MYCO WELL D-ONE: Can a new rapid diagnostic detect *Ureaplasma* spp. and *Mycoplasma hominis* in genitourinary medicine samples?

A thesis submitted in candidature for the degree of

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Summary of Thesis

The genital mycoplasmas – *Mycoplasma hominis, Ureaplasma parvum* and *Ureaplasma urealyticum* – are frequently associated with symptomatic genitourinary medicine patients. Currently there is no provision to test for these organisms within the National Health Service. *Ureaplasma* spp. are isolated from 11–26% of men with non-gonococcal urethritis. Bacterial vaginosis (BV) – present in 20–30% of females attending – has been repeatedly linked to *M. hominis*. The MYCO WELL D-ONE is a rapid culture-based assay that utilises specialist media to detect these organisms in samples collected as part of routine sexual health screenings. Alongside detection, the MYCO WELL D-ONE concurrently screens organisms for antibiotic resistance. IRAS ethics number 230693 was granted for this study. 983 Samples were collected from 857 patients attending sexual health clinics in Cwm Taf: 528 female patients (121 provided both urine and swabs) and 329 male patients. This is the first clinical validation of the MYCO WELL D-ONE to be carried out in the UK against the current 'gold standard' molecular detection methods. Isolates highlighted as resistant by MYCO WELL D-ONE were subjected to confirmatory antibiotic susceptibility testing (AST) in accordance with the current CLSI guidelines. Isolates confirmed as resistance underwent whole genome sequencing to determine the underlying genetic mechanism conferring their resistance. *Ureaplasma* spp. was isolated from 57.2% of female patients compared to 21.6% of males. For *M. hominis*, 16.3% of females and 4.3% of males were infected. The MYCO WELL D-ONE displayed sensitivity values of 91.8% and 78.2%, alongside specificity values of 96.49% and 98.84%, for the detection of *Ureaplasma* spp. and *M. hominis*, respectively. Antibiotic resistance rates in *Ureplasma* spp. were found to be 0.54% for tetracycline and levofloxacin, with a tetracycline resistance rate of 1% for *M. hominis*. The prevalence of genital mycoplasma in the South Wales Valleys convergence area is significantly higher in the female population, consistent with other studies at different geographic locations. The MYCO WELL D-ONE is a simple, sensitive and specific means of detecting these organisms. It offers clinicians a rapid, easy-to-use and inexpensive method of determining the presence of mycoplasma in symptomatic sexual health patients.

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Abbreviations

ATP	Adenosine triphosphate
AST	Antimicrobial sensitivity testing
bp	Base pairs
BPD	Bronchopulmonary dysplasia
BV	Bacterial vaginosis
C. trachomatis	*Chlamydia trachomatis*
CCU	Colour changing unit
CD	Circular dichroism
CDC	The Centers for Disease Control and Prevention
CDS	Coding sequence
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CR	Complement receptor
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
G + C	Guanine and Cytosine
G. vaginalis	*Gardnerella vaginalis*
GAG	Glycosaminoglycan
G-CSF	Granulocyte-colony stimulating factor
HIV	Human Immunodeficiency Virus
HPV	Human papilloma virus
HRT	Hormone replacement therapy

Ig	Immunoglobulin
IL	Interleukin
IS	Insertion sequence
IVF	*in vitro* fertilisation
Kda	Kilodaltons
M. fermentans	*Mycoplasma fermentans*
M. genitalium	*Mycoplasma genitalium*
M. hominis	*Mycoplasma hominis*
M. penetrans	*Mycoplasma penetrans*
M. pneumoniae	*Mycoplasma pneumoniae*
MAbs	Monoclonal antibodies
MAPK	Mitogen-activated protein kinase
MBA	Multiple banded antigen
M-CSF	Macrophage-colony stimulating factor
MHSM	*M. hominis*-specific media
MLSK	Macrolides-lincosamides-streptogramin group and ketolides
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MSM	Men who have sex with men
MSW	Men who have sex with women
N. gonorrhoeae	*Neisseria gonorrhoeae*
N. meningitidis	*Neisseria meningitidis*
NF-$_K$B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGU	Non-gonococcal urethritis

NO	Nitric oxide
NPV	Negative predictive value
NSC	Non-specific cervicitis
NSU	Non-specific urethritis
Opp	Oligopeptide permease
ORF	Open reading frame
PAI	Pathogenicity islands
sterile saline	Phosphate-buffered saline
PCR	Polymerase chain reaction
PG	Prostaglandin
Pgk	Phosphoglycerate kinase
PHE	Public Health England
PID	Pelvic inflammatory disease
PPROM	Preterm prelabour rupture of the membranes
PPV	Positive predictive value
PRR	Pattern recognition receptors
PTB	Preterm birth
qPCR	Quantitative polymerase chain reaction
QRDR	Quinolone resistance-determining region
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SPTL	Spontaneous preterm labour
STD	Sexually transmitted disease
STI	Sexually transmitted infection

SV	Serovar
T. vaginalis	*Trichomonas vaginalis*
TK	Thymidine kinase
TLR	Toll-like receptor
TNF- α	Tumour necrosis factor-α
tRNA	Transfer ribonucleic acid
U. parvum	*Ureaplasma parvum*
U. urealyticum	*Ureaplasma urealyticum*
UPEC	Uropathogenic *Escherichia coli*
USM	*Ureaplasma*-specific media
Vaa	Variable adherence-associated protein
VEGF	Vascular endothelial growth factor

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CHAPTER 1
INTRODUCTION

1. Introduction

1.1 Background: The Mollicutes

The *Mollicutes*, of which there are eight genera: *Mycoplasma*, *Ureaplasma*, *Acholeplasma, Spiroplasma*, *Mesoplasma*, *Anaeroplasma, Asteroleplasma* and *Canditatus phytoplasma,* are a unique class of bacteria comprising over 200 species (Figure 1.1), the bulk of which are *Mycoplasma.*[1,2] Sixteen species of *Mollicutes* have been isolated from humans, typically colonising the respiratory or genitourinary tracts.[2] *Mycoplasmas* and *Ureaplasmas* isolated from humans are often characterised according to the substrate they utilise for ATP generation, arginine, glucose or urea (the latter unique to *Ureaplasma*). The name *Mollicutes* is derived from the Latin *mollis* (meaning soft or pliable), and *cutis* (meaning skin), reflecting the fact that bacteria categorised within this class lack a cell wall and are Gram-neutral (Figure 1.2).[3] *Mollicutes* are likely derived from lactobacilli, bacilli or streptococci via regressive evolution and genome reduction. [4] Wolf *et al.* show a monophyletic origin for *Mollicutes* within the *Firmicute* clade through comparative analysis of amino acid sequence data for the highly conserved 'housekeeping' enzyme phosphoglycerate kinase (Pgk). [5] This proposed origin is strengthened by the low G+C content of *Mollicutes*, harping back to their evolutionary past as gram-positive *Firmicutes*. The majority of *Mollicutes* display host and tissue specificities, with colonisation of such tissues being a prerequisite for replication and, ultimately, survival. To enable colonisation, they have developed several molecular mechanisms that help facilitate the evasion of host immune systems, which subsequently permit host-host transmission. Examples of which include molecular mimicry through the presentation of antigenic epitopes shared by *Mollicute* and host cells, phenotypic plasticity, alongside the modulation of and survival within phagocytic and non-phagocytic host immune-cells.[6] Whilst evolving alongside their Eukaryotic hosts, eventually leading to their now parasitic or saprophytic lifestyle, *Mollicutes* gradually lost cell-wall synthesis encoding genes. This unique aspect of their physiology results in *Mollicutes* being inherently resistant to families of antibiotics that inhibit cell wall biosynthesis – the

β-lactams, such as penicillins and cephlasporins.[7] Moreover, this lack of a cell wall results in *Mollicutes* being osmotically fragile, exhibiting plasticity and pleomorphism, leading to a reliance on host eukaryotic cells for osmotic stability.[8,9] Alongside this, *Mollicutes* lost genes encoding enzymes responsible for the generation of certain metabolites, amino acids, nucleotides, and lipids that were plentiful in host cells. Folic acid, for example, an essential carbon-donating cellular metabolite involved in the *de novo* synthesis of purine and pyrimidine (base precursors of DNA synthesis), can no longer be manufactured or utilised by *Mollicutes*.[10] The lack of this biosynthetic pathway confers an inherent resistance to antibiotics that inhibit folate generation — the sulphonamides and trimethoprim.[7,10] Instead, a nucleotide salvage pathway is employed. Thymidine kinase (TK) initiates the retrieval of pyrimidine deoxynucleosides through catalysing the transfer of a gamma phosphate group from ATP or other nucleoside triphosphates to the 5'-hydroxyl group of a pyrimidine deoxyribonucloside.[11] The genomes of *Mollicutes* are markedly smaller than that of model bacteria such as *Escherichia coli*. Typical *Mollicute* genomes range from 580–1,840Kbp, accounting for between 482 and 2050 coding sequences (CDS), whereas model organisms such as *E. coli* have genome and coding sequence sizes of 4,215–4,639Kbp and 4,176–4,320 CDS, respectively.[12] Nevertheless, despite this massive genome loss and consequent minimalistic physiology, *Mollicutes* have retained the ability to synthesise DNA, RNA and the essential proteins required for self-replication. A novel transcription mechanism of note is the use of UGA as a codon for tryptophan, whereas it classically functions as a stop codon in other bacteria[13]. This leads to difficulties in studying *Mollicute* gene expression through classic transgenic and transformative means using model organisms such as *E. coli*, resulting in the expressed *Mollicute* proteins becoming truncated.[14] However, many can be effectively and repeatedly cultured in axenic conditions in specific media that typically contains ~20% serum, [4] which provides the sterols integral to the lipid bi-layer cell membrane structure.[15] Only the haemoplasmas, such as *Mycoplasma haemofelis* and the *Candidatus* phytoplasma species have yet to be cultured *in vitro.* [16,17] *Mollicutes* have been accepted as the smallest and simplest autonomously self-replicating organisms. [18] That said, this 'simplicity' is

