consensus sequence SSLSDFDKTPTPKLDSKVINEYN is missing from the *tet*M carrying ICEs of all the isolates available for analysis and furthermore were not present anywhere in the rest of the genome for any of the *tetM* positive isolates. Meygret et al. also reported that there were no antimicrobial

resistance genes associated with these reported MICE, which further conflicts with the observed presence of *tet*M-mediated tetracycline resistance. In related research, mini-transposons have been used, albeit artificially, to deliver tetracycline resistance genes (Rideau et al. 2019). This is further backed by the frequent occurrence of HGT that occurs in mollicutes and prokaryotes in general (Johnson and Grossman 2015) and the phenomenon's ability to confer antimicrobial resistance (Bottery et al. 2017) as well as the presence of genes in the ICE region of *M. hominis* such as the aforementioned serine recombinase and Tn*916*integrase, and including ArdA, a known facilitator of gene mobility (Melkina et al. 2016).

Integrases are a mechanism for horizontal gene transfer, whereby this family of genes can regulate not just the insertion but also the excision of gene cassettes (Escudero et al. 2015). Integrases are suspected to originate from genomic insertions by bacteriophage (She et al. 2004) and they are a common facilitator of genetic adaptation and evolution in a wide range of pathogens (Engelstädter et al. 2016). In particular, the integrase in the *tet*M ICE region detailed here is part of Tn*916*, a categorised transposon cassette, but notably lacks ORFs 18-24 (but notably retains critical VirB4 and transmembrane segregation mediating ORFs), which have been found to mediate the conjugation of the transposon. Wright & Grossman (2016) noted the presence of tetracyclines stimulated the expression of these ORFs, which encoded for the self-excision of Tn*916*. Genetic drift in sub-inhibitory levels of tetracycline could explain the loss of these genes, as well as genetic damage during or after insertion.

The other main component of the ICE region previous reported by Calcutt and Foecking (Calcutt and Foecking 2015) is a serine recombinase common to the ICESp2905 (originally described as a *tet*(O)- and *erm*(Tr)-carrying ICE) identified in *Streptococcus pyogenes*, but identified as a common ancestor to ICEs across streptococci (Giovanetti et al. 2012). Serine recombinase elements in streptococci mediate the expected site-specific insertion into the 3' end of the *rum*(A) gene observed in *M. hominis* here, consistent with most mobile genetic elements targeting specific hotspots of bacterial genomes (Ambroset et al. 2016). The main difference between the Tn916 transposon in *M. hominis* and other pathogens is the usage of Opal codon, which instead is well-established to encode for Tryptophan in mycoplasma (Yamao et al. 1985). This is vitally important within the context of a transposon, as it may explain the lack of mobility seen in mycoplasma tn916 transposons as compared to similar transposons observed in *E. coli* (Babakhani and Oloomi 2018) or *Enterococcus faecalis* (León-

Sampedro et al. 2019). The mobility of tn916 also explains dual-resistant isolates such as MH15-3, which as elaborated upon previously, most likely gained fluoroquinolone resistance through the independent mechanism of stress-mediated point mutations.

Still focusing on MH15-3, the isolate harboured inherent resistance to both 14 and 15-membered macrolides as shown in our antimicrobial resistance susceptibility analysis and in addition to resistance to levofloxacin, moxifloxacin, and tetracycline, but could be inhibited by josamycin (a 16-membered macrolide) and tigecycline (a third generation (glycyl)tetracycline). In fact, indistinguishable MICs for tigecycline were observed between *tet*M-positive and -negative isolates (Figure 6). There are issues with the practical application of both these drugs. Currently there is no published guidance in the British National Formulary for dosage and approved use of josamycin in the UK, while it is routinely used in other countries such as France (Kayem et al. 2018) or Russia (Guschin et al. 2015). Tigecycline is available for use in a UK clinical setting, but currently can only be administered IV in a hospital (Ingram et al. 2016). sharply limiting its utility.

Even when pathogens are susceptible to a wider range of tetracyclines, this is with the provision that their well-known chelating properties prevent their administration to pregnant women and neonates (Sánchez et al. 2004; Pöyhönen et al. 2017) two of the demographics often identified for treatment of *M. hominis* infections. While neonatal, pregnancy and sexual health samples accounted for 50/115 of the isolates investigated (Appendix Table 8-1) there were a significant number of surgical complication (four wound drains, four wound swabs, two surgical wounds, one heart valve and four tissue samples) and invasive infection (two knee aspirates, three pleural fluids, two peritoneal fluids, one ascitic fluid, five blood cultures, seven CSFs and two cerebral samples) where IV therapeutics would be possible as part of standard care. Furthermore, while tetracyclines are known to cause these sorts of harsh side-effects, the dosage required to arrest growth is lower than many other pathogens that are routinely treated with these drugs. The tigecycline threshold for resistance in *Escherichia coli* is 8mg/L (Babaei and Haeili 2021), well under the 2mg/L seen in the least-susceptible isolates tested, with *Acinetobacter baumannii* showing 92% susceptibility when assessed with this same threshold of resistance (Jo and Ko 2021). The presence of some resistance and the issues with administering the drug does mean I would not recommend it as a first-line treatment, but especially in cases of surgical complication it could be a valuable tool when used in

tandem with AMR testing. Considering these invasive infections and especially considering the risk of multi-drug resistant isolates such as MH15-3 that can be derived from an adult blood culture, it is imperative to further our understanding of the mechanisms behind the development of AMR and continue surveillance to monitor AMR prevalence. When also considering the resistance documented in this chapter and other studies more generally, provision of up-to-date guidance from NICE/British National Formulary on use of antibiotics for invasive *M. hominis* infection would be beneficial for neonatal patients and those invasive infection, including those with post-operative infection and for immunocompromised patients. Improved resistance testing services, studies, and surveillance on resistance for *M. hominis* would be beneficial.

6 Mechanisms of *tet*M mediated Tetracycline Resistance in *Ureaplasma spp.*

6.1 Introduction

The difficulty in developing clinically viable detection methods complicates our ability to diagnose and study Ureaplasma and as such its relevance as a pathogen is debated, but there is evidence to link it with nongonococcal urethritis and infertility (Beeton et al. 2019), along with further links to pre-term labour and chronic lung disease of prematurity in neonates (Waites et al. 2009). The structural refinement of Ureaplasma also renders *Ureaplasma spp*. immune to many forms of treatment, such as the lack of a cell wall rendering beta-lactams and glycopeptides ineffective (Beeton et al. 2016a). Of the remaining antimicrobials available for treatment, the main classes of antibiotic that are effective against Ureaplasma are tetracyclines (Meygret et al. 2018), along with fluoroquinolones and macrolides (Zhao et al. 2020). While effective at treating susceptible variants of *Ureaplasma* spp., tetracyclines have a harsh set of side-effects including chelating properties that can contraindicate their use in children (Pöyhönen et al. 2017) that can lead to the discolouration of bone and teeth (Sánchez et al. 2004; Judge et al. 2018). Due to both the growing frequency of resistance in these bacteria and the discretion required when utilizing what treatments remain, there is a growing need to understand the mechanisms and regulation of tetracycline resistance in these pathogens.

Tetracycline normally produces a bacteriostatic effect in Ureaplasma by binding reversibly to the 30S subunit of bacterial ribosomes, blocking the binding of tRNA and preventing protein synthesis, leading to cell death (Sheykhsaran et al. 2019). Tetracycline resistance is mediated in *Ureaplasma spp*. by the *tet*M gene, which functions by altering the conformation site of the 30S such that tetracyclines cannot bind to the 30S subunit, ensuring continued protein synthesis (Ahmadi 2021; Dumke 2021). In *Ureaplasma* spp., *tet*M mediated tetracycline resistance is in turn mediated via the Tn*916* transposon. The transposon functions as a mobile genetic element that independently mediates its replication and transmission between host genomes (Speer et al. 1992; Wright and Grossman 2016). Transposons are a well-known mechanism of virulence factors that can confer antimicrobial resistance to a variety of antimicrobials (Speer et al. 1992; Chopra and Roberts 2001; Rideau et al. 2019; Durrant et al. 2020), and the Tn916 transposon and its close relatives have been determined to function as both species-specific and/or cross-species mediators of resistance in a variety of pathogenic bacteria (Santoro et al. 2014; Wright and Grossman 2016; Lunde et al. 2021).

In this chapter the mechanisms of tetracycline resistance were interrogated and characterised in thirteen resistant strains containing the *tet*M gene, with 11 of these demonstrating *tet*M-mediated resistance. Two susceptible isolates contained non-functional tetM genes, with one of these isolates being induced into resistance via culture in sub-lethal concentrations of tetracycline. This interrogation focused on the Tn*916* transposon present in all thirteen isolates, an Integrated Conjugated Element (ICE) region that contained a *tet*M accessory gene. The ancillary transposon genes surrounding the *tet*M genes in these regions were further characterised, as well as how they relate to the occurrence of *tet*M-mediated tetracycline resistance observed in these isolates.

6.2 Results

6.2.1 Analysis and Detection of *tet*M

The 13 *Ureaplasma* spp. isolates (eight *U. urealyticum* and five *U. parvum*) outlined in Appendix Table 8-2 were confirmed to contain the *tet*M gene via PCR screening and full genomic sequencing, as well as 11 of the 13 isolates growing in the presence of >2mg/L tetracycline. Isolate DF28U was an outlier amongst the resistant isolates as it demonstrated an MIC of 128mg/l, two-fold higher than HPA71 and HPA111, and 64-fold higher than the other resistant isolates. A mechanism behind this abnormally high level of resistance could not be determined, but it is worth noting that all isolates with MICs above 2mg/L were derived from two cohorts of Welsh isolates, suggesting a potential geographic link. Significantly, DF145 and HPA71 did not meet the MIC breakpoint of 2mg/L despite PCR and genomic sequencing confirming the presence of a Tn*916* transposon with a *tet*M accessory gene. As such, both isolates were repeatedly challenged with sublethal concentrations of tetracycline to induce resistance, with HPA71 displaying full tetracycline resistance after 5-8 consecutive passages (repeated twice), while DF145 remained susceptible for up to 20 consecutive challenges.

The original sequence of HPA71 showed that the *tet*M gene present carried an Adenine insertion at nucleotide 814 of the gene that led to a frameshift (CAG -> TAG) nonsense mutation and premature truncation of the *tet*M gene with subsequent a loss of function and thus full susceptibility to tetracyclines. Resequencing of HPA71 following post-challenge induction of tetracycline resistance found that the extra adenine in the poly-A stretch had been lost resulting in restoration of the correct translation frame. In contrast, the original sequence of DF145 showed a deletion of a guanine nucleotide at nucleotide 1231 of the tetM accessory gene, leading to upstream frameshift mutations that truncated the gene with a TAG stop codon at amino acid 411. As resistance in DF145 could not be induced, this isolate was not re-sequenced.

Table 6-1 - Summary of 13 Ureaplasma isolates analysed in this chapter. HPA71 has two entries in this table as MIC values were initially 1 (fully susceptible) pre-selection but were 64 (resistant) post-selection when cultured in sublethal-conditions. Serovars marked with an asterisk have not been conclusively serotyped at time of writing and so all potential serovars of their species are listed.

Isolate Name	Species	Serovar	Tetracycline MICs (mg/l)
HPA71 (pre-challenge)	*U. urealyticum*	4/12/13*	1
HPA71 (post-challenge)			64
HPA111	*U. urealyticum*	4/12/13*	64
France UUg1	*U. urealyticum*	4/12/13*	2
Vancouver	*U. urealyticum*	9	2
France UUa	*U. urealyticum*	2/5/8*	2
DF034	*U. urealyticum*	4/12/13*	2
DF471	*U. urealyticum*	4/12/13*	16
DF314	*U. urealyticum*	4/12/13*	16
DF145	*U. parvum*	6	2
HPA23	*U. parvum*	6	64
Cuba 681	*U. parvum*	1	2
Cuba 211	*U. parvum*	3	2
DF28U	*U. parvum*	3	128

6.2.2 Genomic variation of Tn916 and surrounding genomic regions in *U. urealyticum*

In the eight *U. urealyticum* isolates the full Tn*916* complement was maintained, with negligible levels of genomic variation observed between isolate transposons, notwithstanding the shared variation between the reference *E. faecalis* and *U. urealyticum tet*M genes. Excluding the Vancouver reference strain, all seven other *U. urealyticum* isolates demonstrated an elevated level of homology both upstream and downstream of Tn*916* demonstrated in Figure 6-1 suggesting a precise and consistent insertion point.

Regarding genomic content immediately up- and downstream of the Tn*916* transposon, UUg1 and Vancouver demonstrated genomic heterogenicity from the other six *Ureaplasma urealyticum* isolates. UUg1 contained a much shorter length of DNA due to the deletion of a length of genome containing an S8 family peptidase and ATP-binding protein. Another notable variation shown in Figure 6-1 is the insertion of a highly irregular 8bp length of DNA (TGAACTGG) after amino acid 157 of the ORF, leading to a frameshift mutation at amino acid 177 causing a TAG stop codon to truncate the gene.

Uncharacteristically for a reference strain, the genome of Vancouver was markedly different in composition from the other seven isolates seen in Figure 6-1, with a highly heterologous DNA composition upstream of Tn916 as well as the downstream inversion of a 30.4kbp length of DNA including the DEAH/DEAD box helicase, a gene vital for regulation of nucleotide topology in the host cell. The heterogenicity of Vancouver when compared to isolates of the same species across different geographic locations and serovars is unusual for a reference strain and calls into question the usage of Vancouver as a reference for future genomic analysis.