ostensible and superficial. If viewed from an evolutionary standpoint it is easy to argue that the *Mollicutes* are 'more evolved' than other prokaryotes. Refining the 'genetic tool-kit', *Mollicute* evolution has engineered elegant solutions to the difficulties of their unusual cellular compactness and facilitated adaptation to numerous and varied environments. For example, Fisunov *et al.*[19] detail a novel group of transcriptional regulators (specifically C-proteins) that control restriction-modification systems in *Mollicutes*. These regulators can bind to several promoters, thus facilitating the streamlining of the restriction-modification system's regulatory network. Such evolutionary advancements are made to conserve energy through regressive evolution, resulting in multi-purpose proteins, making these organisms distinguished, adaptable and fascinating. However, many would counter this viewpoint as the loss of useful biochemical pathways these organisms have endured as a result of regressive evolution could be viewed through the lens of an extended and steady decay toward extinction. In a sense, these are organisms formulating last-ditch survival strategies to avoid extermination, rather than organisms exploiting the niche environments within which their existence is confined. Either way, they provide a unique insight into, and form the basis of the 'minimal cell', identifying the fundamental genetic elements of self-replicating organisms.[20] This may underpin the development of 'artificial life' – one of today's major challenges in Biology.[21] They facilitate a view into the fundamental foundations of the prokaryote-eukaryote interaction and as such, there is an imperative need to study and better understand the *Mollicutes* in all their capacity.

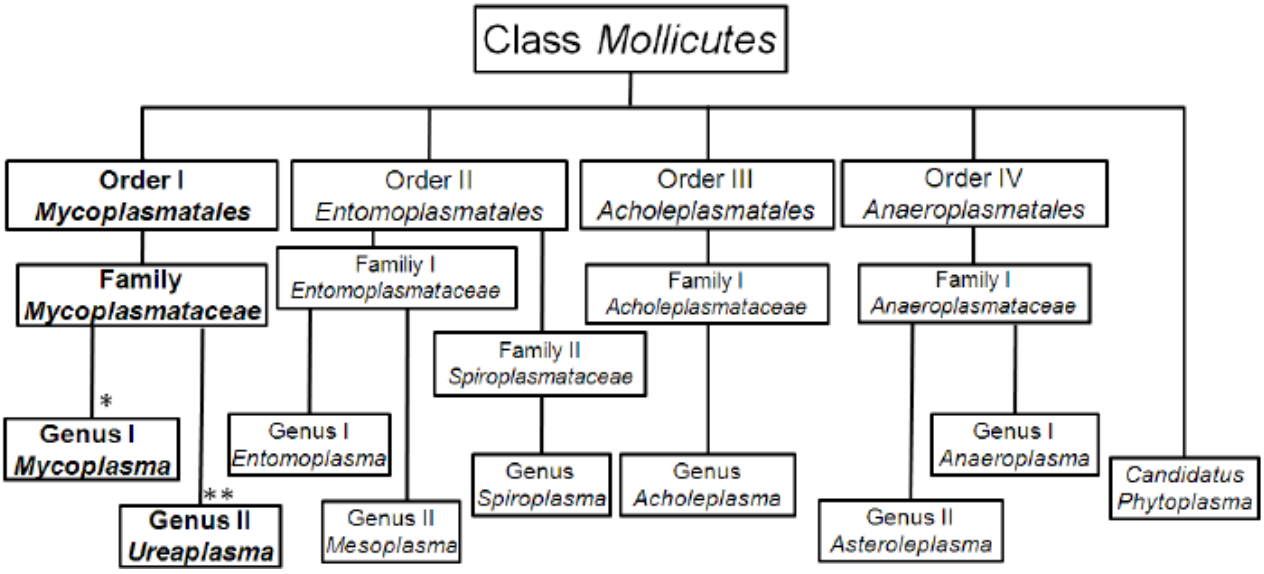

Figure 1.1 Schematic representation of the Mollicute phylogeny. Mycoplasmas and Ureaplasmas are part of the Mycoplasmataceae family.

Source: Giono-Cerezo *et al.* (2012)[22]

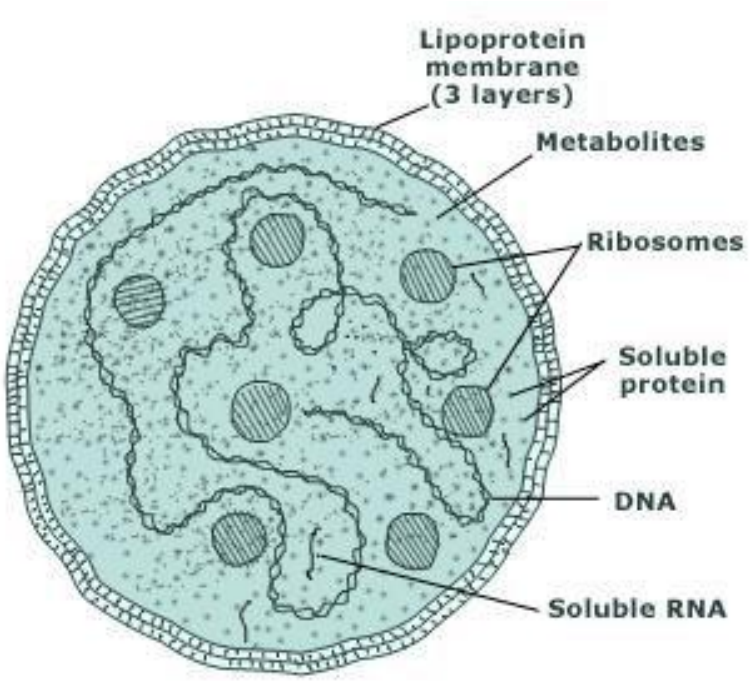

Figure 1.2 Diagram of the mycoplasmal cell. The image highlights the absence of a bacterial cell wall. Displays the fluid triple-layered lipoprotein membrane that encases the mycoplasmal cytoplasm.

1.2 Mycoplasma hominis

1.2.1 Overview

The first *Mollicute* to be isolated from a human in association with a pathological condition was likely *M. hominis* (gaining its current name 10 years later), isolated from a Bartholin's gland abscess in 1937.[23] *M. hominis* is one of four genital mycoplasmas, alongside *M. genitalium*, *M. penetrans* and *M. fermentans*, [24] and is regarded as a commensal component of the flora colonising the lower genitourinary tract, particularly in women, and the respiratory tracts of neonates.[25] Though typically considered an innocuous bacterium, specific pathophysiological mechanisms enable *M. hominis* to act as an opportunistic human pathogen. It is associated with numerous and varied pathological conditions and intrauterine infections: bacterial vaginosis (BV),[26] pyelonephritis,[27] endometritis/pelvic inflammatory disease (PID),[28] and preterm birth or other adverse pregnancy outcomes such as the transmission and infection of neonates. As a typically sexually-transmitted bacterium, barrier methods of contraception are putatively the best preventative measure.[29,30] However, *M. hominis* can be transmitted via extragenital means, being isolated from ~3% of adult upper respiratory tracts, infrequently presenting complications such as pharyngitis and pneumonia.[31] In isolated cases *M. hominis* has been detected as the underlying cause of life-threatening conditions in patients who are hypogammaglobulinanemic, immunocompromised or have endured surgery/trauma, with documented cases of septicaemia, wound infections, meningitis, brain abscesses, arthritis and mediastinitis.[32–35]

1.2.2 Physiology

Due to gene loss, *M. hominis* lacks the genetic components required for ATP generation via traditional energy-generating pathways, i.e. the Kreb's cycle or oxidative phosphorylation. Alternatively, *M. hominis* generates ATP through substrate-level phosphorylation utilising the arginine dihydrolase pathway.[36] Arginine is combined with N-dimethyl-arginine, catalysed by arginine deiminase and N-dimethylarginine dimethyl-aminohydrolase to form citrulline. Subsequently, citrulline is converted to carbamoyl-phosphate by ornithine carbamoyl transferase, resulting in ornithine being released. Finally, ATP is generated via carbamate kinase on carbamoyl-phosphate. The reaction is mediated by an ornithine antiporter (ArcD) requiring no ATP for arginine acquisition, thus making the pathway a maintainable energy-generating option in the absence of glycolytic metabolism.[37] This pathway is useful when broth culturing *M. hominis* due its pH increasing properties. A colour-changing pH indicator (typically phenol red) can be added to *M. hominis* specific media to signify presence/absence detecting increased pH (red colour for phenol red), as organismal growth cannot be determined by visual turbidity. Furthermore, culture of most other prokaryotes (other than urease-containing bacteria) result in an acidification of the growth medium, including *M. fermentans* and *M. pneumoniae*, resulting in phenol red turning bright yellow.

When grown on solid media, *M. hominis* colonies appear in a 'fried-egg' morphology between 200 and 300µm in diameter – individual cells are approximately 200–300nm in diameter.[36] *M. hominis* grows and divides through filament formation, generating chains of multinucleic filaments that subsequently divide into coccoid cells or smaller viable filaments (Figure 1.4).[36,38] The 'fried-egg' morphology is owed to the cells burrowing into the solid media on which they grow, whereby the central portion of bacterial growth penetrates downwards into the agar with growth permeating thinly around the periphery (Figure 1.3).[39]

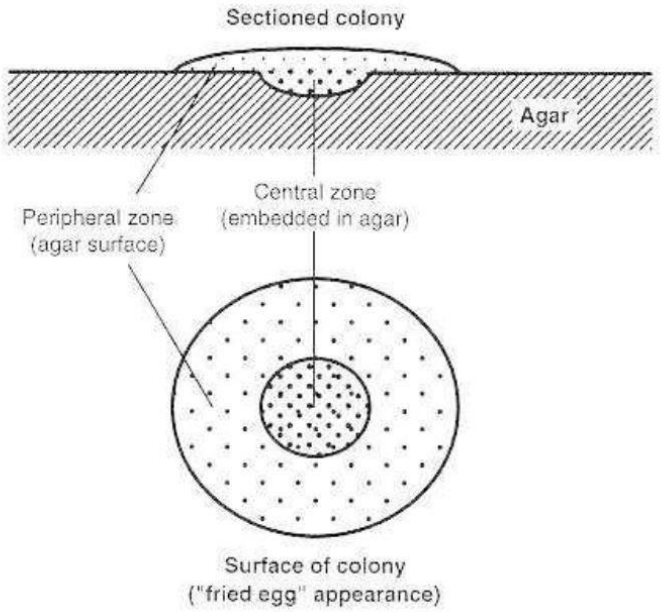

Figure 1.3 Colony morphology of the classic 'fried egg' shape colonies characteristic of Mycoplasma. A dense, burrowing central zone that penetrates the agar, surrounded by the peripheral zone on the agar surface

Source: Razin (1996)[40]

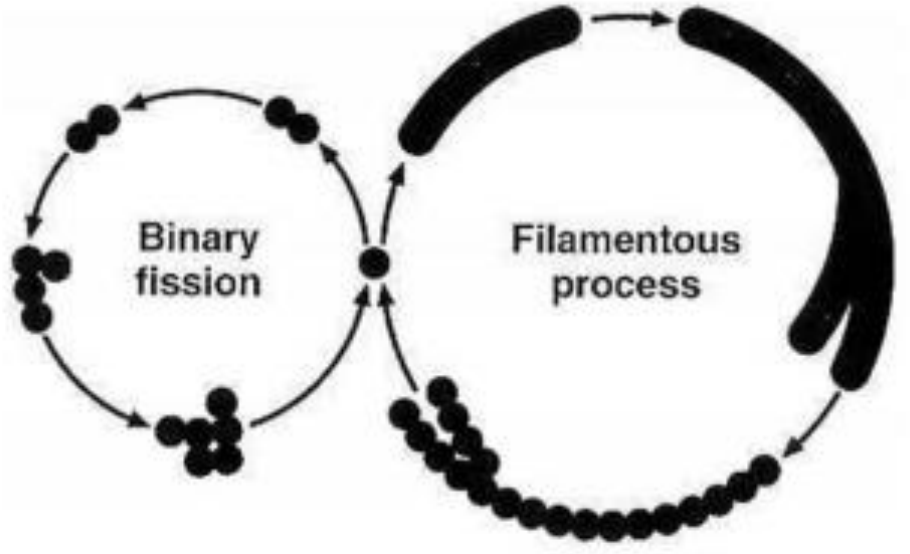

Figure 1.4 Schematic diagram outlining the mycoplasma replication process. Cells can divide by one of two ways, either by binary fission or through a process involving filamentous elongation and subsequent coccoid body formation.

Source: Razin (1996)[40]

1.2.3 Adhesion

To colonise and infect host mucosal surfaces, typically of the urogenital tract, *M. hominis* must first adhere to those surfaces. Many *Mycoplasmas*, such as *M. genitalium* and *M. pneumoniae,* concentrate adhesin proteins into a unique membrane-bound extension of the cytoskeleton named a terminal organelle, facilitating cytadherence to epithelial cells through adhesin-sulphated glycolipid or adhesin-sialoglycoprotein interactions.[41] However, *M. hominis* lacks such a cytoskeletal structure and instead of dedicated organelles, an array of various surface antigens (proteins and lipoproteins) have been identified by monoclonal antibody inhibition assays as playing a role in *M. hominis* adhesion.[42] The core cytoadhesive proteins involved in adhesion are P80 secretin, variable adherence-associated (Vaa) protein/P50, P60 and OppA.[42–46]

1.2.3.1 Surface Proteins

P80 is a membrane-bound surface protein that functions in conjunction with P60 as a cytoadhesin enabling *M. hominis* to attach to host cells. This is evidenced by the reduced capacity of *M. hominis* to adhere to HeLa cells when P80 is masked by three monoclonal antibodies (MAbs) – BE4, LF8 and NB12.[42] Moreover, it is evidenced that P80, following signal 1 peptidase cleavage, is released from the membrane and into the surrounding environment.[45] The precise role of secreted P80 is unclear. If we consider its previously stated immuno-reactive properties, coupled with the existence of P80 homologs in related species such as *Mycoplasma agalactiae* (also highly immune-reactive against polyclonal anti-sera raised in small ruminants),[47] it could play a pro-inflammatory role. Together with this, the secretion of P80 into the environment from its membrane bound P60-P80 complex precursor would likely lead to an alteration of the cell surface structure. With such structures being the typical targets of host immune cells, the change in membrane composition following P80 secretion could potentially interfere with the host effector response, and mediate immune cell

evasion.[42,45] Interestingly, Saadat *et al.* have developed a novel chimeric protein that binds to the conserved antigenic portions of P80, for use in a sero-diagnostic immuno-assay technology for the detection of *M. hominis* infection.[48]

1.2.3.2 Membrane-bound lipoproteins

Deplete of cell walls, *Mycoplasma's* versatility and variability in surface-expressed protein structures is crucial to, and underlies, the adaptability of *Mycoplasma spp.* to colonise the niche environments of host tissues and evade host immune systems. Classically, cell-walled bacteria have developed several mechanisms for dealing with varying and unfavourable environments, founded on a form of gene regulation whereby morphological adaptations are governed by gene activation/repression feedback loops.[49] Such cascade systems are energetically expensive, requiring numerous regulatory and sensing genes that are absent in the *Mycoplasma* genome. An alternative strategy is to generate diversity through high-frequency random mutation, resulting in the production of heterogeneous clonal variants that can survive in the given environment. Although such a strategy bears significant downside in that it requires innovative repair mechanisms that prevent the deletion of essential genetic elements.[50] Again, the limited genome of *Mycoplasma spp.* does not permit such an approach. Instead *Mycoplasma* have evolved a specialised strategy of rapid and reversible modifications to restricted subsets of genes encoding surface proteins, resulting in a multitude of cell-surface variants within a population.[51] This can be achieved through either phase variation, generally referring to an on/off expression of various genes, or via antigenic variation, whereby the bacteria expresses functionally conserved moieties that are antigenically distinct.[52]

Analysis of surface-expressed lipoprotein profiles for *M. hominis* indicates a high degree of heterogeneity, displaying a range of size and phase variants.[53,54] This observed variance in the many characterised and isolated *M. hominis* lipoproteins (Vaa, OppA etc.) is a consequence of complicated genetic systems such as phase variation via homopolymeric repeats. This is where small regions of reiterated bases provide favourable targets for the insertion or deletion of nucleotide sequence

resulting in the aforementioned on/off phase switching.[4] Transient misalignment during DNA replication, a processed termed 'slipped-strand mispairing',[55] is thought to govern the insertion or deletion of nucleotides. The position of the reiterated bases within the regulatory or structural coding region determines the level at which regulation occurs. The antigenic variance observed in *M. hominis* Vaa for example, occurs at the translational level of DNA replication. It has been shown that a single nucleotide oscillation, involving the insertion and deletion of a nucleotide into a short sequence of homopolymeric adenine residues, results in a translational frameshift regulating Vaa generation.[56] Antigenic variation may also be achieved via repetitive domains. This has been demonstrated as the mechanism underlying the observable size variation in *M. hominis* Vaa within an isogenic population. Wise describes size-variant alleles of single copy *vaa* gene, housing between one and four tandem repetitive units of 121 amino acids. To gain or lose one or more of these repetitive sections results in variable sizes of Vaa lipoprotein being expressed.[57]

1.2.3.3 *Mycoplasma hominis* Variable adherence-associated antigen (Vaa)

In 1991, Vaa was first described as a surface-expressed lipoprotein and highlighted as a potential adhesin of *M. hominis* 1620. This inference was made following demonstrable adherence inhibition, utilising monoclonal antibodies.[43] It has been isolated by several other researchers in various forms: as a 49kDa protein from *M. hominis* PG21 and as a 50kDa adhesin in strain FBG termed p50.[58] Isolation of this highly variable protein under differing nomenclature could possibly lead to confusion when reviewing the literature. As the heterogeneity of the protein has subsequently become understood, and the molecular mechanisms that regulate the variability has been elucidated to, standardising the name to Vaa is recommended. Moreover, the variation may be used as a mechanism to further sub-categorise strains of *M. hominis.*[59]