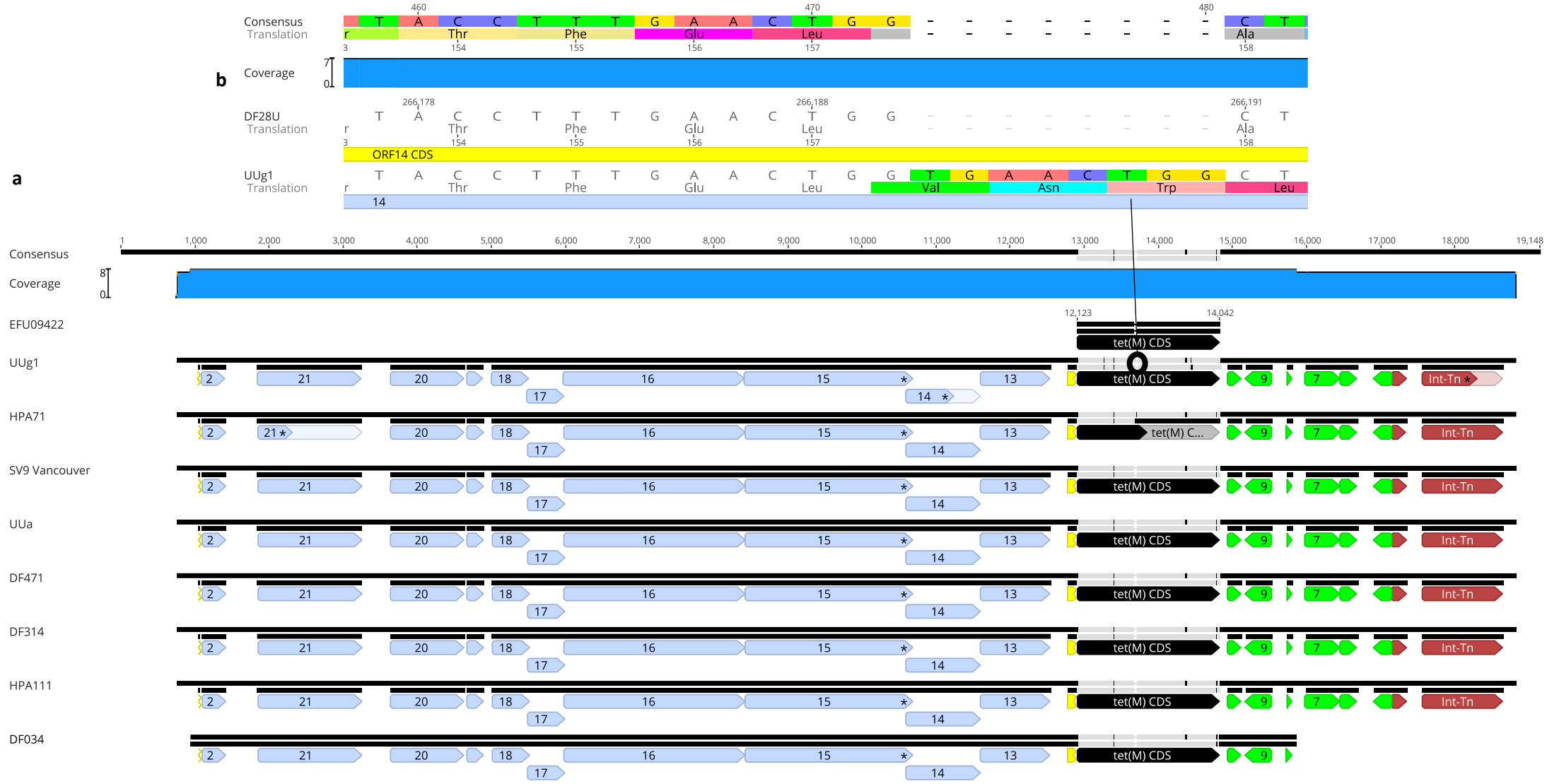

Figure 6-1 – An alignment of the Tn916 transposon of all eight *Ureaplasma urealyticum* isolates. Section **(a)** is an alignment of isolates mapped against the *tet*M gene of a reference strain of Enterococcus faecalis DS16 (Accession no. EFU09422). Asterisks indicate the position of a stop codon leading to a gene truncation. Section **(b)**

Figure 6-2 – Insertion sites for the Tn*916* transposon in *U. urealyticum*. Aligned relative to the common *tet*M gene, the DEAH/DEAD box helicase is marked in red, and the common insertion site is marked with purple arrows, demonstrating the inversion of genomic content present in the Vancouver reference strain.

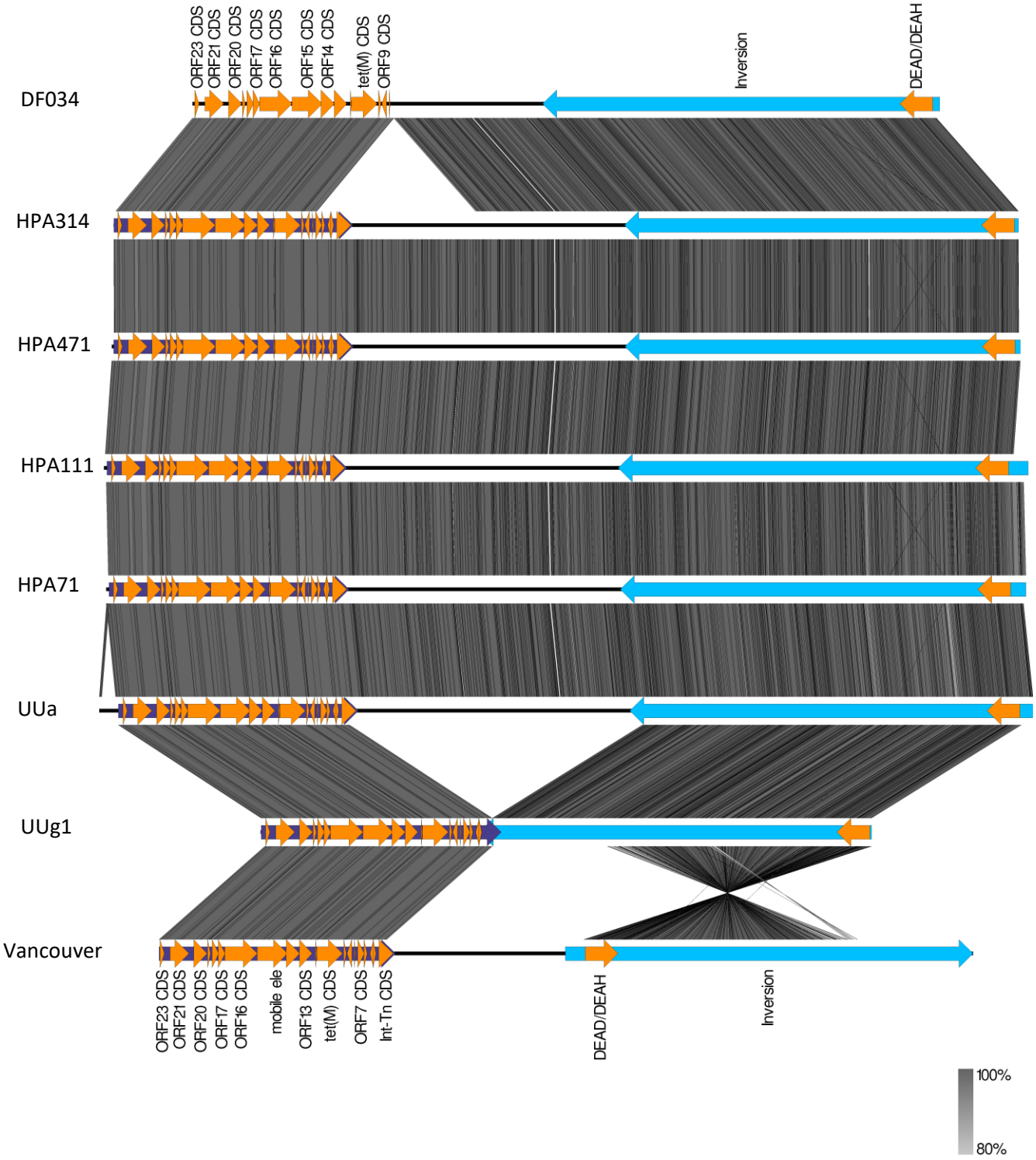

Figure 6-3 – Alignment of Tn916 and downstream genomes of *U. urealyticum* isolates. Genes are marked in orange, with homologous regions marked in purple for the Tn916 transposon, and blue for the section of genome between the transposon and the DEAH/DEAD box helicase gene. Black-grey lines indicate BLAST identity percentage, as per the figure legend.

6.2.3 Genomic variation of Tn916 and surrounding genomic regions in *U. parvum*

In comparison, four of the five *U. parvum* isolates (Cuba 211, Cuba 681, HPA23 and DF145) demonstrated severe but homologous genomic recombination, with the full deletion of all conjugative transfer ORFs across all four Tn916 elements while simultaneously maintaining a common complement of core host genes upstream of the truncated transposon composed of ser(S), fts(H), hpc, uvr(A) and three conserved core hypothetical coding regions as seen in Figure 5 7, once again suggesting a common insertion point for Tn916. The variation between the four isolates negligible, with only 1 to 4 nucleotide variations between any of the four U. parvum isolates.

Regarding genomic content immediately up- and downstream of the Tn916 transposon, U. parvum demonstrated high levels of genomic heterogenicity between each other indicating a common insertion site. This excludes DF28, which appears to have inserted itself into a completely different section of the genome (see REF). There were no notable gene truncations present in any of the five U. parvum isolates, with point mutations leading to truncations in the hypothetical coding regions of DF145 and the ORF15 and ORF14 regions of DF28U.

It is well-established that within mycoplasmas as a clade, UGA ("Opal") does not function as a stop codon and instead codes for tryptophan, potentially affecting the coding regions of a transposon as it moves between clades with disparate coding structures (Neyrolles et al. 1996). To interrogate the possibility of "hidden" read-through mutations accrued when the Tn*916* transposon moved between species, the entire Tn*916* transposon from *Enterococcus faecalis* reference strain DS16 was analysed to compare its coding regions when using the standard bacterial and mycoplasma amino acid code. This analysis seen showed that when converting from a typical bacterial coding structure to a mycoplasma coding structure, readthrough mutations are present in ORF 14 and 13, as well as ORF 7 and 8. While the exact effects of these mutations are unknown, it is likely that these read-through mutations could drive compensatory gene mutations to truncate these ORFs.

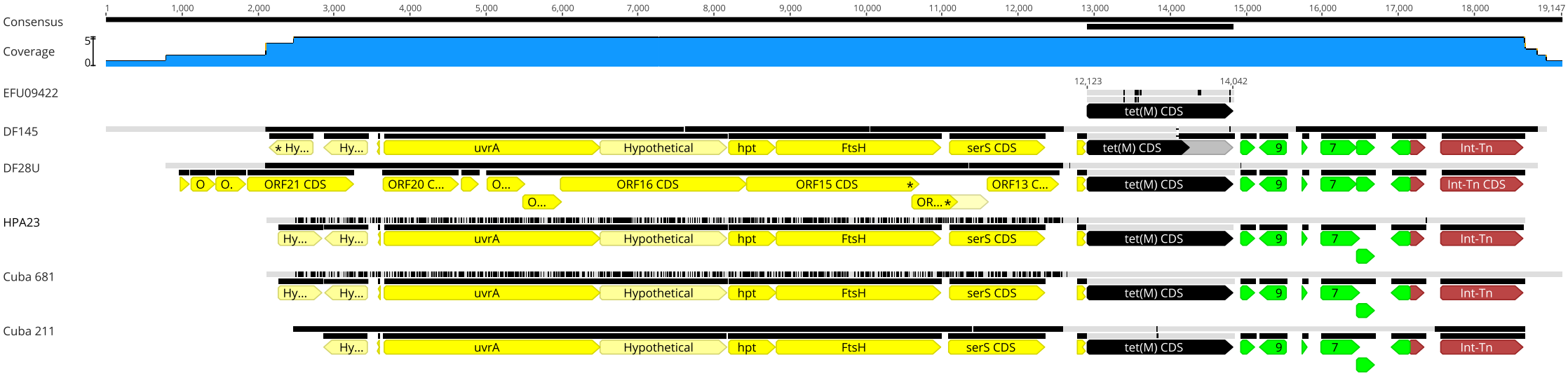

Figure 6-4 - An alignment of five *Ureaplasma parvum* isolates. The tetM accessory gene is labelled in black, with truncation labelled in grey. All conjugative transfer genes normally upstream of the *tetM* accessory genes in Tn*916* are absent in all but DF28U, which contains the full complement of ORFs but also a truncation at ORF 14, marked with an asterisk.

6.2.4 Analysis of Potential Gene Insertion Sites

Using the first sequenced human Ureaplasma isolate (ATCC 700970) as a reference, *tetM* cassette insertion was mapped by locating the position of the first 5' conserved homologous gene as seen in Figure 6-5. All *U. parvum* isolates, except DF28U, aligned to genes encoding for *ser*(S) and an ATP-binding cassette protein, while DF28U was located at a completely novel insertion site in the genome approximately 260,000 bp away. The *TetM* insertion sites for all *U. urealyticum* isolates were also conserved at a site 380,000 bp away from the conserved *U. parvum* insertion site. DF28U by comparison was found inserted in a completely different location in the genome as shown in ref. Furthermore, the Tn916 composition of DF28U differed from this similarity by retaining the full complement of conjugative transfer genes missing from the other four isolates required for the function of the Tn916 transposon, implying a recent insertion. ORF 14 contained a stop codon at amino acid 162 of the gene, which notably is the only genomic mutation present in any of the genes in the Tn916 transposon, which suggests that further movement of the transposon could be abrogated. This indicates that apart from DF28U, both insertion events occurred independently in separate ancestral strains and have evolved independently between species. However, the significant variance in the gene order observed for the Vancouver strain that was isolated by Denys K Ford in Vancouver in 1967 (Ford 1967), relative to the other *U. urealyticum* isolates indicates significant local recombination and remodelling within the Vancouver insertion site.

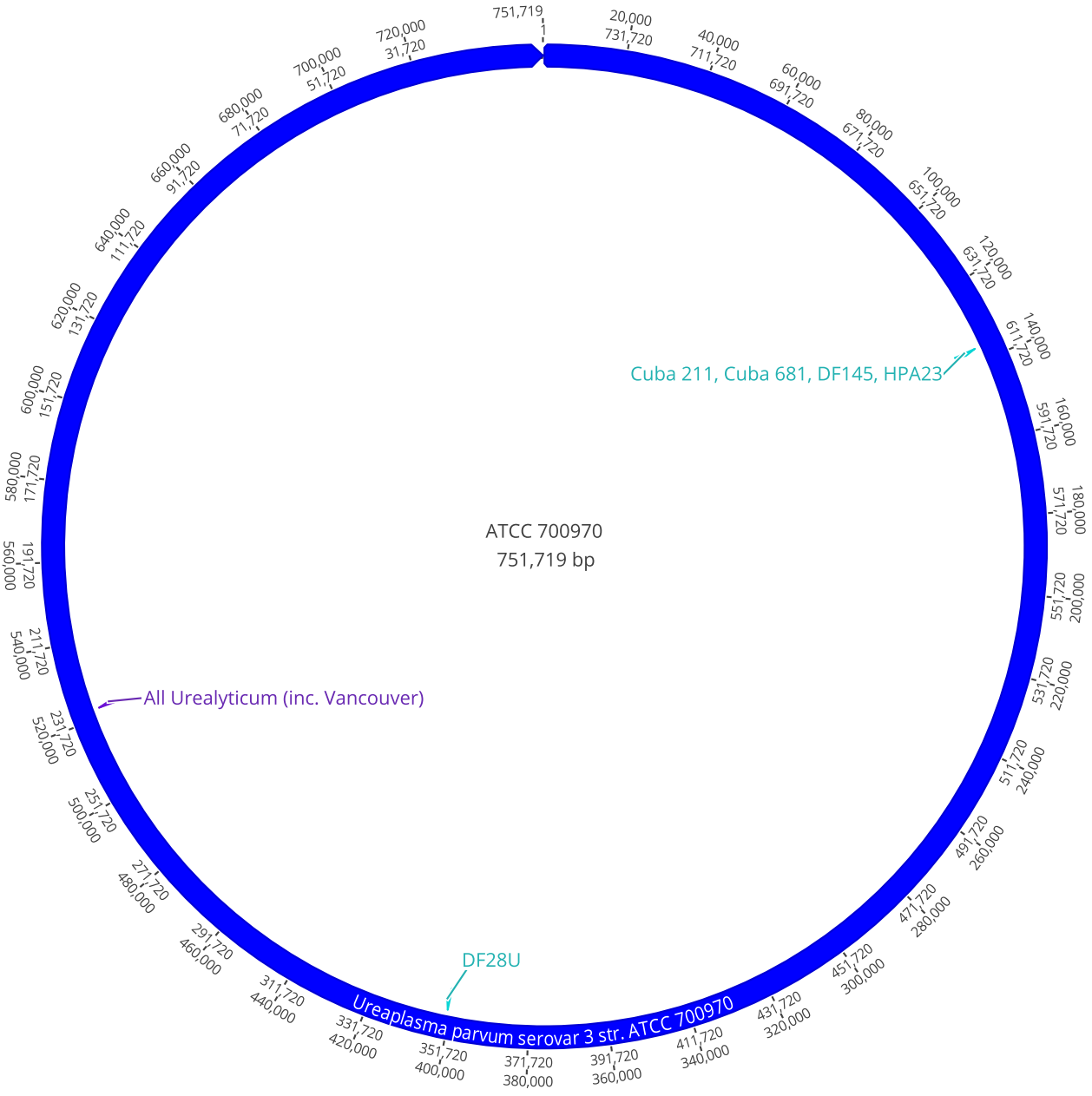

Figure 6-5 – Insertion sites for the Tn*916* transposon in *U. parvum* & *U. Urealyticum*. Only DF28U has a different insertion site when compared with the other four parvum isolates, which all have the same insertion site and are flanked by the same regulatory genes. All *U. urealyticum* isolates have the same insertion site, accounting for the severe recombination present in Vancouver.