Determination of Vaa length across variants has revealed a range of between 28 and72 Kda. With examination of *M. hominis* 1620 Vaa initially revealed three size variants: Vaa-2, Vaa-3 and Vaa-4.[54]

Subsequent sequencing noted that differences in *vaa* gene length between variants was owed to the number of 363bp intragenic tandem repeats present. The number of repeats is reflected in the strain nomenclature, with the number applied to the Vaa variant corresponding to the number of repeats present – Vaa-2 contains two repeats, and so on.[57] The described repetitive fragments form the basis of grouped sequences – termed 'modules' – the arrangement of which can be used to categorise clonal lineages of Vaa into one of six categories (1-6). Analysis of 42 strains of *M. hominis* *vaa* genes utilised 82% sequence homology as a threshold to qualify regions as identical modules.[60] To date, all *vaa* genes sequenced and analysed begin with adjacent modules I and II coupled with one of two module II variants – II' or II''. Modules I and II form the N-terminal region of the protein. Module I (amino acids 1-27) encodes the prolipoprotein signal peptide of the precursor protein. Module II (amino acids 28-104) encodes a conserved N-terminal region in final protein, though a lack of sequence homology between *vaa* variants results in the divergent names of module I' and II'' being applied to the subsequent sequence. Alongside this, module VI is conserved among all variants encoding a 10 amino acid sequence in the C-terminal portion of the protein. Variation is achieved through modules III, IV, V, VII & VIII forming interchangeable and rearrangeable cassettes of sequence. Differences in cassette number and arrangement correspond to observed Vaa variation (Figure 1.5).[60] Further analysis by Boeson *et al.* deduced high sequence homology between cassettes (44-78% overall), particularly high homology was observed between modules V and VII (67-78%), indicating modules are derived from a common precedent sequence.[60] This sequence homology coupled with the cassette-driven variation suggests a process by which homologous recombination results in the insertion or deletion of whole cassettes. This is supported by the use of homologous recombination as a means to explain the mechanism underpinning antigenic variation and mosaic gene structure of class 1 outer membrane protein in *Neisseria meningitidis.*[61] Furthermore, this antigenic variation through homologous recombination plays a pivotal role in the pathogenicity and antigenicity of an organism, again this pathoadaptive mechanism was eluded to in the case of *N. meningitidis*.[62] Additionally, a repeated and stable sequence motif was noted in Vaa module II

consisting of four amino acids (SFKE), later extended to ELESFKE found in all interchangeable cassettes.[60] This motif is thought to play a part in the adhesive role of the Vaa protein. The vaa gene, or sequences homologous to it, have yet to be isolated from other *Mycoplasma spp.* indicating that intra-species genetic transfer is the causal factor of the currently documented collection of Vaa variants.[59]

Protein models constructed from the previously listed sequence analysis shows that Vaa can be categorised as a monomeric microbial surface exposed coiled-coil protein, with a high degree of similarity to Staphylococcal Protein A (Figure 1.6). Boesen *et al*., through circular dochroism (CD) spectroscopy, show Vaa to display ~80% α-helical content. Furthermore, axial shape ratios indicate an elongated C-terminal region juxtaposed with a globular N-terminal. When CD data is coupled with the sequence data to predict three proline containing coiled α-helices, plus the 1:4 N-terminal axial ratio, this indicates a triple helical coiled-coil structure.[63] The coiled-coils of this N-terminal domain, though predicted to be longer, are like those observed in Staphylococcal Protein A.[64] This triple coiled-coiled N-terminal binds to a membrane bound lipid anchor, binding to a cysteine residue. These helices are separated by looping region comprised of two β-sheets, followed by the C-terminal helix that folds backward toward the elongated helical structure. The Boesen *et al.* model predicts an N-terminal base that platforms a spiked C-terminal that projects outward from, and loops back toward, the surface of *M. hominis*.[60]

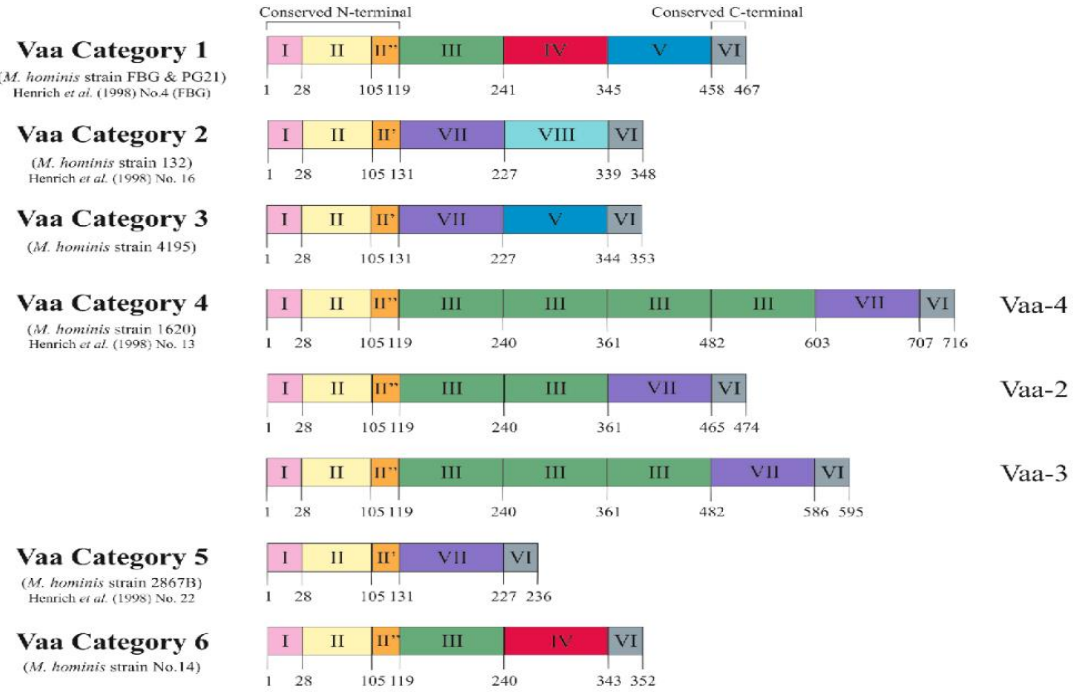

Figure 1.5 A graphical representation of the 6 known forms of Vaa gene constructed from sequence analysis of various M. hominis strains. The proteins are built of modules (1-8) with homologous modules having >82% sequence similarity. The conserved N-terminal is comprised of modules 1 and 2, the conserved C-terminal home to module 6. Consequently modules 3, 4, 5, 7 and 8 form the interchangeable cassettes

Source: Brown, Chalker and Spiller (2014)[59]

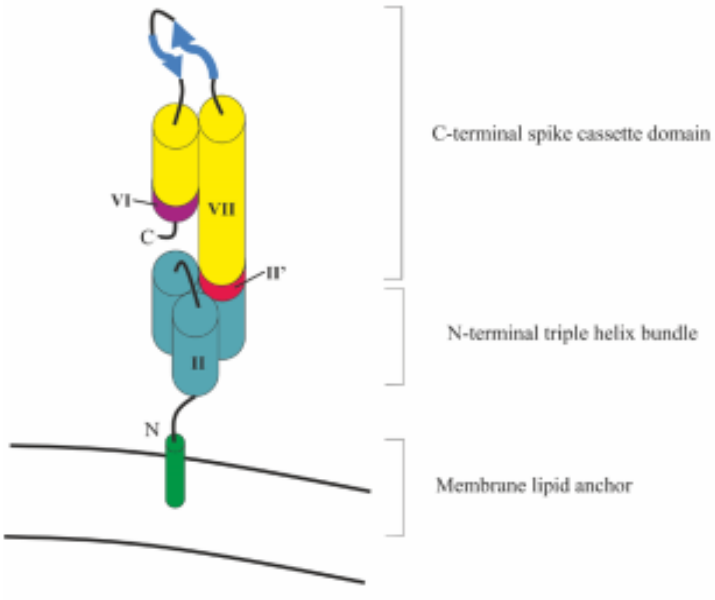

Figure 1.6 Representative model of the protein structure for Vaa category 5 with N-terminal bound to the lipid membrane. Module numbers correspond to those shown above in Figure 1.5.

Source: Brown, Chalker and Spiller (2014)[59]

1.2.3.4 OppA

OppA is a component lipoprotein of the oligopeptide permease (Opp) transport system, responsible for the transfer of oligopeptides into a bacterial cell. An Opp transport system has been identified in *M. hominis*, though it displays little sequence similarity with the corresponding domains of other species. Nonetheless its functionality remains. It is composed of four domains: OppBCDF, alongside the cytoadherence-associated, substrate-binding lipoprotein P100/110 – OppA.[65] OppB and OppC are two integral membrane-bound proteins, whereas OppD and OppF are two nucleotide-binding proteins. OppA was designated as a surface-bound membrane molecule after it was demonstrated that it was proteolytically digested by trypsin following treatment of intact *M. hominis* cells.[42]

OppA has been shown to have multiple functions that extend beyond its use as a substrate-binding domain of the oligopeptide permease system. It performs as an immunogenic cytoadhesin, though binding is inhibited (to HeLa cells) following the application of monoclonal antibody BG11[42], and uniquely, it serves a further function as the main- Mg^{2+} dependant ecto-ATPase hydrolysing extracellular ATP.[66] Numerous functions for ecto-ATPases in a variety of cell types have been elucidated to: for defence against the cytolytic effect of extra-cellular ATP,[67] regulation of ecto-kinase substrate concentration,[68] signal transduction,[69] and also cellular adhesion.[70] In fact, OppA-mediated cytoadhesion in *M. hominis* has been shown to be regulated by its ATPase. Hopfe *et al.* utilised recombinant OppA mutants to demonstrate that ecto-ATPase activity and adherence to HeLA cells are inter-dependent functions of OppA.[71] Additionally, OppA has been demonstrated to facilitate the release of ATP from HeLa cells. Subsequent ATP hydrolysis via ecto-ATPase results in apoptosis of host cells. This is thought to contribute to the survival of extra-cellularly colonising *M. hominis* through nutritional supplementation.[72] Moreover, this indicates that OppA plays an integral role in the pathophysiology of *M. hominis* colonisation.