6.3 Discussion

In this chapter, it was demonstrated that all thirteen isolates sequenced contained the *tetM*-tetracycline resistance gene, associated with components of the Tn*916* transposon, a known mediator of tetracycline resistance in multiple species of pathogenic bacterium. The composition of the Tn*916* transposon, while varied between species and isolates, broadly follows the common structure identified in previous works, which allows us to extrapolate which aspects of the transposon have been rendered inactive over subsequent generations. More specifically, the novel findings of this work are evidence to suggest that the insertion of tn916 into the Ureaplasma genome is mediated by horizontal gene transfer that is then clonally inherited by subsequent isolates. Furthermore, this work provides evidence that the insertion site is based on gene targets rather than specific locations in the Ureaplasma genome, and that the transposon becomes fixed in the Ureaplasma genome due to asymmetrical codon functions between conventional bacteria and mycoplasmas.

In *U. urealyticum* the Tn*916* transposon is mostly intact, with the variation between isolates being restricted to the contig length of DF034 and more broadly the truncation of genes across the transposon. A common truncation is caused by mutation to avoid read-through between ORF 15 and ORF 14 which exists in all 8 isolates. This truncation is expected, as multiple other works have also observed variance within ORF 15 within Tn*916* (Santoro et al. 2014; Sirichoat et al. 2020) and other highly similar transposons (Ciric et al. 2011). UUg1 contained an 8bp nucleotide insertion in ORF 14 which led to a downstream truncation of the gene, but notably this insertion did not lead to a frameshift mutation downstream of ORF 14 which meant that the accessory *tetM* gene present still retained its function as a resistance-encoding gene, suggesting some sort of robust genomic structuring existing in the transposon.

Higher levels of genomic variance could be observed in the genomic regions flanking the transposon, with transposon insertion sites varying slightly between isolates around the transposon but sharing the same broad location target downstream of the same DEAH/DEAD box helicase gene. There was greater insert organisation variation in the Vancouver strain, which is unfortunate for a dedicated reference strain for serovar 9. The DEAH/DEAD box helicase downstream of the Tn*916* transposon in all seven other isolates was inverted relative to the Vancouver strain. However, *U. urealyticum* contains two copies of this gene, which suggests that this gene was carried by a larger ICE (as it is often required as an accessory gene) that had acquired the Tn*916* element prior to inserting into an ancestral *U. urealyticum* isolate. It is also notable that the Vancouver strain carries the MBA specific for serovar 9, and the remaining isolates all carry the MBA that is common to serovars 4, 12 and 13, suggesting independent evolutionary divergence post-acquisition of a common *U. urealyticum* ancestor. As none of the *U. parvum* isolates demonstrate this Tn*916* co-located DEAH/DEAD box helicase it lends further evidence to suggest that *U. parvum* and *U. urealyticum* acquired *tetM* after separating into two separate species.

This variation is surprising due to how established Vancouver is as a reference strain (Lin and Kass 1980), but equally as its discovery precedes molecular methods this could suggest more modern and representative reference strains should be used to assess genomic variability within cohorts of isolates. This significant divergence is especially relevant as Vancouver is the

agreed reference strain for *U. urealyticum* susceptibility testing quality control (CLSI 2011). While Vancouver still contains a fully functional *tetM* gene its overall genomic composition immediately surrounding the gene is significantly different, which could very well hamper the use of Vancouver as a reference strain in genomic analysis. However, other *tetM*-positive serovar 9 isolates would be needed to confirm this. For general genotyping and resistance profiling, the Serovar 3 reference strain Seattle (ATCC 27815) would be an adequate substitute, as would most other reference strains currently available on open platforms.

Comparison between *U. parvum* isolates found that four of the five isolates (excepting DF28U) were very similar, and contained the same deletion of all ORFs upstream of *tetM* and its leader peptide as seen in Figure 6-4 as well as the same complement of genes immediately upstream of *tetM* (*ser*(S), *fts*(H), *uvr*(A) and hypothetical protein coding regions). This identical deletion and gene complement is mirrored by the common transposon insertion site shared by all four isolates, flanked by a *ser*(S) gene and a gene coding for an ATP-binding cassette protein. Furthermore, no truncations exist in any genes of these four isolates, although as the neighbouring genes are important for the regulation of genomic regulation and metabolic homeostasis, it is worth noting that truncations may regularly occur that damage or frameshift these genes and that due to their deleterious effect cannot be detected by culture. What is surprising, is that the absence of ORFs 13-24 (the entire conjugative gene cassette in Figure 16) indicating no possibility of mobility of this remnant of Tn*916* – is found in three separate serovars of *U. parvum*. This calls into question the previous assertion from the previous chapter for *M. hominis* that variation of VAA between *tetM*-positive strains with a lack of ORFs 18-24 still substantiated that the serine recombinase was mediating movement between strains. However, the previous *tet*M for *M. hominis* is part of a composite ICE with a different transposase responsible for directed insertion into the *rum*(A) gene. For *U. parvum*, given the absence of *cis*- or *trans*- conjugation gene cassette presence is that the serovar determining MBA region is capable of independent mobility by some yet undetermined mechanism.

DF28U was observed to have a variety of unique characteristics when compared to the other four parvum isolates studied in this analysis. It demonstrated the full complement of ORFs 13-24 (i.e., an intact Tn*916* transposon), albeit with a point mutation at amino acid 491 of ORF 14 (TCA -> TAA) that truncated the gene. This observation of a truncation at a conjugative transfer gene is significant as it offers an explanation to why Tn*916* may become “fixed” in *Mollicutes* genomes once acquired. Mycoplasmas as a clade are well-established to undergo elevated levels of genomic adaptation via horizontal gene transfer, antigenic variation, and genomic recombination (Zimmerman et al. 2011; Aboklaish et al. 2016; Oliveira et al. 2017; Waites et al. 2022). As such, my observation of the rapid truncation of ORF 14 is unsurprising. Another feature of note observed in DF28U is the significantly different insertion site it demonstrated, flanked by hypothetical protein coding regions 264kbp away from the insertion site of the other four parvum isolates, in a genome 751kbp in length.

This variation in insertion site is tempered by the commonality of neighbouring genes – across four of the five different insertion sites (excluding DF28U), only five genes make up the flanking neighbour genes. Aspects of tRNA function are well-established to be regulated by

the virulence factor genes encoding for ATP-binding cassette domain-containing protein (Davidson et al. 2008) *ser*(S) (Vincent et al. 1995) and *phe*(T) (Ibba et al. 1994), while *pol*(C) is known to function as a 3' – 5' endonuclease (Barnes et al. 1994). The DUF1410 domain-containing proteins and the hypothetical protein coding regions flanking DF28U are the only genes of unknown function, but the DUF1410 genes are a common gene structure across many species of Ureaplasma (Yang et al. 2020a) and associated with the *mba* genes, another virulence factor due to its function as a medium for antigenic variation (Aboklaish et al. 2016; Sweeney et al. 2017b). This means that while the physical region of insertion varies between isolates and species, the function of the genes targeted by the Tn*916* transposase share commonalities in DNA regulation and replication, of high importance to a self-regulating and thus self-replicating mobile genetic element.

Regarding the *tet*M gene specifically, a significant observation was the variability in reversibility of function loss between HPA71 and DF145. Both isolates contained frameshift mutations in their *tet*M coding regions that led to a premature truncation of the protein. However, only HPA71 demonstrated a rescue reversion to tetracycline resistance, while DF145 failed to revert even after a significant repeated exposure to sub-inhibitory tetracycline levels. When we look at the exact mechanisms of truncation in each gene, HPA71 contained an adenine insertion in a poly(A) tract such that AAG became AAA G at amino acid codon 262 that led to a stop codon at amino acid 272 truncating the gene, while DF145 contained a guanosine insertion in a poly(G) tract such that GGA -> GGG A at amino acid codon 392 truncating the gene with a stop codon at amino acid 441. In the context of a gene 1921bp long with 640 codons, both truncations occur early in the synthesis of the gene product, resulting in tetracycline susceptibility.

Overall, this work demonstrates the existence of significant variation in the Tn*916* transposon gene structures between *U. parvum* and *U. urealyticum*. Apart from DF28U, *Ureaplasma parvum* exhibited far higher levels of recombination than *U. urealyticum*, which contained the full suite of Tn*916* genes, albeit with truncations that might preclude the motility of the transposon. In comparison, *U. parvum* exhibited complete loss of all conjugative transfer genes upstream of the *tet*M accessory gene in four of the five isolates, with only DF28U retaining these ORFs. Both species interrogated contained exceptions that differed from the consensus alignments of their Tn*916* transposon and lacked a recombinase-directed insertion site into the wider genome, as well as smaller-scale recombination such as point mutations and gene truncations. This high degree of variance makes it hard to describe species-level differences but is consistent with the genomic data we have on Ureaplasma and Mycoplasmas more generally, due to the elevated level of genomic variation observed in these bacteria (Zimmerman et al. 2013; Oliveira et al. 2017; Rideau et al. 2019; Hakim et al. 2021). Although the transposon insertion point is inconsistent between species, consistency is always seen in the genes which neighbour the point of insertion. This suggests that the transposon opportunistically inserts near genes that carry a specific function that can facilitate the replication and further transmission of additional transposons.

Another mechanism of insertion to consider is the variance of genetic code between conventional bacterial genomes and Mycoplasmas. The usage of the 'opal' codon (TGA) as

tryptophan in mycoplasma differs from its usage as a stop codon in other prokaryotes. Mobility genes utilizing an Opal codon as a stop codon would not function in Mycoplasmas, as they would suffer from read-through mutations and invariably incur loss-of function. Within the context of a mobile genetic element that can move between these clades, this offers a potential reason the mobile genetic element is able to move into Mycoplasma, but not move out. In the case of Tn*916*, the transposon observed in *Enterococcus faecalis* suffers from two readthrough mutations in the conjugative transfer ORFs 14 and 13 contained upstream of *tet*M, which would certainly preclude further generation and transmission of the transposon as a mobile genetic element. An interesting finding is the alteration in coding regions immediately surrounding the *tet*M gene and its leader peptide. While *tet*M as the gene is not truncated, the CDS of its leader peptide was elongated by an additional 30bp. While this attenuation loop does not encode for a gene and is merely a regulatory structure, the alteration of this structure could have a yet-unknown effect on the regulation and expression of the *tet*M gene. The disruption of *tet*M expression may in turn influence the fitness costs usually incurred by *tet*M expression, but until the full effects of these ORF alterations are understood with further work, it is difficult to describe how this relates to the species-specific costs of Tn916 between species.

One main area in which this work could be explored further is via an expanded sample size. With five *U. parvum* and eight *U. urealyticum* samples, it is difficult to determine how significant the anomalous isolates (DF28U and Vancouver) are, although this is less applicable with *U. urealyticum* and more so with *U. parvum*. In addition, attempts to induce the transmission of a Tn*916* transposon from *Enterococcus faecalis* to either *Ureaplasma* species had been attempted several times and were unsuccessful, and as such was not able to be reported here. An ability to observe the transmission of Tn*916* in “real time” would be of immense importance to understanding the effects of variant genetic codes and the effects on *tet*M gene expression, but it is likely to be a significant experimental undertaking.

The increase in tetracycline resistance across several STIs (Beeton and Spiller 2017; Jiang et al. 2017; Truong et al. 2022) is of immense concern for academics and clinicians due to the sharply decreasing set of tools left to treat these infections. As such, utilizing our knowledge of shared mechanisms between these adaptations is important to improve our chances of overcoming these developments in antimicrobial resistance and retain our ability to treat these common infections.

7 General Discussion

In this study, I utilized a variety of methods to assess the antimicrobial resistance of urogenital *Mollicutes*, interrogating both their clinical incidence and the genomic mechanisms underlying the development of resistance in these pathogens. The study of mycoplasmas as sexually transmitted diseases and more broadly as pathogens has been hampered by the lack of accurate and reliable tools for their detection and analysis. However, commercially available, and clinically validated molecular tests are available that can facilitate the rapid detection of these bacteria while simultaneously assessing levels of antimicrobial resistance in these bacteria. In other medical areas such as fertility and the management of post-transplant infection, mycoplasma such as *M. hominis* and *U. urealyticum* are acknowledged and surveyed pathogens, but there has been a reluctance to share this view in the field of sexual health. The accurate detection of these pathogens in symptomatic patients complements the established body of evidence linking these pathogens to symptomatic disease and heavily suggests the need for further surveillance of these pathogens. This study also expands on pre-existing knowledge on the mechanisms of resistance in these pathogens, noting their similarities to mechanisms of resistance shared with other sexual health pathogens such as *N. gonorrhoea*. This work also further compared the incidence rates of *N. gonorrhoea*.

The genomic analysis contained in this work focused on the mechanisms of tetracycline and fluoroquinolone resistance common to these pathogens. Specifically, *tet*M-mediated tetracycline resistance was studied in both *M. hominis* and *Ureaplasma* spp. to further elucidate the conjugative transfer of Tn*916* theorized to facilitate the propagation of tetracycline resistance in closely related bacteria. Additionally, *gyrA*/*parC*-mediated fluoroquinolone resistance was studied in *M. hominis*, with the identification of an intermediate mutant strain that elucidates the link between the two gyrase genes in the mediation of resistance. Together, this provides a better understanding of the molecular aspects of resistance and contributes to the development of the improved surveillance assays required to rapidly and accurately detect and therefore treat these pathogens. Returning to the overarching hypothesis, this work has identified methods that can rapidly and effectively detect antimicrobial resistance and its genomic mechanisms. This has been achieved using modern molecular methods that have studied the link between mycoplasmas and symptoms of disease with both established links with mycoplasma infections, as well as symptoms not yet associated clinically with mycoplasma infections such as *M hominis*.