1.2.4 *M. hominis* pathophysiology

As previously listed, *M. hominis* is associated with a plethora of wide-ranging and varied pathologies. Conditions associated with and influenced by *M. hominis* colonisation will be explored further in this section.

1.2.4.1 Bacterial Vaginosis (BV)

Bacterial vaginosis is a complicated, nuanced and common polymicrobial clinically diagnosed pathology, which typically occurs in women of all ethnicities between the menarche and menstruation.[73] BV is the result of hydrogen peroxide-producing lactobacilli that comprise the normal vaginal flora, being superseded by increasing concentrations of other vaginal organisms such as *Gardnerella vaginalis*, mobiluncus species, anaerobic rods (generally prevotella, porphyromonas, and bacteriodes), peptostreptococcus species, and *M. hominis.*[74] Increases in these bacterial populations will result in the loss of the usual low vaginal pH.[26] Presentable symptoms such as mucosal inflammation; vaginal discharge, itching and burning; a lack of leuokocytic exudate; redness and swelling, are all indicative of BV.[75] Laboratory diagnosis of BV currently relies on gram staining, followed by the application of a score between 1 and 10, termed the Nugent score.[76] This score estimates the proportion of healthy to detrimental bacterial morphotypes present on a vaginal smear stain. However, as *M. hominis* lacks a cell wall they cannot be visualised on a gram stain and as such are not typically considered when clinicians are diagnosing BV.

Developing BV can lead to an increased risk of preterm birth and pelvic inflammatory disease,[75] along with an increased risk of contracting HIV.[77,78] Moreover, Beverly *et al.* have demonstrated that the bacterial load of *M. hominis* positively correlates against female genital-tract HIV RNA load.[79] An explanation of this correlation is offered through *M. hominis* ability to activate long-terminal repeat transcription in HIV-infected cells through a NF-$_{K}$B-dependent mechanism.[80] Another mechanism by

which *M. hominis* can upregulate HIV expression is through the stimulation of tumour necrosis factor-α production (TNF- α), as has been displayed in a murine macrophage cell line.[81]

Taylor-Robinson and McCormack first eluded to the association between BV onset and *M. hominis*.[82] Since then, multiple researchers have highlighted and studied this association in both pregnant women,[83,84] and non-pregnant women.[84–86] Utilising culture-based methods, researchers reported *M. hominis* was isolated from 24-75% of BV cases, compared with 13-22% of women without BV.[87] Detection rates were comparable when PCR was substituted for culture-based detection techniques. Keane *et al*. reported *M. hominis* to be detected in 53% of women suffering from BV, as opposed to zero *M. hominis* detected in women with healthy vaginal flora. Further investigation by Taylor-Robinson and Rosenstein report that *M. hominis* was present in the healthy vagina, however, in those that developed BV, the concentration of *M. hominis* populations increased by a factor of 10,000.[88] In line with this, studies using quantitative PCR have revealed that gene copy number of *M. hominis* present in cervicovaginal lavage samples correlate with Gram stain criteria for BV, the previously mentioned Nugent score.[89] Though the role of *M. hominis* in the pathology of BV is frequently determined to be unclear, it has been shown that toll-like receptors (TLRs) are involved in the pathology of BV. The fluids expressed by the genital mucosa of women with BV act as stimulators of leukocytes, prompting secretion of TNF-α, alongside TLR4 and TLR2 mRNA expression.[90] Following this, Hasesbe *et al.* suggest a mechanism whereby p50, an *M. hominis* cytoadhesin, indirectly stimulates TLR2 expression in macrophages[91], signifying a potential role of *M. hominis* p50-related TLR2 expression in women with BV. The topical application of metronidazole or clindamycin results in decreased *M. hominis* colonisation in women with BV.[92]

Furthermore, the proposal of a symbiotic relationship between *M. hominis* and another BV-related microorganism – *Trichomonas vaginalis* – increasingly solidifies the *M. hominis*-BV association. Researchers have shown, *in vitro*, that *T. vaginalis* is able to act as a vector facilitating the infection of mycoplasma-free trichomonad isolates and human cervical cells.[93] Moreover, *M. hominis* is

capable of residing and replicating within trichomonad cells.[94] As mentioned previously, *M. hominis* metabolises arginine for ATP generation. Similarly, *T. vaginalis* utilises arginine as an energy source under anaerobic conditions.[95] Morada *et al.* demonstrated increased arginine metabolism for co-cultures of *M. hominis* and *T. vaginalis*.[96] Furthermore, the researchers suggest that the rapid removal of cytoplasmic arginine of *T. vaginalis* by *M. hominis*, coupled with an increase in *M.hominis*-exported ornithine would drive putrescine formation by the *T. vaginalis* ornithine decarboxylase. Thus supplying *M. hominis* with a constant source of putrescine, which *T. vaginalis* is incapable of synthesising. The benefit to *T. vaginalis* of this relationship is not as clear, however, increased arginine scavenging may be of importance. This is because it would reduce the amount of freely-available arginine available for nitric oxide (NO) generation by host macrophages, thereby inhibiting an important host defence mechanism.[97,98] Additionally, the synergistic upregulation of inflammatory mediators (IL-1β, TNF-α and IL8) of human macrophages by symbiotically-associated *T. vaginalis* and *M. hominis* has been established *in vitro*.[99] Interestingly, further advantages to the endosymbiosis have recently been elucidated to, with *M. hominis* being shown to down-regulate metronidazole susceptibility-associated genes in *T. vaginalis*.[100]

1.2.4.2 Pelvic Inflammatory Disease (PID)

Pelvic Inflammatory Disease (PID) is a term used to encompass a wide range of clinical manifestations: endocervicitis, endometritis, salpingitis, and peritonitis, for example. It can be broadly characterised as an infection-induced inflammation of the female upper reproductive tract.[101,102] Both sexually transmitted and commensal vaginal flora are isolated from the inflamed tissues, suggesting PID is the result of the ascension of microorganisms from the lower genital tract to the endometrium and fallopian tubes.[103] The major concern surrounding PID is the potential for severe adverse sequelae, such as fallopian tube and reproductive organ damage. Consequently, infertility rates among women suffering acute PID were shown to be 13.3% higher than those without it.[104]

M. hominis is often implicated in PID, being cultured from 8% of fallopian tube samples of women with acute salpingitis. Moreover, 62% of cervix samples from the 50 women with PID diagnosed by lacroscopy were *M. hominis* culture positive. 34 PID negative women absent of laparoscopic signs were all culture negative for *M. hominis*.[105] Additionally there is serological evidence that supports the role of *M. hominis* in PID, with the presence and elevation of increased *M. hominis* antibodies in around 25% of patients diagnosed.[106] The etiological role of *M. hominis* was seemingly further solidified by experimental infection of grivet monkey fallopian tubes by direct inoculation with *M. hominis* D1887. Within 3 days, all monkeys developed parametritis and salpingitis due to swelling and hyperaemia of the tubes, with the parametria becoming oedematous.[107] That said, *M. hominis*' role has been reduced to that of a secondary pathogen by some researchers. This is because whilst *M. hominis* recovery declined following symptom-resolving treatment for patients symptomatic for PID, it persisted in significant numbers of asymptomatic patients' post-treatment. It was found that 71.2% of patients with PID had positive endometrial cultures for *M. hominis* on admission. However, ~29% remained *M. hominis*-positive following treatment with antibiotics that resolved symptoms to which *M. hominis* were determined to be typically sensitive.[108]

1.2.4.3 *M. hominis* and pregnancy

The role of *M. hominis* in adverse pregnancy outcomes is well documented. There are several adverse pregnancy outcomes linked to *M. hominis* such as ectopic pregnancy, preterm labour, abortion, stillbirth, low birth weight, and postpartum or postabortal fever.