7.1 Fluoroquinolone resistance in *M. hominis*

The first set of findings outlined is that 14.8% of *M. hominis* samples tested positive for tetracycline resistance and 2.4% tested positive for fluoroquinolone resistance – while useful as an absolute survey of resistance in archival isolates, it not straightforward to quantify the relative meaning of this incidence due to range of resistances reported in the literature. These wide ranges in resistance appear to relate to geographical regions – in Bordeaux, France, *M. hominis* fluoroquinolone resistance rates were at a comparable 1.6% - 2.7% in 183 isolates tested by Meygret et al. (2018), far lower than the likewise comparable rates of tetracycline resistance in the same cohort at 14.7% of isolates. This concordance of results is despite the

significant variation in sample size, providing evidence of the effectiveness of the molecular techniques that were shared between the two studies. Nonetheless, as we consider wider geographical ranges for comparison, resistance rates vary significantly. A recent study conducted in Zhejiang, China by Yang et al. (2020b) found sharply higher rates of resistance with 84.62% of *M. hominis* samples showing resistance to both levofloxacin and moxifloxacin while another study conducted in Franceville, Gambon found that 100% of all *M. hominis* isolates tested were resistant to azithromycin (Lendamba et al. 2022). Although these studies had relatively low sample sizes (9 and 13 isolates respectively), previous observations demonstrate that we can make confident comparisons between these different sample sizes. While these rates are significantly different, this does make sense in line with different health systems providing significantly different antibiotic regimen, specifically the over-reliance and over-prescription of particular kinds of (more) affordable antibiotics and their subsequent selective effect for resistance (Pokharel et al. 2019).

Due to the fastidious nature of *M. hominis*, molecular assays such as the ones used in this work will be required to develop standardized clinical surveillance systems and this work demonstrates both the feasibility of doing so and the deeper understanding of antimicrobial resistance development that can be gained from this. Examples of this are the finding that co-mutations in *gyr*A and *par*C produce significantly higher levels of resistance (MICs) in *M. hominis* than singular mutations in *gyr*A alone, and that intermediate strains induced into full resistance only demonstrate the complementary *par*C mutation when subjected to sublethal concentrations of fluoroquinolones. This is especially interesting as *M. genitalium* displays a purely *par*C mediated resistance (Bodiyabadu et al. 2021), showing variation in resistance mechanism even between two mycoplasma occupying similar environmental niches as urogenital pathogens. Looking further afield, work on *H. pylori* demonstrating the absence of *par*C and *par*E genes observed in *M. hominis* (Rhie et al. 2020) and another study by Ostrer et al. (2019) demonstrates that these mutations can be used as the sole predictors of fluoroquinolone resistance in *Acinetobacter baumannii*, *Escherichia coli*, *Staphylococcus aureus*, but not *Klebsiella pneumonias* or *Pseudomonas aeruginosa*.

As such, understanding the epistatic relationship between these two genes helps develop precise methods of assessing resistance. The observation of a *gyr*A mutant/*par*C wild type isolate being induced into full resistance upon exposure to fluoroquinolones also emphasize the need for the careful interpretation intermediate or inconclusive results that are often produced by molecular assays. The further work that could be conducted in this area would most likely be in exploring regulatory relationship between *gyr*A and *par*C as they relate to the mediation of fluoroquinolone resistance in *M. hominis*, and potentially identifying additional partial mutant isolates that could confirm the findings here.

7.2 *tet*M-mediated tetracycline resistance in *M. hominis*

The resistance mechanism of tetracycline resistance in *M. hominis* are also an area which I investigated, to expand upon pre-existing observations and theories. The underlying question the work sought to interrogate surrounded the mobility of the Tn*916* transposon, which in turn carries the resistance mediating *tet*M gene. While the mobility of Tn*916* itself is well-established (Mardassi et al. 2012; Santoro et al. 2014; Sirichoat et al. 2020; Lunde et al. 2021),

the source of the transposon and aspects of the genomic insertion into the *M. hominis* genome were not fully understood, which this work sought to explore.

Within *M. hominis*, five approximate groups were observed of gradually shorter length, as genes encoding for the transport and incision of the transposon became truncated and lost, suggesting that the rate of recombination that occurs once the ICE inserts itself into the host genome is relatively rapid. This is in line with previous observations of *M. hominis*, which has been observed to undergo rapid recombination in other areas of the genome such as the incorporation of other transposons (Rideau et al. 2019), the modulation of virulence genes that are used to form multi-locus sequence typing schemes (Boujemaa et al. 2018) and the aforementioned spontaneous mutations that facilitate fluoroquinolone resistance in *M. hominis*.

The malleability observed in *M. hominis* is so extensive that one recent study even found variants able to reorganise their metabolic pathways to facilitate and prioritise the metabolization of deoxyribonucleosides (particularly thymidine) over arginine or ribonucleosides (Fisunov et al. 2022). The recombination that we can observe in *M. hominis* is coupled with a lack of random point mutations that was observed in my own work when MH-12 was isolated in triplicate, suggesting that the recombination that we do observe in *M. hominis* is genetically mediated in response to environmental pressures. Furthermore, there was no observed correlation between the presence of *tet*M, which provides evidence that the Tn*916* transposon we observe in these isolates was not a historical insertion that was then propagated through clonal inheritance, but a mobile element that regularly inserts itself into *M. hominis* in response to selection pressures. This has been theorized to function whereby ICEs insert themselves into sections of genome of lower genomic regulation (Citti et al. 2020), creating “genomic islands” of highly conserved genes vital to cellular function flanked by ICEs, either intact or eroded by recombination. While this does not entirely disagree with the common conception of mycoplasmas as having a minimal genome (Grosjean et al. 2014; Danchin and Fang 2016), it does emphasize the dynamic nature by which these pathogens are able to adapt to environmental challenges (Antczak et al. 2019; Dordet-Frisoni et al. 2019).

Developing antibiotic resistance to last-resort antibiotics is a topic of growing concern, with the tools available to clinicians for treatment becoming more sharply limited as pathogens adapt faster than new treatments can be developed. As such, this section of my work examined resistance not only to current tetracyclines in use for *M. hominis* but also newer antimicrobial variants such as glycylcyclines, which are currently limited to tigecycline. Tigecycline was shown to be fully effective at preventing growth of the resistant isolates due to interacting with the ribosomal complex in a different orientation than conventional tetracyclines, as well as also interacting with the helix H34 subunit of the 30S ribosomal subunit as well as the A site (Olson et al. 2006). This alternative form of interaction means that it subverts both the efflux pumps encoded by resistance genes such as tet(A) and tet(B) (Kumar and Varela 2012) and the conformational changes to the 30S subunit encoded by *tet*M. The only effective mechanism for resistance to tigecycline that has been identified is the *tet*(X) family of genes, which encode for a protein that hydroxylates Tigecycline at Carbon 11a to produce 11a-Hydroxytigecycline, a far less effective antimicrobial that can be expelled

from the cell via efflux pump (Moore et al. 2005). While *tet*(X) has not been observed in any *Mollicutes* species, it is carried by a plasmid that occurs in common bacteria such as *E. coli* and therefore could jump species (Sun et al. 2019), although this is purely speculative. The other prominent mechanism of resistance currently understood is via structural changes in the 16S region of the rRNA structure, induced via point mutation after repeated or otherwise sublethal challenge (Gerrits et al. 2002). Neither mechanism has been observed in a *Mollicutes* pathogen at time of writing, but potentially both the *tet*(X) family of genes and 16S point mutations are worth investigating to see if either could be artificially introduced to *M. hominis in vitro*.

7.3 *tet*M-mediated tetracycline resistance in Ureaplasma spp.

In contrast to *M. hominis*, the investigation of *tet*M in *Ureaplasma. spp*. was more complex in practice for several reasons. The prominent reason was due to the relative dearth of information on the clade, as the two species of urogenital Ureaplasma (*U. parvum* and *U. urealyticum*) were not formally categorized until 2002 (Robertson et al. 2002). As such, the culture-based knowledge that underpinned aspects of my work on *M. hominis* was not as developed. The wealth of reference strains that were available for my investigations into *M. hominis* were not mirrored in either species of *Ureaplasma* due to the pandemic making it difficult to acquire additional strains from academics seconded to Covid research. Nonetheless, this was not a significant issue as inter-species comparisons were in of themselves a significant topic of investigation, but studies into species-specific mechanisms might require additional work to build a cohort of isolates for analysis. Of the 13 isolate genomes available for analysis, all contained the same mechanism of resistance – a Tn*916* transposon of varying composition containing a *tet*M gene. This concurred with previous studies into the composition of *tet*M-mediated resistance in other pathogens such as oral streptococci and *E. coli* (Poyart et al. 1995; Lunde et al. 2021). In *U. urealyticum*, the transposon was shown to be mostly intact across each of the eight genomes analysed, with minimal variations between each of the ORFs. One isolate (UUg1) contained an 8bp insertion that truncated its ORF 14, but even this did not create a frameshift mutation in any other gene up- or downstream of the ORF.

UUg1 and the reference strain Vancouver significantly diverged in terms of surrounding genomic structures. While they shared a common proximity to a 30.4kbp sequence of DNA, in the case of both UUg1 and Vancouver there was a full deletion of the genomic content between Tn916 and the common length of DNA. Vancouver contained the even more severe recombination of said length of DNA being inverted. This is especially unusual as the 30.4kbp length of DNA contained the DEAD/DEAH box helicase, a gene vital for the regulation and function of the bacterial genome. As discussed in the results chapter, Vancouver demonstrates a uniquely high level, of heterology that calls into question its value as a reference strain. The strain was identified before in-depth genomic analysis was available (Robertson and Stemke 1979) and before the designation of *U. parvum* and *U. urealyticum* as species, so to some degree this is not surprising. Nonetheless, its usage as a reference genome could not be recommended until more in-depth analysis of its genome was conducted. This is due to these, and many other irregularities contained in its genome and is relative to the other *U. urealyticum* genomes available for research.

Another interesting finding was the inability of two isolated strains (HPA71 and DF145U) to grow in the presence of tetracycline as expected, and the subsequent induction of resistance in HPA71 but not DF145. Both isolates presented as positive carriers of the *tet*M gene via qPCR analysis and were found to carry a *tet*M but differed in the composition of their respective *tet*M genes. DF145U carried a deletion at nucleotide 1231 that caused a frameshift mutation and subsequent loss-of-function that could not be rectified by repeated challenge, while HPA71 carried a similar frameshift mutation at 814, but this was due to an additional adenine that was lost after exposure to tetracyclines, resulting in a gain-of-function mutation that conferred resistance. Variations in phenotype due to mutations undetected by qPCR could complicate the usage of qPCR and other NAATs as a diagnostic tool, as if either of these isolates were detected in a clinical setting this could constitute a false positive. In practice, the usage of such a test precludes the existence of a second- or last-line treatment that could be administered instead, and therefore such false positives could be accounted for. This observation could also suggest that the expression of *tet*M is regulated by genomic machinery to avoid the fitness costs that *tet*M is known to impose on carriers (Dunai et al. 2019; Koonin et al. 2020).

Additionally, I observed that the *tet*M gene and surrounding transposon was present in a different insertion site in one *U. parvum* isolate, DF28U. In this isolate, the *tet*M gene was located approximately 260kbp downstream of the insertion site of the other four *tet*M transposons, indicating that it had been inserted into the genome in a separate event to the other four, which may have concurrently gained the *tet*M gene via insertion at the same site or inherited the gene via clonal expansion. The surrounding transposon present in DF28U carried the full complement of conjugative genes, with a truncation present in ORF 14 as explanation for the transposon's inability to move from the genome. I theorize that the isolates that demonstrates a common insertion site likely gained resistance from clonal inheritance, but DF28U demonstrates a capacity for *U. parvum* to gain resistance from environmental sources of *tet*M.

The ability for *U. parvum* to rapidly develop tetracycline resistance (Beeton et al. 2016a) is another factor in why regular antimicrobial surveillance is required for this pathogen, as this work shows that *U. parvum* can gain resistance rapidly via horizontal gene transfer, conserve this resistance mechanism through clonal inheritance and then modulate this resistance in line with selection pressures and fitness costs. The phenomenon of poly(A) tract mutations mediating "transient silencing" has been observed to occur in other species of bacteria with similar mechanisms of resistance, such as tet(K)-mediated tetracycline resistance in *Staphylococcus aureus* (Kime et al. 2019). Furthermore, it has been noted more broadly that plasmids and ICEs can themselves carry genes that act to silence xenogeneic genes (Navarre 2016; Duan et al. 2021), which in turn mediates the insertion's integration into the wider genome. This relates back to the mediation of fluoroquinolone resistance I observed in *M. hominis*, whereby specific and/or complementary point mutations were required for MIC levels denoting resistance, emphasizing the need for in-depth whole-genome sequencing of these pathogens to fully understand the mechanisms of resistance and the molecular targets of antimicrobial surveillance assays.

7.4 Clinical Interventional Study

The final set of results I presented in this work relates to a clinical study undertaken in collaboration with the Cwm Taf Morgannwg NHS Health Board, analysing the presence and antimicrobial susceptibility of urogenital *Mollicutes*. This part of my research was the most affected by the Covid-19 pandemic, with secondary aspects of the analysis such as patient surveys and follow-up analyses unavailable due to the pandemic and its subsequent restrictions. Nonetheless, 81 patients were recruited pre-pandemic (20 male patients and 61 female patients) and 79 patients were recruited post-pandemic (28 male patients and 51 female patients). By combining these two cohorts (where appropriate), I was able to ensure that my inferences were able to carry a degree of statistical relevance.

Of the 160 patients, *U. parvum* had the highest prevalence amongst patients at 22.5%, followed by *M hominis* at 11.25%, *U. urealyticum* at 8.1%, *N. gonorrhoea* at 4.38% and *M. genitalium* at 2.5%. A meta-analysis conducted to look at the epidemiology of *M. hominis*, *M. genitalium* and *Ureaplasma urealyticum* in Iran (Moridi et al. 2020) found higher levels of *U. parvum* than *U. urealyticum* but similarly lower levels of *M. hominis*. In addition, Moridi and colleagues noted that *U. urealyticum* had a higher prevalence than *U. parvum* or *M. hominis* in infertile men and women. Another study in Shenyang, China (Shao et al. 2021) looked at the prevalence of *U. parvum* and *M. hominis* in suspected UTIs and found 37.5% of patients carried either, with 27.5% of patients carrying U. parvum, 2% carrying *M. hominis* and 7.6% carrying both as a co-infection. While I did not find a statistically significant relationship between any combination of co-infection, Shao and their colleagues observed seasonal trends in co-infection and altered resistance profiles, demonstrating the benefit of analysing these pathogens as a cohort.