Ectopic pregnancy is typically the result of prior PID in which the fallopian tubes have become damaged. As previously mentioned, *M. hominis* has been shown to be an etiological agent involved in the development of PID. Consequently, it is believed that specific cases of ectopic pregnancy can be attributed to *M. hominis* infection.[109] This has been demonstrated to be the case through serological methods, for *M. genitalium*.[110]

As ectopic pregnancy is linked to PID, preterm labour is similarly associated with BV.[111] Preterm birth (PTB) encompasses various clinical presentations, typically: spontaneous preterm labour (SPTL) (~50%), preterm prelabour rupture of the membranes (PPROM) (~30%), and medically-induced PTB due to feto-maternal indications (~20%).[112] The cause of PTB is often multifactorial, however, several bacteria including *M. hominis* are frequently linked to the pathogenesis of preterm birth.[113] As such, *M. hominis* is considered a significant risk factor concerning PTB in pregnant women, and screening for the presence of this organism in the genitourinary tract is recommended.[114] The pathogenic means by which *Mycoplasma* may initiate SPTB or PPROM has not been fully resolved. However, it has been displayed experimentally in other *Mycoplasma spp.* and proposed that the primary mechanism by which *M. hominis* damages host cell membranes is via oxidative stress, through hydrogen peroxide and superoxide radical generation (Figure 1.7).[4,115] Alongside this, the previously described membrane-bound lipoproteins act as further inflammation-inducing cellular components. Evidence of this is given by their ability to induce the maturation of dendritic cells in a TLR-2 dependant manner. This leads to IL-23 secretion, promoting the skewing of $CD4^+$ T helper cells to IL-17 secretion (T_h17).[116] Consequently, IL-17 secretion results in stromal cells (epithelial, endothelial and fibroblasts) upregulating prostaglandin (PG) E_2 production, alongside other cytokines such as IL-

6, IL-8 and granulocyte-colony stimulating factor (G-CSF).[117] Inoue *et al.* showed endogenous PGE_2 was able to regulate the production of proinflammatory cytokines –IL-6, macrophage-colony stimulating factor (M-CSF) and vascular endothelial growth factor (VEGF) by IL-1β-stimulated human synovial fibroblasts –through the activation of EP_2 and EP_4 receptors.[118] PGE_2 is used clinically to induce labour and promote cervical ripening, which could explain *M. hominis* association with SPTB.[119] This is supported by Novy *et al.* whereby intrauterine infection with *M. hominis* in rhesus monkeys initiated a robust pro-inflammatory response, an upregulation of pro-inflammatory cytokines, and prostaglandin (E2 and F2α), all of which contributed to preterm labour and subsequent neonatal infection.[120] Moreover, PGE_2 can also bind to and modulate EP_3 receptor isoforms $EP_{3\text{-}v}$ and $EP_{3\text{-}vi}$ in human uterine cells, resulting in the activation of $G_{\alpha i}$ and $G_{\beta/\gamma}$ subunits of G protein.[121] Activation of the $G_{\beta/\gamma}$ stimulates the mitogen-activated protein kinase (MAPK) pathway, which in turn, is associated with increased production of matrix metalloproteinase 9 (MMP-9).[122] MMP-9 degrades foetal membranes and down regulates glycosaminoglycan (GAG) synthesis, which ripens the uterine cervix, offering a possible mechanism for the association of *M. hominis* with PPROM.[119,121] Additionally, and certainly of note, is that unwanted stimulation (overexpression) of the MAPK pathway has been linked to several malignant neoplasms.[123,124] Moreover, with relevance to *M. hominis* as a genital mycoplasma, Branca *et al.* linked MAPK pathway expression to cervical cancer, though determined it to be an early carcinogenesis marker rather than a predictor of disease outcome.[125] Additionally and regardless of MAPK pathway stimulation, genital mycoplasma colonisation has been determined to be a cofactor in human papilloma virus (HPV) infection,[126] associated with increased human papilloma virus loads, persistent infection, abnormal cervical cytology and abnormal cervical histology.[127] Oncological research has unequivocally demonstrated that HPV infection is the most significant risk factor surrounding cervical cancer.[128]

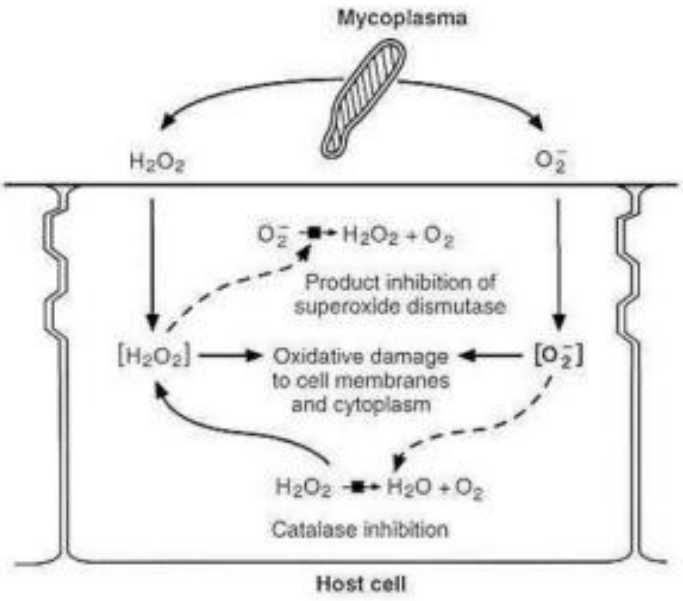

Figure 1.7 A suggested mechanism by which Mycoplasma may cause tissue damage through oxidative stress.

Source: Razin (1996)[40]

1.2.4.4 Female sex hormones: influence on colonisation

It has been shown in mouse models that female sex hormones have a significant effect on the vaginal colonisation of *M. hominis*. For example, *M. hominis* was recovered from 90% of mice treated with oestradiol, with infections persisting for >200 days, compared with a transient infection rate of 10% in untreated mice.[129] Interestingly, treatment with progesterone did not achieve similar levels of colonisation. Furthermore, the contrast in the effect of the two hormones was highlighted when infected mice were treated with progesterone. This resulted in the reproductive cycle entering the dioestrus phase; consequentially the *M. hominis* infection was rapidly resolved with an efficacy comparable to antibiotic treatment.[130]

1.2.4.5 *M. hominis* in men

The role of *M. hominis* in urogenital pathologies remains unclear. However, numerous studies regarding male infertility and *M. hominis* colonisation in Chinese males indicates detection of *M. hominis* in semen samples is associated with low sperm concentration and abnormal sperm morphology.[131,132] It is suggested that *M. hominis* may attach directly to the spermatozoa, and via cellular interactions, negatively affect their vitality, motility, morphology, cellular integrity or molecular structure. The link between non-gonococcal or non-specific urethritis (NGU/NSU) and *M. hominis* is somewhat tenuous, with numerous researchers presenting conflicting opinions on its ability to induce urethritis. Though it is worth noting that *M. hominis* is isolated from ~20% of males suffering from acute NGU/NSU.[133]

1.2.5 *M. hominis* treatment: antibiotics, modes of action and resistance

Infections and colonisation with *M. hominis* are treated using antibiotic therapy. However, their inherent resistance to a panel of commonly prescribed antibiotics restricts the number of appropriate therapeutic agents to the tetracyclines, lincosamides and fluoroquinoles.[134] Though restricted, this is still a seemingly varied range of antibiotics available for the treatment of *M. hominis* infections. However, the list of available antibiotics significantly bottlenecks when treating *M. hominis* infection in neonates and children, whereby administering tetracyclines and quinolones is prohibited. Tetracycline prescription is contraindicated for children younger than 8 due to its affinity for calcifying tissues, possessing the ability to chelate calcium ions and become deposited into, and permanently stain, developing bones.[135] Similarly, quinolones are contraindicated in pre-adolescent individuals due to their potential to negatively impact developing tissues, the cartilage of weight-bearing joints in this case.[136] There exist two mechanisms of inherent resistance to antibiotics in *M. hominis*, the previously described lack of cell wall and β-lactam resistance that applies to all *Mollicutes*, and the species-specific resistance to 14- and 15-membered ring macrolides and telithromycin. The mechanistic underpinning of this novel and inherent resistance was elucidated to through comparison of accumulated, radio-labelled erythromycin in both *M. hominis* and *Mycoplasma pneumoniae*. A marked reduction in accumulated radio-labelled erythromycin in *M. hominis* suggested the presence of an active efflux pump, with subsequent ribosomal affinity for erythromycin being demonstrated. Further sequence analyses of the ribosomal operons revealed a G2057A transition in its 23S rRNA sequence of *M. hominis*[137], a mutation, known to result in macrolide resistance in other species. However, *M. hominis* typically remains susceptible to 16-membered ring macrolides, such as josamycin, alongside lincosamides and ketolides. Nevertheless, several methods of acquired antibiotic resistance have been elucidated to.

1.2.5.1 Fluoroquinolones

Fluoroquinolones are a class of antibiotics that inhibit specific bacterial DNA synthesis enzymes, known as topoisomerases, which are unique to prokaryotes. Topoisomerases are the enzymes responsible for the separation of duplex strands of bacterial DNA; following separation they insert and seal a new strand of DNA into the originally separated strands.[138] They are able to add both negative and positive supercoiling to strands of DNA through either cutting both strands (topoisomerase II) or through acting upon a single strand (topoisomerase I), before twisting the DNA clockwise (topoisomerase I) or anticlockwise (topoisomerase II) and resealing it. Their primary function is to package and unpackage DNA. The two topoisomerase enzymes fluoroquinolones inhibit are known as DNA gyrase and topoisomerase IV.