Looking at the wider body of works on this topic, I found significantly more works analysing these pathogens in single sex cohorts. This is likely due to the wider acceptance of these bacteria having a role in gynaecological and obstetric complications focusing research efforts towards studying these bacteria in female patients. Furthermore, I observed a significantly higher prevalence of *U. parvum* and *M. hominis* in female patients, suggesting a sex-based component to pathogenicity. The relationship between sex and pathogenicity is not entirely understood but well-established through observational studies, with *U. parvum* acknowledged to be far more likely to produce symptoms in female patients (Ma et al. 2021) and the role of U. urealyticum in male NGU acknowledged even by the otherwise-dismissive European guidelines on its treatment (Jensen et al. 2016). This could suggest that U. parvum is an opportunistic pathogen in women and a commensal in men, with the inverse true for U. urealyticum. If so, I would hypothesize such a variance would be underpinned by aspects of biochemistry and cytology not yet understood. This was briefly investigated via differing concentrations of hormone levels, but culturing both *U. parvum* and *U. urealyticum* in varying levels of both beta-oestradiol and testosterone did not produce any observably consistent differences in growth. As previously described, the mechanisms of pathogenicity are likely secondary in nature and relate to hormone-mediated signalling mechanisms such as cytokines (Bereshchenko et al. 2018; Garza et al. 2021) and the interactions.

Examining the usage of real-time, quantitative PCR for the analysis of patient samples, the in-house qPCR assay and accompanying methodology demonstrated a 100% concordance with both the SpeeDx commercial molecular assay and the standardized NHS culture methods for the detection of all urogenital mollicute pathogens, with only the detection of *N. gonorrhoea* suffering from issues with culture failures and the presence of contaminants. It should be noted that the in-house molecular assay had a 100% concurrence with the commercial assay, demonstrating the increased species-specificity of molecular methods. The successful concordance of pathogen detection is tempered with the failure of the assay to accurately determine species-specific tetracycline resistance, due to the ubiquity of *tet*M as a resistance gene and insufficient specificity of that aspect of the assay. This emphasizes the need for culture-based methods to provide confirmatory results and one of the major barriers to the implementation of molecular assays such as this one – the time and cost required to develop and implement such assays. Another barrier is developing the skills required to interpret the results of an assay within the context of a patient's treatment (Messacar et al. 2017; Hu et al. 2019), absent from many online postal self-sampling (OPSS) STI services available. Nonetheless, it has been demonstrated that these methods are a rapid and accurate means to detect these pathogens and their resistance profiles. Furthermore, I have provided evidence of relatively high levels of resistance in *N. gonorrhoea*, *M. genitalium* and *M. hominis,* emphasizing the need for further incidence and AST surveillance.

7.5 Summary

In this study I examined the levels of antimicrobial resistance and its genomic mechanisms in urogenital mollicutes, to determine whether they could be rapidly and effectively identified using modern molecular methods through the link between mycoplasmas and symptoms of disease, utilizing both established links with mycoplasma infections and symptoms not yet associated clinically with mycoplasma infections such as *Mycoplasma hominis*. In summary:

Tetracycline resistance was found in 12 out of the 81 *Mycoplasma hominis* isolates acquired from PHE (14.8%), uniformly mediated by *tet*M, and carried in a Tn*916* transposon. Genomic analysis of these isolates showed high levels of recombination of the Tn916 genes surrounding *tet*M, confirmed by supplementary testing of isolates grown in parallel. Furthermore, isolates carrying this transposon did not demonstrate common ancestry, suggesting that the transposon was not inherited clonally, but regular became fixed in strains via horizontal gene transfer. This transposon was also shown to confer resistance to tetracycline, but not josamycin or tigecycline, providing alternative means of treatment for these strains.

Mycoplasma hominis was observed to carry fluoroquinolone resistance in 4.1% of the 72 isolates analysed, mediated by linked point mutations in the *gyr*A and *par*C topoisomerase IV genes. SNP frequencies were highest amongst *gyr*A genes and varied broadly between isolates, but non-synonymous mutations were drastically lower. As observed with tetracycline resistance, fluoroquinolone-resistant strains were shown to lack common lineage, suggesting the most likely source of resistance was spontaneous mutation induced by short-term selection pressures. Additionally, isolates carrying *gyr*A mutations but not the corresponding *par*C mutations were observed to lack the resistance phenotype. When the

resistance phenotype was induced via repeated challenge, the resulting strains developed the *parC* mutations seen in other resistant isolates, suggesting a causal link between these two mutations.

13 *Ureaplasma* spp. isolates found to be resistant to tetracycline were also observed to carry a Tn*916* transposon of varying composition, with resistance mediated by the transposon's accessory *tet*M gene. The transposons present in *U. urealyticum* were found to be mostly intact, with mutations confined to SNPs such as a poly(A) mutation present in HPA71 that truncated the *tet*M gene until repeated challenge induced a gain-of-function mutation. In comparison, four out of five *U. parvum* isolates were found to have undergone significantly more recombination, including the full deletion of all genes upstream from *tet*M and a truncation in the *tet*M gene of DF145 that could not be rescued by repeated challenge. DF28U was an outlier in that it contained a fully conserved Tn*916* transposon comparable to U. urealyticum with point mutations at ORF 14, suggesting a mechanism for the transposon's loss of horizontal transmission.

In total, 160 patients were recruited as part of an interventional clinical study – 81 pre-pandemic (20 male patients and 61 female patients) and 79 post-pandemic (28 male patients and 51 female patients). Of the 160 patients, 36 (22.5%) carried *U. parvum*, 13 (8.1%) carried *U. urealyticum*, 17 (11.25%) carried *M. hominis* (MH), 7 (4.38%) carried GC and 4 (2.5%) patients caried MG. No significant difference in the incidence of any pathogen was found before and after pandemic restrictions. After AST, three out of four cases (75%) of MG were found to be azithromycin resistant, three of the seven cases of GC (41.7%) were found to be ciprofloxacin resistant, and seven out of nineteen MH (37%) were determined to be tetracycline resistant. 100% concordance was observed between the in-house qPCR assay, the commercial SpeeDx kit and traditional culture-based detection, all of which were able to be used in parallel pre-pandemic. Likewise, 100% concordance was observed between molecular AST and culture AST for *M. hominis*, *U. parvum* and *U. urealyticum*, but a concordance of only 50% concordance was observed for *N. gonorrhoea*. This disparity was interrogated by supplementary qPCR testing and genomic sequency and found to be a contaminant and further emphasized the role of molecular testing to supplement and enhance traditional, culture-based methods.

7.6 Future Work & Recommendations

The findings presented in this study have offered insight into a series of mechanisms that mediate antimicrobial resistance in urogenital mycoplasmas. However, there are a series of questions that are still unanswered that require further experiments to elucidate.

Firstly, much of the work here has been focused on the Tn*916* transposon that mediates tetracycline resistance in *Mycoplasma hominis* and *Ureaplasma spp*. While isolate-specific and species-specific differences were observed, there was a common structure observed in both studies that suggest the occurrence of horizontal gene transfer in both. Nonetheless, the next obvious investigation would be to observe the movement of the Tn*916* transposon and its associated *tet*M gene from a donor to a host genome, and in doing so elucidate the precise mechanism of transfer.

Another potential avenue of investigation could be to further our understanding of tetracycline resistance in *M. genitalium*. Isolates demonstrating resistance have been recently described (Le Roy et al. 2021) but there has been some difficulty in pinning down the mechanisms of resistance (Chua et al. 2022) and this area of research may help us understand why doxycycline has such a poor rate of cure (Jensen et al. 2022).

The second obvious avenue for further work is in the clinical aspect of this work. While a cohort of 160 patients is far lower than the original target of 1000 patients, the sample size was nonetheless large enough to draw some inferences on the correlations between demographics and urogenital mycoplasmas. Furthermore, the disruption caused by the pandemic meant that a full cost-efficiency analysis was not possible. As such, the next step in understanding the benefits of rapid diagnostics in a clinical sexual health setting would be to expand the study conducted here with a larger cohort of participants, and ideally more demographic data. Once these associations have been better characterized, we could move to target those demographics most at-risk with better tailored treatments to produce better patient outcomes.

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A. Appendix

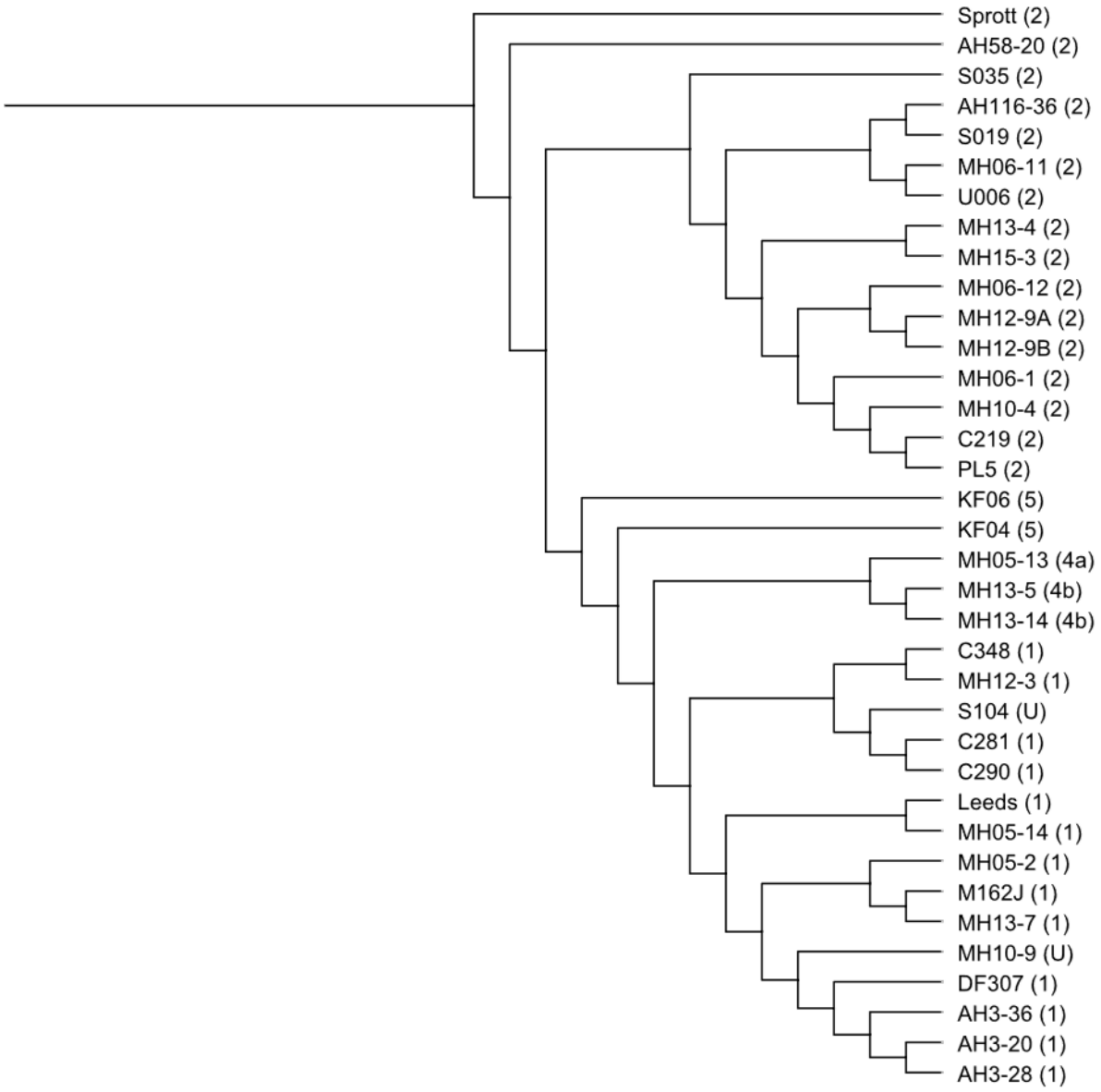

Figure 8-1 – A phylogenetic tree of 72 *Mycoplasma hominis* isolates analysed (and two induced resistant strains linked by arrows). Tree was constructed via concatenations of all four topoisomerase genes (*gyr*A, *gyr*B, *par*C and *par*E). The outgroup used was Mycoplasma pneumoniae M129, listed on the tree under its accession number (NC000912). Naturally occurring resistant strains are identified by arrowheads.

Table 8-1 – Meta-Data of *Mycoplasma hominis* isolates analysed in this work.

Isolate Name	Sample Origin	Isolation Date	Patient Type
ah3-20	Australia	2014	pregnant female
ah3-28	Australia	2014	pregnant female
ah3-36	Australia	2014	pregnant female
AH58	Australia	2014	pregnant female
AH116	Australia	2015	pregnant female
CJ02	Cuba	2015	plural effusion
CJ05	Cuba	2015	plural effusion
CJ10	Cuba	2015	plural effusion
CJ12	Cuba	2015	plural effusion
CJ13	Cuba	2015	plural effusion
CJ20	Cuba	2015	Plural effusion
CJ21	Cuba	2015	Plural effusion
JMM19	Cuba	2015	Plural effusion
N219	Cuba	2014	Endocervical swab following spontaneous abortion
N281	Cuba	2014	Endocervical swab, vaginitis
N290	Cuba	2016	Endocervical swab, antenatal screening
N348	Cuba	2016	Endocervical swab, antenatal screening
Leeds	England	2017	Prosthetic joint infection
M162b	England	2013	Endotracheal secretion, preterm neonate 1st week of life
M162e	England	2013	Endotracheal secretion, preterm neonate 3rd week of life
M162j	England	2013	Endotracheal secretion, preterm neonate 6th week of life
MH05-1	England	2005	PHE archival strain (information unavailable)
MH05-13	England	2005	PHE archival strain (information unavailable)
MH05-14	England	2005	PHE archival strain (information unavailable)
MH06-01	England	2006	PHE archival strain (information unavailable)
MH06-11	England	2006	PHE archival strain (information unavailable)
MH06-12	England	2006	PHE archival strain (information unavailable)
MH08-5	England	2008	PHE archival strain (information unavailable)
MH10-04	England	2010	PHE archival strain (information unavailable)
MH10-09	England	2010	PHE archival strain (information unavailable)
MH10-15	England	2010	PHE archival strain (information unavailable)
MH11	England	2012	Peritoneal fluid
MH12	England	2013	Peritoneal fluid
MH12-3	England	2012	PHE archival strain (information unavailable)
MH12-9	England	2012	PHE archival strain (information unavailable)
MH13-04	England	2013	PHE archival strain (information unavailable)
MH13-5	England	2013	PHE archival strain (information unavailable)
MH13-7	England	2013	PHE archival strain (information unavailable)
MH15-03	England	2015	PHE archival strain (information unavailable)
MH17	England	2004	PHE archival strain (information unavailable)
MH18	England	1993	Cervical swab

MH20	England	1990	Cervical swab
MH21	England	1986	Knee aspirate
MH23	England	2005	Neonate; 25 weeks gestation
MH26	England	2008	Cerebral abscess
MH27	England	2008	Renal transplant
MH28	England	2004	Post-hysterectomy complication
MH43	England	2013	Post-operative wound infection
ATCC 21134	France	1989	Neonatal
S019M	Serbia	2019	Sexual health sample for clinical trial
S019M2R	Serbia	2021	Induced levofloxacin resistance of S019 after one sub-culture
S035M	Serbia	2019	Sexual health sample for clinical trial
S042	Serbia	2019	Sexual health sample for clinical trial
S048	Serbia	2019	Sexual health sample for clinical trial
S070	Serbia	2019	Sexual health sample for clinical trial
S104	Serbia	2019	Sexual health sample for clinical trial
PL5	USA	2013	GenBank under the accession no. JRXA00000000.1
Sprott	USA	1978	GenBank under the accession no. CP011538
DF28	Wales	2017	Vaginal swab
DF282R	Wales	2017	Laboratory culture
DF284	Wales	2018	Cervical swab
DF289	Wales	2018	Cervical swab
DF290	Wales	2018	Cervical swab
DF307	Wales	2018	Cervical swab
KF06	Wales	2017	Cervical swab
KF08	Wales	2017	Cervical swab
KF09	Wales	2017	Cervical swab
KF12	Wales	2017	Cervical swab
KF14	Wales	2017	cervical swab
U006	Wales	2018	Walk-in sexual health patient
U018	Wales	2018	Walk-in sexual health patient
U156	Wales	2019	Walk-in sexual health patient

Table 8-2 - Meta-Data of *Ureaplasma parvum* and *Ureaplasma urealyticum* isolates analysed in this work.

Isolate Name	Species	Serovar	Tetracycline MICs (mg/l)	Isolation Date	Sample Origin	Patient Type
HPA71 (Pre-challenge)	*U. urealyticum*	4/12/13*	1	2010	England	PHE Archival Strain
HPA71 (Post-challenge)			64	2022	England	Laboratory Strain
HPA111	*U. urealyticum*	4/12/13*	64	2008	England	PHE Archival Strain
UUg1	*U. urealyticum*	4/12/13*	2		France	Cervical swab
Vancouver	*U. urealyticum*	9	2		Canada	Archival type strain
UUa	*U. urealyticum*	2/5/8*	2		France	Knee synovial fluid
DF034	*U. urealyticum*	4/12/13*	2	2017	Wales	Walk-in sexual health patient
DF471	*U. urealyticum*	4/12/13*	16	2018	Wales	Walk-in sexual health patient
DF314	*U. urealyticum*	4/12/13*	16	2018	Wales	Walk-in sexual health patient
DF145	*U. parvum*	6	2	2017	Wales	Walk-in sexual health patient
HPA23	*U. parvum*	6	64	2010	England	PHE Archival Strain
Cuba 681	*U. parvum*	1	2	2015	Cuba	Plural effusion
Cuba 211	*U. parvum*	3	2	2015	Cuba	Plural effusion
DF28U	*U. parvum*	3	128	2017	Wales	Walk-in sexual health patient

Table 8-3 - Amino acid variations present in the gyrA genes of all isolates analysed in reference to ATCC 21134.

Isolate	CDS																													
	21	**22**	**39**	**41**	**65**	**79**	**153**	**157**	**228**	**272**	**273**	**297**	**321**	**450**	**461**	**488**	**514**	**525**	**577**	**745**	**764**	**771**	**798**	**804**	**871**	**902**	**903**	**906**
ATCC 21134	S	H	S	D	R	K	S	E	V	E	I	K	L	E	A	I	N	K	I	D	I	V	A	R	N	A	**E**	L
ah3-20					K																L				S		K	
ah3-28					K																L				S		K	
ah3-36					K																L				S		K	
AH58	G					R																			S	V	K	
AH116					K													R			L				S	V	K	
CJ02																	D								S		K	
CJ05					K																				S		K	
CJ10					K											V							V		S		K	
CJ12					K																				S		K	
CJ13					K																				S		K	
CJ20					K																				S		K	
CJ21	G	Q			K																				S		K	
DF28						R	L																		S		K	
DF282R						R	L																		S		K	
DF284					K																				S		K	
DF289					K																				S		K	
DF290					K																				S		K	
DF307					K																				S		K	
JMM19						R																			S		K	
KF06	G					R																			S		K	
KF08						R																			S		K	
KF09		Y	G		K																	I			S		K	
KF12					K																				S		K	
KF14					K																L						K	
Leeds					K		L																		S		K	
M162b					K													R			L				S	V	K	
M162e					K													R			L				S	V	K	
M162j					K													R			L				S	V	K	
MH05-1					K																				S		K	
MH05-13					K																				S		K	
MH05-14					K																				S		K	

MH06-01					K																		S		S		K	
MH06-11					K								F												S		K	
MH06-12	G					R				K															S		K	
MH08-5					K																				S		K	
MH10-04	G					R			I	K															S		K	
MH10-09					K			G							V										S		K	
MH10-15																									S		K	
MH11					K													R							S		K	
MH12					K													R							S		K	
MH12-3																									S		K	
MH12-9					K													R			L				S		K	
MH13-04					K																				S		K	F
MH13-5					K														M						S		K	
MH13-7						R																					K	
MH15-03					K		L																		S		K	
MH17					K																				S		K	
MH18					K																				S		K	
MH20					K																				S		K	
MH21																					L				S		K	
MH23					K																				S		K	
MH26					K															N					S		K	
MH27					K																				S		K	
MH28					K																				S		K	
MH43					K																				S		K	
N219					K																				S		K	
N281					K													R			L				S		K	
N290					K													R			L				S		K	
N348					K																				S		K	
PL5				E	K																				S	V	K	
S019M					K		L											R						K	S		K	
S019M2R					K		L											R						K	S		K	
S035M					K												D				L				S		K	
S042					K																L				S		K	
S048					K																				S		K	
S070			G		K							R													S		K	
S104	G					R				K															S		K	

Sprott	K									S	K
U006		L			M	D				S	K
U018		R								S	K
U156	K									S	K

Table 8-4 - Amino acid variations present in the gyrB genes of all isolates analyzed in reference to ATCC 21134.

Isolate	**CDS**										
	67	**143**	**194**	**302**	**313**	**385**	**403**	**527**	**552**	**567**	**579**
ATCC 21134	L	K	I	A	T	I	R	F	S	Q	A
ah3-20				S		V					
ah3-28				S		V					
ah3-36				S		V					
AH58											
AH116											
CJ02											
CJ05											
CJ10											
CJ12											
CJ13											
CJ20											T
CJ21										K	
DF28											
DF282R											
DF284											
DF289											
DF290											
DF307											
JMM19					I						
KF06					I						
KF08		R	V								
KF09											
KF12					I			L			
KF14											
Leeds											
M162b											

M162e							
M162j							
MH05-1							
MH05-13							
MH05-14							
MH06-01							
MH06-11							I
MH06-12							
MH08-5							
MH10-04							
MH10-09							
MH10-15							
MH11				I			
MH12				I			
MH12-3							
MH12-9							
MH13-04							
MH13-5							
MH13-7							
MH15-03							
MH17			V				
MH18			V		V		
MH20	S						
MH21			V				
MH23			V				
MH26			V				
MH27		R					
MH28			V			H	
MH43							
N219							
N281							
N290							
N348							
PL5							
S019M							
S019M2R							

S035M	
S042	
S048	
S070	
S104	
Sprott	V
U006	
U018	
U156	

Table 8-5 - Amino acid variations present in the parC genes of all isolates analysed in reference to ATCC 21134 (divided into **(a)**, **(b)** and **(c)** due to size constraints). CDS numbers in the QRDR are highlighted in red.

(a)

Isolate	**CDS**																		
	6	**18**	**64**	**91**	**95**	**144**	**154**	**285**	**322**	**339**	**349**	**373**	**423**	**424**	**484**	**509**	**524**	**535**	**537**	**542**
ATCC 23114	K	E	H	S	E	K	A	A	N	S	N	I	N	K	L	H	K	V	T	S
ah3-20	K					R														
ah3-28	K					R														
ah3-36	K					R														
AH58																				
AH116						R	V							R						
CJ02						R														N
CJ05														R						N
CJ10																				
CJ12						R									F					N
CJ13														R						N
CJ20											S			R						
CJ21																				N
DF28							V		S											N
DF282R					K		V		S											N
DF284						R														N
DF289																				N
DF290																	E			N
DF307							V													
JMM19						R								R						
KF06																				
KF08													D							
KF09																				
KF12						R														N
KF14		K				R														N
Leeds						R														N
M162b							V							R						
M162e														R						
M162j							V							R						
MH05-1																				
MH05-13													D							
MH05-14						R														
MH06-01						R										R				N
MH06-11						R														

MH06-12																				N
MH08-5																			A	N
MH10-04																			A	N
MH10-09					V													I	A	N
MH10-15																				
MH11		Y																		
MH12		Y																		
MH12-3		Y																		
MH12-9						R						T								N
MH13-04						R														
MH13-5						R		S												
MH13-7						R														
MH15-03				I																
MH17																				
MH18																				
MH20						R														N
MH21																		I	A	N
MH23														D						
MH26						R		S												
MH27						R		S												
MH28														D						
MH43						R														
N219																				
N281																				
N290																				
N348																F				N
PL5						R														N
S019M																				
S019M2R					K															
S035M						R														N
S042						R														N
S048						R		S												
S070																				
S104																				
Sprott						R														N
U006				I		R				T										
U018						R														N
U156																				N

(b)

Isolate	**CDS**																			
	558	**591**	**602**	**616**	**623**	**632**	**649**	**661**	**689**	**690**	**717**	**719**	**720**	**737**	**738**	**743**	**767**	**769**	**771**	**776**
ATCC 23114	V	Q	S	N	M	V	M	A	V	G	T	S	K	D	T	S	A	T	S	L
ah3-20				S		I										L				
ah3-28				S		I										L				
ah3-36				S		I										L				
AH58				S												L				
AH116				S	I						A				A					
CJ02				S																
CJ05				S	I			V												
CJ10				S	I															
CJ12				S												L				V
CJ13				S	I			V												
CJ20				S		I									A					
CJ21		K		S																
DF28				S	I			V												
DF282R				S	I			V												
DF284				S																
DF289				S												L				
DF290				S																
DF307				S																

JMM19				S									Q				T	A		
KF06				S	I															
KF08				S															N	
KF09				S																
KF12				S	I	I														
KF14				S	I															
Leeds				S												L				
M162b				S	I									A						
M162e				S	I									A						
M162j				S	I									A						
MH05-1				S	I	I														
MH05-13				S														A	N	
MH05-14				S					A	V			Y	A						
MH06-01				S																
MH06-11				S																
MH06-12				S	I											L			N	
MH08-5				S	I									A						
MH10-04				S	I									A						
MH10-09				S	I							L		A						
MH10-15				S																
MH11				S																
MH12				S																
MH12-3				S																
MH12-9				S		I			A					A		L				
MH13-04			P	S	I	I														
MH13-5				S	I	I														
MH13-7				S		I	I													
MH15-03				S												L				
MH17				S	I	I														
MH18				S	I									A						
MH20				S																
MH21				S	I									A						
MH23				S												L		A	N	
MH26				S	I	I														
MH27				S	I	I														
MH28				S												L		A	N	
MH43	I			S																
N219				S		I														
N281				S		I														
N290				S		I														
N348				S																
PL5				S																
S019M				S																
S019M2R				S																
S035M				S	I															
S042				S																
S048				S																
S070				S	I	I														
S104				S																
Sprott				S																
U006				S																
U018				S																
U156				S																

(c)

Isolate	**CDS**																		
	785	**791**	**804**	**816**	**827**	**828**	**829**	**830**	**840**	**841**	**843**	**861**	**862**	**878**	**886**	**894**	**898**	**908**	**910**
ATCC 23114	A	F	K	I	V	D	E	I	Q	G	I	N	Q	I	P	D	D	V	Q
ah3-20												D					N		
ah3-28												D					N		
ah3-36												D					N		
AH58					A	N							E						
AH116					A												N	I	
CJ02					A							D							
CJ05					A	N	K						E					I	

CJ10					A	N	K												
CJ12				V	A												N		
CJ13					A	N	K						E					I	
CJ20					A	N	K						E						
CJ21					A								E						
DF28					A	N	K						E					I	
DF282R					A	N	K						E					I	
DF284					A							D							
DF289					A	N							E						
DF290					A					E	T								
DF307					A								E					I	
JMM19																	N		
KF06					A							D							
KF08					A							S							
KF09					A														
KF12					A											G			
KF14					A					E	T			M					
Leeds					A					E	T								
M162b					A												N	I	
M162e					A												N	I	
M162j					A												N	I	
MH05-1			R		A											G			
MH05-13					A							S							
MH05-14	S				A	N													
MH06-01					A						T							I	
MH06-11					A						T								
MH06-12					A							S							
MH08-5					A												N		
MH10-04					A												N		
MH10-09					A	N											N		
MH10-15																			
MH11					A						T								
MH12					A						T								
MH12-3					A						T								
MH12-9			R		A											G			
MH13-04					A											G			
MH13-5					A											G			
MH13-7																		I	
MH15-03												D			S		N		
MH17			R		A											G			
MH18					A												N		
MH20					A								E						
MH21					A	N											N		
MH23					A							S							
MH26					A											G			
MH27					A											G			R
MH28					A							S							
MH43					A	N	K						E					I	
N219					A											G			
N281					A											G			
N290					A											G			
N348					A	N							E				N		
PL5					A														
S019M					A								E						
S019M2R					A								E						
S035M					A											G			
S042					A								E						
S048					A														
S070			R		A											G			
S104		I			A			M					E						
Sprott					A							D							
U006					A								E					I	
U018					A				K			D							
U156					A								E					I	

Table 8-6 - Amino acid variations present in the parE genes of all isolates analysed in reference to ATCC 21134.