DNA gyrase is an adenosine triphosphate-hydrolysing topoisomerase essential to prokaryotic replication. This is because, to enable bacterial DNA to exist within the cell, its DNA helix must be extensively coiled, after which the coiled DNA is wound in the opposite direction of the double helix, forming a structure termed a 'negative supercoil'.[139,140] DNA gyrase acts by introducing negative superhelical twists ahead of the replication fork, catalysing the separation of the daughter chromosomes facilitating the initiation of DNA replication by permitting the binding of initiation proteins.[141] Structurally, DNA gyrase is composed of 2 GyrA and 2 GyrB monomeric subunits – A_2B_2 complex – encoded for by the *gyrA* and *gyrB* genes, respectively.[142]

Topoisomerase IV on the other hand controls the unlinking of daughter replicons, facilitating the successful segregation into two daughter cells post-replication – a process termed decatenation.[143] Similarly to DNA gyrase, topoisomerase IV is also made up of four homologous monomeric subunits, 2 ParC subunits and 2 ParE subunits encoded for by the *parC* and *parE* genes.[144]

Fluoroquinolones inhibit both type 2 topoisomerases through interaction with the enzyme-DNA complexes they form, causing conformational changes in the complex, preventing the necessary

enzymatic activity. The newly formed drug-enzyme-DNA complex halts the progression of the replication fork. Consequently, the complete inhibition of DNA synthesis is achieved, resulting in bacterial cell-death akin to apoptosis rather than necrosis.[141,145]

The main resistance-mechanisms to fluoroquinolones that bacteria have developed can be categorised into one of the following: conformational changes in the monomeric proteins of the topoisomerases as a result of mutations in the *gyrA*, *gyrB*, *parC* or *parE* genes; reduction in cytoplasmic accumulation of fluoroquinolone via efflux pumps or restricted drug permeation; and target protection such as plasmid-encoded resistance owed to Qnr proteins that protect target enzymes from quinolone action.[146]

The only described acquired resistance for *M. hominis* is through mutations in target genes, and these occur within regions of genes termed quinolone resistance-determining region (QRDR).[147–149] Characterisation and analysis of the QRDR's for fluoroquinolone-resistance strains of *M. hominis* demonstrated the varying enzymatic targets of different fluoroquinolones. Sparfloxacin, for example, primarily targets DNA gyrase, while pefloxacin, ofloxacin and ciprofloxacin target the topoisomerase of *M. hominis*.[149]

1.2.5.2 Macrolides-lincosamides-streptogramin group and ketolides (MLSK)

MLSKs are a clinically important, chemically heterogeneous group of antibiotics that all act through binding the peptidyl transferase centre of the large ribosomal subunit to inhibit protein synthesis.[150] This ribosomal centre is wholly constructed of RNA and serves to catalyse the formation of peptide bonds during protein elongation.[151,152] Despite the structural variety within the MLSK group, they all appear to interact at the same, or closely related, sites. They bind to nucleotides residing within domain II or IV of 23S rRNA within the eubacterial ribosome. Typically, drug interactions occur within the central loop in domain V where adenosine 2058 (A2058) resides. A2058 and its adjacent nucleotides have been demonstrated to play a significant role in drug binding.[153,154] Though *M. hominis*, as described above, is resistant to 14- and 15-membered ring macrolides such as erythromycin and azithromycin, it is susceptible to 16-membered ring macrolides (josamycin) and lincosamides like clindamycin. Interestingly, it has been demonstrated that *M. hominis* mutants with a C2611U transition on the *rrnB* operon lose their intrinsic resistance to erythromycin, azithromycin and telithromycin.[137]

1.2.5.3 Tetracyclines

Tetracyclines are a family of broad-spectrum antibiotics effective against both gram-positive and gram-negative bacteria. First discovered in the 1940s, they act through preventing the binding of aminoacyl-tRNA to the ribosomal acceptor (A) site of the 30S subunit through the disruption of codon-anticodon interactions between tRNA and mRNA, therefore inhibiting protein synthesis.[155] Tetracycline can bind reversibly to 6 specific binding sites (Tet-1—Tet-6) within 16S rRNA (Table 1.1), of which Tet-1 is principally responsible for the bacteriostatic effects of the molecule.[156] Its primacy is due to its location and the sites higher binding affinity. Tet-1 is in a pocket formed by residues 1054-1056 and 1196-1200 of H34, and 964-967 of H31. 1054 and 1196 protrude from H34 toward

Table 1.1 Table of Tet-ligands, their relative occupancy and locations in the 30S ribosomal structure.

the A-site, and this protrusion permits the hydrophobic attachment of the tetracycline molecule, inducing a small conformational change.

Ligand	Relative Occupancy	Location in 30S Structure
Tet-1	1.0	A964–G966 (H31), G1053, C1054 (H34), A1196–G1198 (H34)
Tet-2	0.7	Lys85, Val92–Leu96 and Leu188 (S4)
Tet-3	0.65	C1162–G1164 (H40), G1172–G1174 (H40)
Tet-4	0.53	G941, G942 (H29), C1342, G1343 (H29), A1349–U1351 (H43), Gln124 (S9)
Tet-5	0.41	U244–G247 (H11), G894–G896 (H27)
Tet-6	0.41	A937–A938 (H28/H29), C1378–U1380 (H28), Arg4, Arg5 (S7), Arg120 (S9)
Edeine	1.0	US190–US192(H24), G926 (H28), U1498, U1505 (H45) G693 × C795[b] (H23 × H24)

Source: Table adapted from Pioletti *et al.* (2001)[156]

Alongside their use as a clinical therapy, tetracyclines have been routinely added at subtherapeutic levels to agricultural animal feeds to act as growth promoters. This practice has raised significant concerns that their utilisation as a growth promoter has contributed to the emergence of tetracycline-resistant human pathogens.[157]

Resistance to tetracyclines is typically mediated through the acquisition of new genes, frequently those with conjugative mobile elements associated with plasmids, transposons, or both.[158] Currently, 38 *tet* and *otr* genes have been described, including 23 genes coding for efflux proteins, 11 genes coding for ribosomal protection proteins, and 3 that code for an inactivating enzyme with the 1 remaining gene having an undetermined resistance mechanism.[159] Well documented examples of the efflux protein-coding genes include *tet*(A) and *tet*(B). These efflux protein-coding genes provide resistance through the production of membrane-associated proteins that function by exporting tetracycline from the cell, lowering intracellular concentrations of the antibiotic, and protecting and preventing attachment to the ribosome.[158]

Ribosomal protection protein-coding genes, such as the well-studied and best characterised *tet*(M) and *tet*(O) genes, confer resistance through the production of cytoplasmic proteins that shield the ribosome from tetracycline molecules.[160] Burdett documented that the Tet(M) protein permits the aminoacyl-tRNA to bind to the ribosomal A-site in the presence of inhibitory concentrations tetracycline. Moreover, Tet(M) protein has been shown to result in ribosomal release of bound tetracycline.[160] Ribosomal protection proteins are homologous to elongation factors EF-Tu and EF-G.[161,162] However, Tet(M) protein has a greater binding affinity for the ribosomal binding sites than EF-G, indicating Tet(M) must be released prior to EF-G binding.[163] Tet(M) acts in a ribosome-dependent GTPase manner, though it has been demonstrated the hydrolysis of GTP is not critical through the use of nonhydrolyzable GTP analogues.[160] Furthermore, Tet(M) contains a loop structure termed loop III residing in domain IV, residues Y506 and Y507 within the loop III complexes with GTP and detects ribosome-bound tetracycline. These bound residues augment the conformation of

nucleotide C1054 within H34 of the 16S rRNA compromising the tetracycline binding site. Additionally, the altered conformation can result in the dissociation of bound tetracycline from the ribosome and prevents rebinding.[164] Calcutt and Foeking characterised the *tet*(M) gene of the clinically isolated Sprott strain of *M. hominis*, revealing it has an incomplete and truncated Tn*916*.[165] Tn*916* is an integrative and conjugative element (ICE) or conjugative transposon. These elements, alongside plasmids, permit horizontal gene transfer intercellularly whereas transposons are restricted to intracellular movement.[166] The researchers also stated that though the truncation precluded Tn*916* from mobility, the larger and uncharacterised transposon within which Tn*916* resides retained its ability for excision and circularisation. This larger transposable element surrounding the tn*916* and *tet*(M) could be the composite unit responsible for the transmission of tetracycline resistance in *M. hominis*. Dordet-Frisoni *et al.* outline the mechanistic underpinnings for the intraspecies dissemination of the previously mentioned, *tet*(M) harbouring, mobile genetic element between bacterial populations.[167] However, *tet*(M) positive strains of *M. hominis* can still be susceptible to tetracycline.[168] One of the two susceptible *tet*(M) positive strains described in this study by Degrange *et al.*, had an insertion sequence (IS) that disrupted the leader peptide sequence, likely prohibiting transcription. However, due to the significant energetic cost involved in expressing *tet*(M) in the absence of tetracycline numerous researchers have demonstrated *tet*(M) priming with sub-inhibitory concentrations of tetracycline is required to induce expression. This results in transcriptional attenuation, a mechanism supported by the presence of several inverted repeats present upstream of *tet*(M). These repeats result in stem loops in mRNA, such stem loops can control the speeds of the RNA polymerase and ribosome transcription. The stem loops act to form termination sites during nascent polypeptide formation, couple this with the scarcity of amino-acyl tRNAs such as Cysteine, Methionine and Histidine and protein translation is paused. Due to the formation of the termination loops, *tet*(M) is not transcribed. However, during exposure to tetracycline, the levels of rare amino-acyl tRNA would rise, permitting the ribosome to closely

readthrough the mRNA relative to the RNA polymerase. Consequently, termination loop formation would be inhibited and Tet(M) translation facilitated.[169]