Isolate	CDS																			
	57	**71**	**107**	**122**	**128**	**182**	**218**	**237**	**249**	**252**	**278**	**309**	**326**	**334**	**371**	**387**	**417**	**468**	**523**	**546**
ATCC 23114	N	V	A	V	K	P	D	I	E	L	T	S	E	T	S	K	V	A	N	L
ah3-20																R				
ah3-28																R				
ah3-36																R				
AH58																R				
AH116															F	R	I			
CJ02																R				
CJ05																R				
CJ10									K							R	I			
CJ12																R	I			
CJ13																R				
CJ20																	I			
CJ21																R				
DF28																R	I			
DF282R																R	I			
DF284	S															R				
DF289																R	I			
DF290																R				
DF307												L				R	I			
JMM19																R	I			
KF06																R	I			
KF08						S							K			R	I			F
KF09																R	I			
KF12																R				
KF14																R				
Leeds																R	I			
M162b																R	I			
M162e																R	I			
M162j																R	I			
MH05-1																R				
MH05-13						S							K			R	I			F
MH05-14					R			F								R				
MH06-01																R	I			
MH06-11							N									R				
MH06-12																R	I			
MH08-5																R				
MH10-04																R	I			
MH10-09																R	I			
MH10-15																R				
MH11																R	I			
MH12																R	I			
MH12-3																R	I			
MH12-9			T											M		R	I			
MH13-04																R				
MH13-5																R	I			
MH13-7										S						R	I			
MH15-03																R				
MH17																R				
MH18																R	I			
MH20											A					R		V		
MH21																R	I			
MH23					R	S							K			R	I			F
MH26																R				
MH27																R				
MH28					R	S							K			R	I			F
MH43		I			R	S							K			R	I			

N219		R		
N281		R		
N290		R		
N348		R	I	
PL5		R		
S019M	A	R		
S019M2R	A	R		
S035M		R	I	
S042		R		
S048		R		
S070		R		S
S104		R		
Sprott		R		
U006		R	I	
U018		R	I	
U156		R	I	

Table 8-7 – A list of SNP frequencies observed across the topoisomerase genes of each of the seventy-four isolates analysed. Tables correspond to SNPs present in gyrA **(a)**, gyrB **(b)**, parC **(c)**, and parE **(d)**.

(a) Isolate	gyrA		
	Synonymous SNP	Non-Synonymous SNP	Total
AH116	92	6	98
ah3-20	44	4	48
ah3-28	44	4	48
ah3-36	44	4	48
AH58	47	6	53
CJ02	22	3	25
CJ05	47	4	51
CJ10	53	5	58
CJ12	40	3	43
CJ13	47	4	51
CJ20	38	3	41
CJ21	46	5	51
DF28	51	5	56
DF282R	51	5	56
DF284	42	3	45
DF289	41	3	44
DF290	38	3	41
DF307	43	3	46
JMM19	43	4	47
KF06	56	5	61
KF08	47	4	51
KF09	42	5	47
KF12	37	3	40
KF14	36	3	39

Leeds	41	4	45
M162b	66	6	72
M162e	66	6	72
M162j	66	6	72
MH05-1	42	3	45
MH05-13	45	3	48
MH05-14	44	3	47
MH06-01	37	4	41
MH06-11	49	4	53
MH06-12	53	6	59
MH08-5	36	3	39
MH10-04	44	7	51
MH10-09	45	5	50
MH11	44	4	48
MH12	44	4	48
MH12-3	0	0	0
MH12-9	49	6	55
MH13-04	43	4	47
MH13-5	44	4	48
MH13-7	39	3	42
MH15-03	43	5	48
MH17	42	3	45
MH18	44	3	47
MH20	45	3	48
MH21	44	4	48
MH23	45	3	48
MH26	30	4	34
MH27	45	3	48
MH28	45	3	48
MH43	39	3	42
N219	48	3	51
N281	52	5	57
N290	52	5	57
N348	43	3	46
PL5	60	5	65
S019M	35	6	41
S019M2R	35	6	41

S035M	33	5	38
S042	43	4	47
S048	43	3	46
S070	41	4	45
S104	50	6	56
Sprott	44	3	47
U006	41	5	46
U018	46	4	50
U156	35	3	38

(b)	gyrB		
Isolate	Synonymous SNP	Non-Synonymous SNP	Total
AH116	32	0	32
ah3-20	22	2	24
ah3-28	22	2	24
ah3-36	22	2	24
AH58	29	0	29
CJ02	19	1	20
CJ05	18	1	19
CJ10	20	1	21
CJ12	18	0	18
CJ13	18	1	19
CJ20	16	3	19
CJ21	25	1	26
DF28	8	0	8
DF282R	8	0	8
DF284	11	0	11
DF289	23	0	23
DF290	27	0	27
DF307	23	0	23
JMM19	18	1	19
KF06	18	1	19
KF08	22	2	24
KF09	1	0	1
KF12	19	2	21
KF14	29	0	29
Leeds	29	0	29
M162b	24	0	24
M162e	24	0	24
M162j	24	0	24
MH05-1	23	1	24
MH05-13	29	1	30

MH05-14	34	0	34
MH06-01	16	2	18
MH06-11	9	1	10
MH06-12	6	1	7
MH08-5	22	0	22
MH10-04	29	0	29
MH10-09	18	0	18
MH11	19	1	20
MH12	19	1	20
MH12-3	19	1	20
MH12-9	5	0	5
MH13-04	21	0	21
MH13-5	20	1	21
MH13-7	22	2	24
MH15-03	22	2	24
MH17	23	1	24
MH18	19	1	20
MH20	23	3	26
MH21	19	1	20
MH23	29	1	30
MH26	30	1	31
MH27	18	1	19
MH28	30	2	32
MH43	20	0	20
N219	17	0	17
N281	30	0	30
N290	30	0	30
N348	21	0	21
PL5	25	0	25
S019M	26	0	26
S019M2R	26	0	26
S035M	1	0	1
S042	25	1	26
S048	25	0	25
S070	18	1	19
S104	27	0	27
Sprott	21	1	22
U006	5	0	5

U018	14	0	14
U156	27	0	27

(c)	parC		
Isolate	Synonymous SNP	Non-Synonymous SNP	Total
AH116	57	11	68
ah3-20	17	7	24
ah3-28	17	7	24
ah3-36	17	7	24
AH58	26	5	31
CJ02	29	6	35
CJ05	26	12	38
CJ10	19	6	25
CJ12	31	10	41
CJ13	26	12	38
CJ20	32	10	42
CJ21	24	6	30
DF28	30	13	43
DF282R	30	14	44
DF284	27	6	33
DF289	34	6	40
DF290	28	7	35
DF307	29	6	35
JMM19	28	8	36
KF06	28	6	34
KF08	54	8	62
KF09	23	4	27
KF12	25	8	33
KF14	35	10	45
Leeds	27	7	34
M162b	34	10	44
M162e	31	9	40
M162j	34	10	44
MH05-1	28	7	35
MH05-13	54	8	62
MH05-14	32	10	42
MH06-01	31	9	40
MH06-11	36	7	43
MH06-12	48	8	56

MH08-5	31	10	41
MH10-04	31	10	41
MH10-09	52	13	65
MH11	47	7	54
MH12	47	7	54
MH12-3	0	0	0
MH12-9	31	11	42
MH13-04	27	8	35
MH13-5	25	8	33
MH13-7	20	6	26
MH15-03	22	6	28
MH17	28	7	35
MH18	43	6	49
MH20	30	6	36
MH21	52	11	63
MH23	54	8	62
MH26	25	8	33
MH27	25	9	34
MH28	54	8	62
MH43	26	9	35
N219	16	5	21
N281	16	5	21
N290	16	5	21
N348	39	8	47
PL5	23	5	28
S019M	33	4	37
S019M2R	33	5	38
S035M	31	8	39
S042	33	7	40
S048	25	5	30
S070	30	8	38
S104	29	6	35
Sprott	27	6	33
U006	23	8	31
U018	27	7	34
U156	31	6	37

(d) Isolate	parE		
	Synonymous SNP	Non-Synonymous SNP	Total
AH116	59	3	62
ah3-20	0	0	0
ah3-28	19	1	20
ah3-36	19	1	20
AH58	26	1	27
CJ02	0	0	0
CJ05	0	0	0
CJ10	0	0	0
CJ12	0	0	0
CJ13	0	0	0
CJ20	0	0	0
CJ21	0	0	0
DF28	0	0	0
DF282R	0	0	0
DF284	22	2	24
DF289	28	2	30
DF290	25	1	26
DF307	25	3	28
JMM19	32	2	34
KF06	22	2	24
KF08	38	6	44
KF09	18	2	20
KF12	21	1	22
KF14	0	0	0
Leeds	0	0	0
M162b	20	2	22
M162e	20	2	22
M162j	20	2	22
MH05-1	0	0	0
MH05-13	0	0	0
MH05-14	0	0	0
MH06-01	0	0	0
MH06-11	0	0	0
MH06-12	0	0	0
MH08-5	0	0	0
MH10-04	0	0	0

MH10-09	0	0	0
MH11	25	2	27
MH12	25	2	27
MH12-3	0	0	0
MH12-9	0	0	0
MH13-04	0	0	0
MH13-5	0	0	0
MH13-7	0	0	0
MH15-03	0	0	0
MH17	20	1	21
MH18	13	2	15
MH20	21	3	24
MH21	23	2	25
MH23	38	6	44
MH26	29	1	30
MH27	28	1	29
MH28	38	6	44
MH43	28	6	34
N219	18	1	19
N281	15	1	16
N290	19	1	20
N348	21	2	23
PL5	13	1	14
S019M	15	2	17
S019M2R	0	0	0
S035M	21	2	23
S042	0	0	0
S048	0	0	0
S070	0	0	0
S104	30	1	31
Sprott	21	1	22
U006	0	0	0
U018	0	0	0
U156	0	0	0

Table 8-8 - Bacterial cell count values of *Ureaplasma parvum* isolates grown in media with varying concentrations of sex hormones. U-values and p-values were calculated via Mann-Whitney U-Test.

		HPA5									ATCC-27815									F2								
		Time (Hours)									Time (Hours)									Time (Hours)								
		0	2	4	6	8	10	12	p-Value	U-Value	0	2	4	6	8	10	12	p-Value	U-Value	0	2	4	6	8	10	12	p-Value	U-Value
Beta-oestradiol	0	412	2894	2623	6981	6692	40188	37526	_	_	1664	732	865	2385	3794	15303	28162	_	_	418	147	305	1505	3348	20957	5877	_	_
	62.5pg/ml	1649	1460	2589	8862	12812	114189	123394	0.80	27	735	832	1033	2994	5389	13691	34507	1	25	452	829	1143	1090	3353	106233	245799	0.46	31
	300pg/ml	1692	814	2832	7246	14364	61699	65943	0.71	28	650	781	927	3060	4120	11608	109766	1	24	1434	1326	1138	2799	6381	23649	136275	0.32	33
	1000pg/ml	1652	1295	4283	7681	11866	148577	101054	0.62	29	568	556	2015	2998	4768	27388	27736	1	24	528	577	984	2073	3337	10567	231950	0.54	30
Testosterone	0	7	235	637	1572	4232	50240	45958	_	_	57	169	124	1145	940	2525	8322	_	_	719	5100	6827	9567	9959	30815	56941	_	_
	0.5nmol/L	281	405	876	2796	5822	23111	25967	0.90	26	217	14	84	375	411	51703	35903	1	24	747	1895	1588	3502	5161	5186	6597	0.07	10
	1nmol/L	239	110	775	824	1481	10196	21276	0.62	20	444	72	187	174	693	2554	6008	1	25	260	1794	4812	4644	7241	10972	161221	0.46	18
	2nmol/L	45	139	286	782	2068	18052	58374	0.90	23	134	166	175	536	917	1802	4828	1	24	108	522	871	2884	3030	49193	30784	0.21	14
	8nmol/L	413	32	87	322	911	2211	44212	0.46	18	194	148	67	373	703	4272	7941	1	24	85	421	1644	2381	3479	228426	10574	0.26	15
	30nmol/L	79	28	100	112	151	46758	51960	0.46	18	33	35	64	450	1008	16093	10201	0.80	22	284	280	998	930	982	53406	5131	0.10	11

Table 8-9 - Bacterial cell count values of *Ureaplasma urealyticum* isolates grown in media with varying concentrations of sex hormones. U-values and p-values were calculated via Mann-Whitney U-Test.

	(a)	HPA71									Vancouver								
		Time (Hours)									Time (Hours)								
		0	2	4	6	8	10	12	p-Value	U-Value	0	2	4	6	8	10	12	p-Value	U-Value
Beta-oestradiol	0	3860	2182	2462	10010	15435	16540	24555	_	_	2504	4209	1927	15621	9672	28825	9228	_	_
	62.5pg/ml	41	6528	4129	9017	14245	6761	22620	0.80	22	97	1995	2817	3342	10260	8721	13634	0.46	18
	300pg/ml	1947	1481	6130	8315	14522	2765	18739	0.46	18	142	1340	3863	8532	11642	17024	33955	0.90	23
	1000pg/ml	2216	911	3979	9536	16990	3375	46069	0.80	22	925	1245	5312	8951	13237	25368	12548	0.80	22
Testosterone	0	359	743	1602	5407	10074	39818	45958	_	_	57	169	124	1145	940	2525	8322	_	_
	0.5nmol/L	295	264	575	3133	3922	11639	25967	0.38	17	685388	438962	607361	884492	1104824	1238825	1825342	0.90	26
	1nmol/L	914	373	718	4007	9340	29516	21276	0.80	22	357698	531483	598648	821683	1985083	1930638	2183225	0.71	21
	2nmol/L	159	594	806	3569	7559	43631	58374	0.80	22	523998	494235	585862	874936	724970	1249492	1586916	0.46	18
	8nmol/L	511	146	495	1126	26540	111769	44212	0.71	21	273418	412198	538590	625239	783171	1759930	1594968	0.80	22
	30nmol/L	631	1154	6598	15862	34123	113895	51960	0.62	29	146998	430438	504562	781164	1517648	1790861	1583131	1	24

	(b)	UUg1								
		Time (Hours)								
		0	2	4	6	8	10	12	p-Value	U-Value
Beta-oestradiol	0	2150	1109	4069	10436	18877	7794	9762	_	_
	62.5pg/ml	8436	722	2167	7534	14962	30767	32133	0.62	29
	300pg/ml	1698	565	1466	7788	15257	2189	30633	0.62	20
	1000pg/ml	396	1361	3188	9879	25819	31163	74842	0.62	29
Testosterone	0	390148	641355	902494	1239473	1651327	2311658	2638640	_	_
	0.5nmol/L	170388	616094	806870	1217712	1526931	2187910	2531904	0.71	21
	1nmol/L	196730	598507	764935	1162229	1446166	1643835	2832725	0.71	21
	2nmol/L	138264	443264	800616	1229754	1471282	1955663	2605212	0.71	21
	8nmol/L	141180	538559	756895	1077898	1615575	1771173	2341583	0.71	21
	30nmol/L	231193	435782	761732	804500	1707192	1543945	2157229	0.71	19

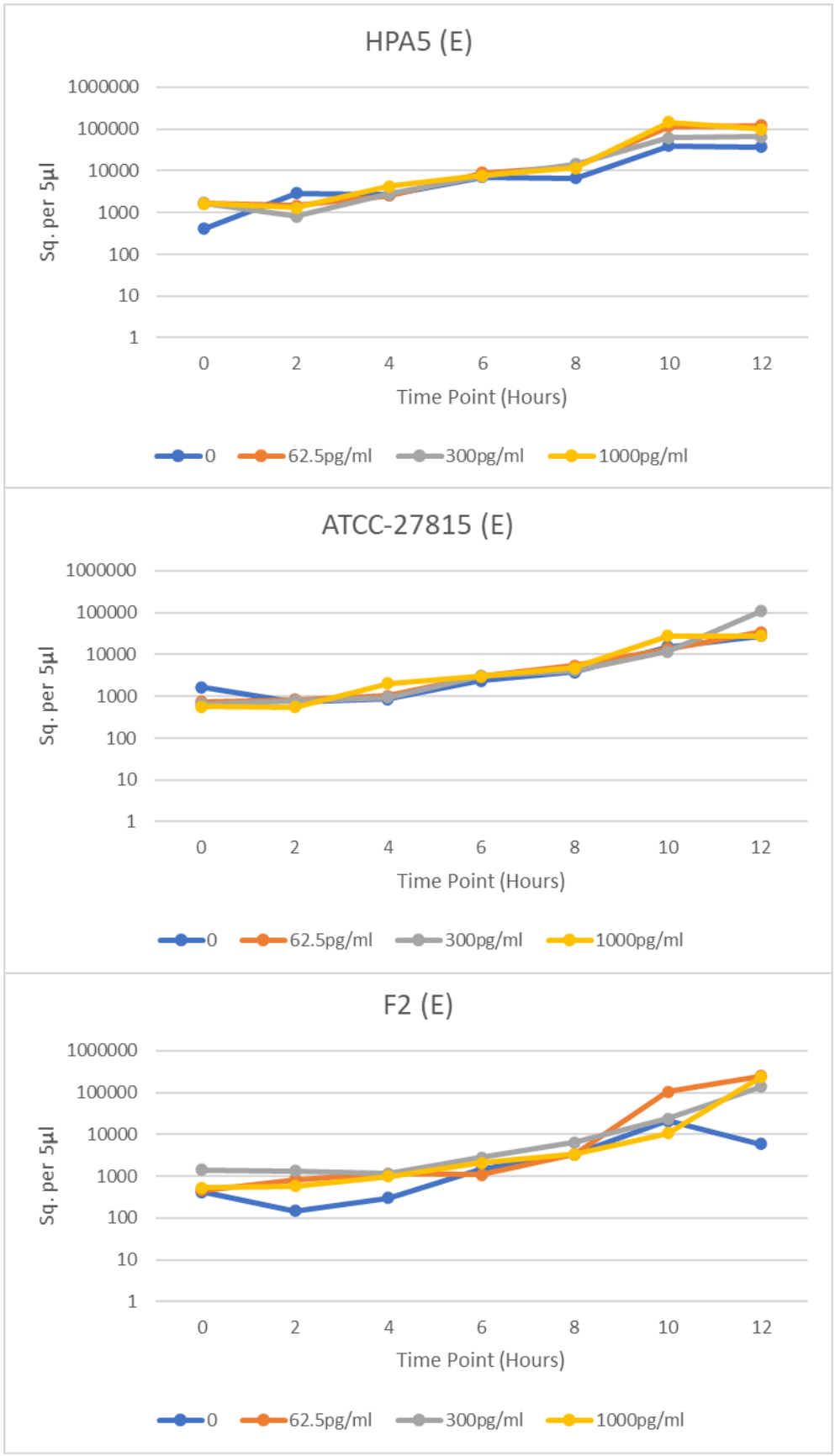

Figure 8-2 – Growth curve graphs for three *U. parvum* isolates cultured in beta-oestradiol. Graphs are plotted exponentially across the 12-hour culture time.

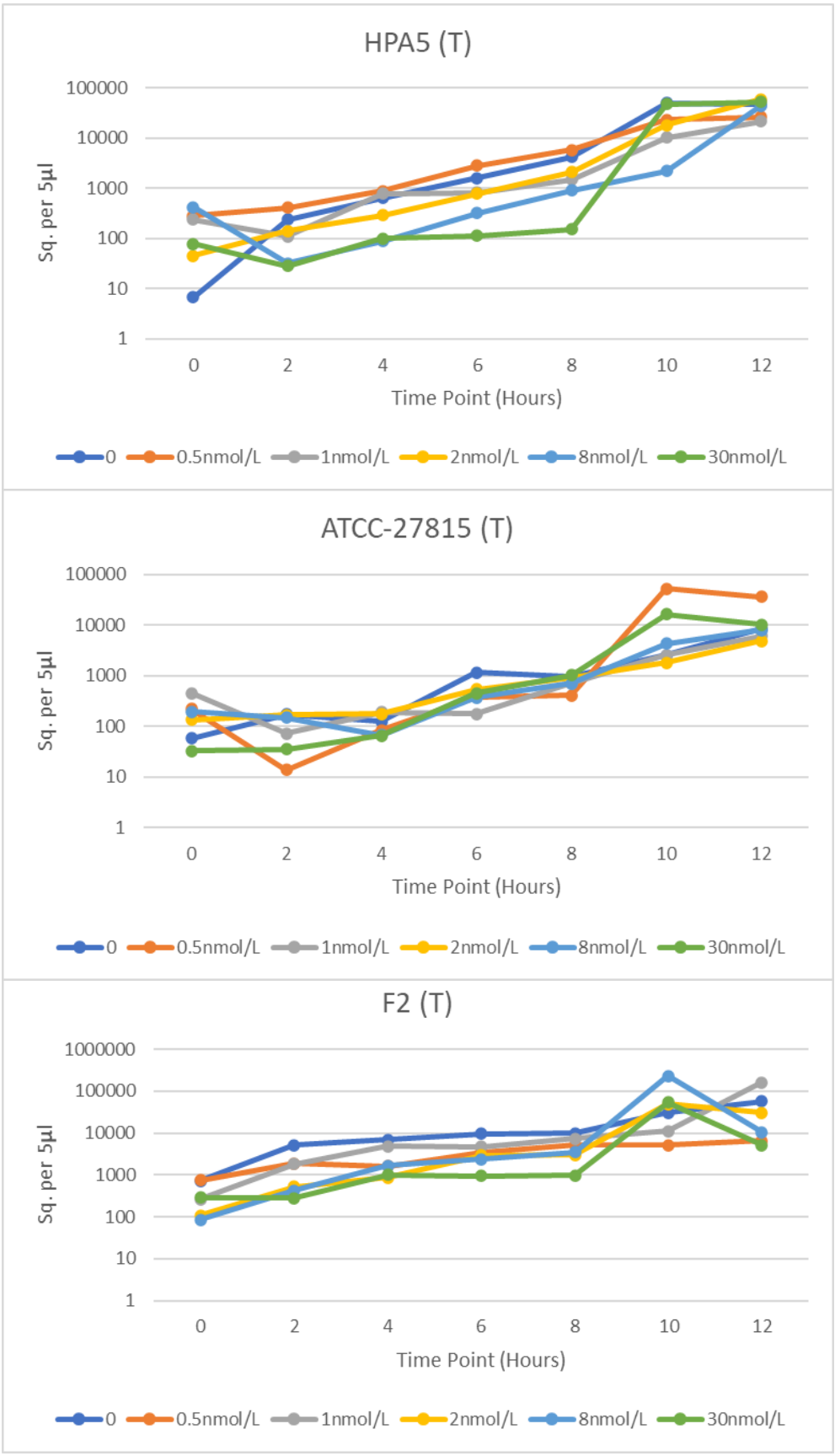

Figure 8-3 - Growth curve graphs for three *U. parvum* isolates cultured in testosterone. Graphs are plotted exponentially across the 12-hour culture time.

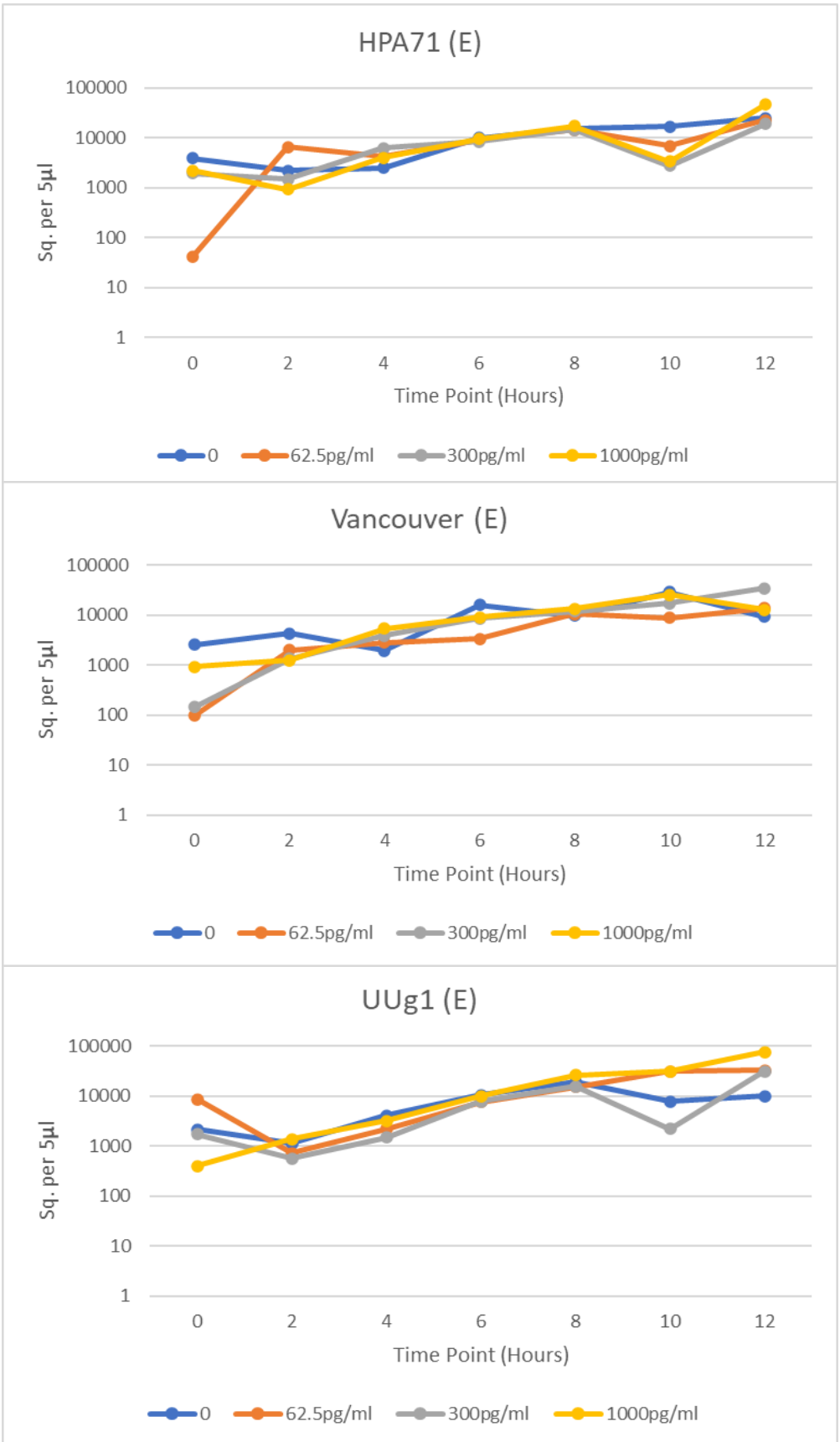

Figure 8-4 - Growth curve graphs for three *U. urealyticum* isolates cultured in beta-oestradiol. Graphs are plotted exponentially across the 12-hour culture time.

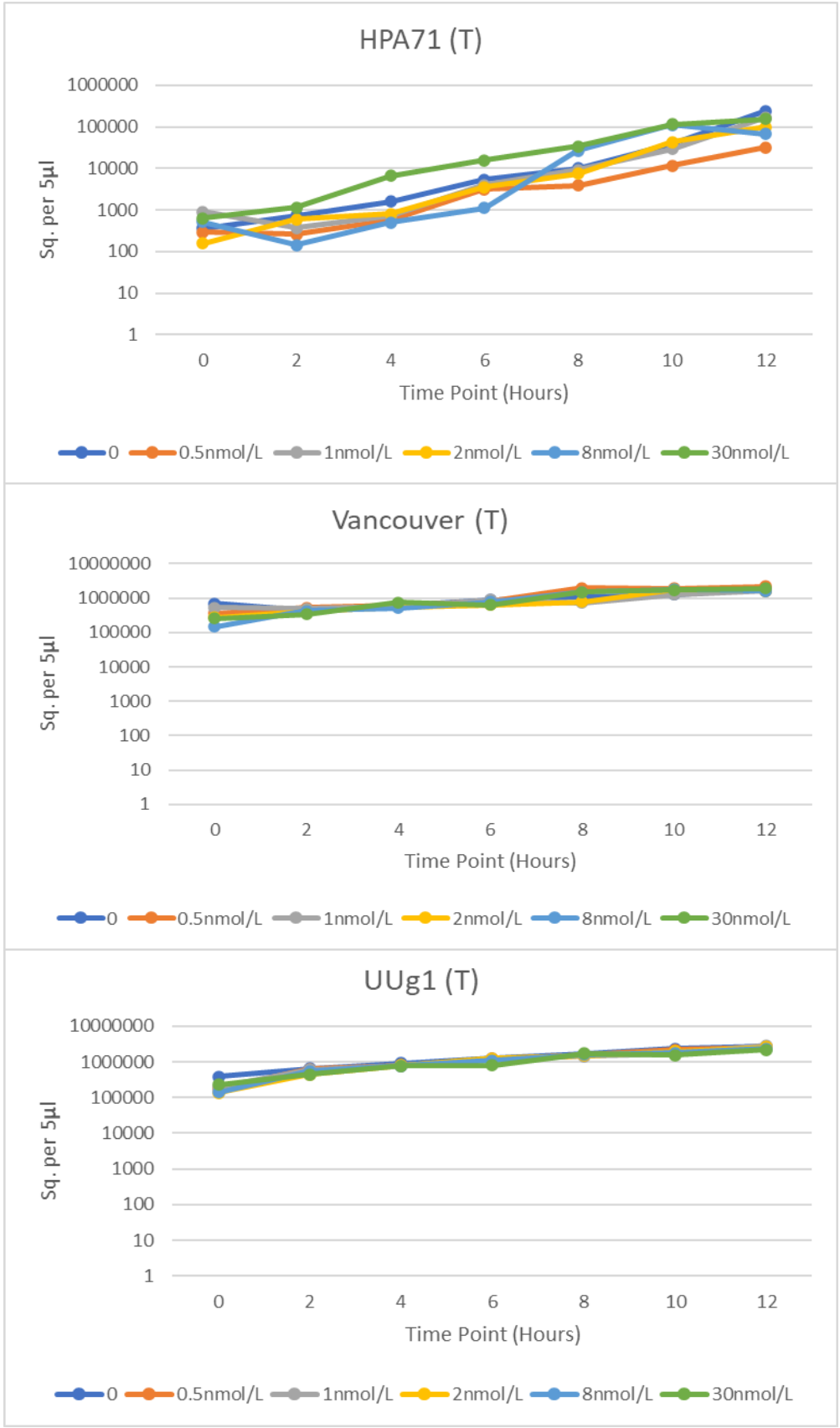

Figure 8-5 - Growth curve graphs for three *U. urealyticum* isolates cultured in testosterone. Graphs are plotted exponentially across the 12-hour culture time.