1.3 Ureaplasma spp.

1.3.1 Overview

First isolated by Shepard in 1954, *Ureaplasma spp.* was recovered from men suffering with non-gonococcal urethritis (NGU).[170] Subsequently, in 1962 their status as self-replicating biological entities was confirmed.[171] Following their discovery, *Ureaplasma spp.* were initially described as T-mycoplasmas (tiny mycoplasmas) due to their production of extremely small colonies, that typically range from 5µm-20µm in diameter. Though much of their physiology is shared with the mycoplasmas their unique ability to hydrolyse urea and utilise it as a primary energy source, a separate *Ureaplasma* genus was proposed to taxonomize these organisms. This genus consisted of 14 serovars, determined through metabolic inhibition and indirect epifluorescence assays, all designated within *Ureaplasma*.[172] Pre-2002, the human-related ureaplasmas consisted of a single species: *Ureaplasma urealyticum*, with the 14 serovars divided into 2 biovars; biovar 1 consisted of serovars 1, 3, 6 and 14, biovar 2 consisted of 2, 4, 5, 7, 8, 9, 10, 11, 12 and 13. However, in 2002 Robertson *et al*. proposed the reclassification of the two biovars into two separate species – *Ureaplasma parvum* (previously biovar 1) and *Ureaplasma urealyticum* (previously biovar 2).[173] The species vary in coding capacity, *U. parvum* has the smaller genome typically ranging from 0.75-0.77Mb, compared with *U. urealyticum* with 0.83-0.94Mb. Prototype strains for each species are serovar 3 (strain 27) and serovar 8 (strain T960) for *U. parvum* and *U. urealyticum*, respectively.[173] The pleiomorphic cells of the ureaplasmas are around 0.2µm, which permits their transfer through filters typically used to sterilise solutions.[174]

1.3.2 Urease activity

As ureaplasmas possess no cytochromes and lack quinones, coupled with their lack of enzymes, and incomplete panel of tricarboxylic acid cycle enzymes, oxidative phosphorylation and many types of substrate-level phosphorylation are not possible.[175] Though mycoplasmas typically generate ATP through the fermentation of glucose, or the hydrolysis of arginine, *Ureaplasma*s lack both of these metabolic pathways, and instead, hydrolyse urea.[176] The hydrolysis of urea by *Ureaplasma* spp. is achieved through a cytosolic urease, which generates an increase in intracellular ammonia, along with this, a concomitant increase in proton electrochemical potential Δ*p* is utilised to power *de novo* ATP synthesis.[177] The generation of a transmembrane electrochemical potential, through urease activity and its role in ATP synthesis was further solidified with reports of proton pump inhibitors omeprazole and lansoprazole markedly reducing ATP synthesis in *U. urealyticum*.[178] Moreover, urease activity is not only linked with energy generation but also the pathogenesis of *Ureaplasma* spp. colonisation of the upper urinary tract, and the formation of kidney stones more specifically.[179,180] In addition to this, the hydrolysis of urea can result in ammonia toxicity. The effects of ammonia toxicity, as result of ureaplasmal urease was demonstrated in murine models. Intravenous and intraperitoneal injections of ureaplasmal urease displayed characteristic symptoms of acute neurotoxicity, consistent with ammonia intoxication, resulting in death within 5 minutes. Researchers also displayed that pre-treatment of the *Ureaplasma* with a urease inhibitor (flurofamide) prevented death in another group of injected mice. Unequivocally displaying the cause of mortality in these animals was due to urease-induced ammonia toxicity. However, it should be noted that the doses of bacteria injected into the mice was not a true reflection of levels of bacteria present in naturally occurring infections.[181]

1.3.3 *Ureaplasma* spp. virulence factors

Though not recognised as a notoriously virulent bacterium, as they are routinely isolated as commensals,[182] *Ureaplasma* spp. are regularly determined to be the cause of invasive infections, particularly in neonates.[134] Determining their status as pathogens or commensals is marred in controversy and contradiction, and it would seem the answer lays somewhere in between. For example, *Ureaplasma* spp. are the microorganisms most routinely isolated from the amniotic fluid and placentae of pregnant and postpartum females, this prevalence occurs either in the presence or absence of chorioamnionitis. [183,184] Additionally, they have been linked to spontaneous abortion,[185] preterm birth,[186] chorioamnionitis,[184] and preterm premature rupture of membranes (PPROM).[186] However, given the list of associated conditions, together with *Ureaplasma spp.* accompanying 42% of premature pregnancies, the pathogenesis of this species is difficult to elucidate; especially as not all those colonised experience the adverse outcomes outlined.[187] To resolve this issue, researchers have suggested that virulence may be restricted to specific strains or serovars. For example, it has been demonstrated that *U. parvum* serovar 3/14 and T960 biovar were significantly associated with symptomatic patients with disrupted vaginal flora (reduction in lactobacilli), whereas serovar 6 was significantly related to asymptomatic females with healthy vaginal flora.[188] On the contrary, other studies have not shown any significance between disease and serovar type, with reports linking serovar and overall disease outcome remaining largely inconsistent, possibly due to the size variation of antigens observed within serovars.[189,190] A number of potential virulence factors have been proposed and characterised, which will be discussed here:

1.3.3.1 Multiple banded antigen (MBA)

The MBA is a surface-expressed, immunodominant antigen of *Ureaplasma spp.*, it activates NF-$_{K}$B, resulting in the production of cytokines through the stimulation of TLRs 1, 2 and 6.[191] Watson *et al*. documented how human sera from *Ureaplasma*-infected patients bound to a 71kDa band of

Ureaplasma antigens.[192] Subsequent immunoblot analysis using monoclonal antibodies against MBA, detected a varied and 'laddered', multiple banding pattern, hence the name applied to this antigen.[193] The MBA protein consists of a 5' N-terminal region containing a single peptide and acylation site (a membrane lipoprotein lipid attachment site at a cysteine), with the C-terminal region consisting of multiples of six-amino-acid (encoded for by 18 nucleotides) tandem repeats that are surface-exposed, within which, serovar-specific epitopes are contained.[194] It is this C-terminal domain that is antigenic, binding to antibodies and initiating a host response during *Ureaplasma* infection.[195] The variation in the number of tandem repeats present in the C-terminal region is also responsible for the size variation observed between different MBA.[193,195] Moreover, the N-terminal region was shown to be conserved through all 14 *Ureaplasma* serovars, making it an ideal target for species detection via PCR, though conserved, species-specific polymorphisms allow speciation based on PCR product size.[194]

Evasion of host immune systems is often a prerequisite to colonisation and an adaptive tactic adopted by most pathogenic bacteria. Similarly, *Ureaplasma*s have generated several mechanisms by which they evade host immune defences. For example, much like the Vaa in *M. hominis* previously described, varying antigenicity is achieved through phase variation and phase switching. For *Ureaplasma*s, the first described method of MBA variation was phase variation.[195] It was demonstrated that a clinically isolated SV3 strain from neonatal lung tissue produced two major bands at 53 and 68.5 kDa through immunoblotting. Following this, the isolate was sub-cloned with the resultant clones 1 and 21 expressing the 53 kDa protein and clones 16 and 19 expressing the 68.5 kDa protein. Successive PCR analysis revealed no difference in N-terminal region fragment, evidencing the conservation of the N-terminal region, however, clones 1 and 21 had smaller C-terminal PCR products relative to 16 and 19, indicating the deletion of the tandem repeating units via slipped strand mispairing (previously described). Alternatively, *Ureaplasma* has also been shown to employ an alternative mechanism of phase variation for the expression of the MBA – on/off switching. Monecke *et al*. imposed a selective pressure against cytadherence through the co-

incubation of *U. parvum* and *U. urealyticum* with either erythrocytes or HeLa cells and in the presence of polyclonal antibodies. Immunoblotting profiles of parental cells, compared with those of the generated non-adherent displayed not a reduction in antigen size, but instead, a complete loss of antibody reactivity—the protein was not expressed.[196] Total removal of all repeating units was dismissed as a possibility, supported by the failure of PCR to amplify the non-repetitive, conserved region of *mba* gene in the non-reactive progeny. Later, the genetic underpinning and molecular background of this expression profile was elucidated. The on/off expression of the MBA protein is regulated by phase switching in the *mba* locus. There have been well-described mechanism of phase switching in related *Mycoplasma* spp.; the *vsp* genes of *Mycoplasma bovis*[197] and the *vsa* genes of *Mycoplasma pulmonis*.[198] In both these species the switching off of one gene is coupled with the switching on of other genes within a gene family or locus, with the switching occurring through site-specific inversions within the loci. Zimmerman *et al.* demonstrated that site-specific DNA inversions (specifically the promoter) within the *mba* locus of *Ureaplasma* alter the expression of the *mba* gene (UU375), resulting in the expression of the adjacent gene UU376, producing an alternate transmembrane lipoprotein termed the *Ureaplasma* phase-variable membrane protein 376 (Upvmp376).[199] Furthering this work beyond the *mba* gene, investigation into the MBA N-terminal paralogue UU172 (Figure 1.9) demonstrated at both the genomic and protein level that DNA inversion is responsible for alternating the expression of other open reading frames (ORF).[200] Researchers also highlighted another orthologue and potential inversion site at UU114, where it is hypothesised chimeric genes can evolve. This substantiates the idea that site-specific recombination events as dynamic, and frequently involved strategy, employed by *Ureaplasma* spp. to generate antigenic variation on a wider scale and better evade host immune systems. Identification of the potential tyrosine recombinase thought to be responsible for the mediation of these site-specific DNA inversions followed, and was determined to be, XerC. The model organism *M. pneumoniae* M129 was co-transformed with a recombinase *xerC* gene and *mba* locus adjacent to an active promoter region with subsequent DNA inversion occurring in the *mba* locus.[201]

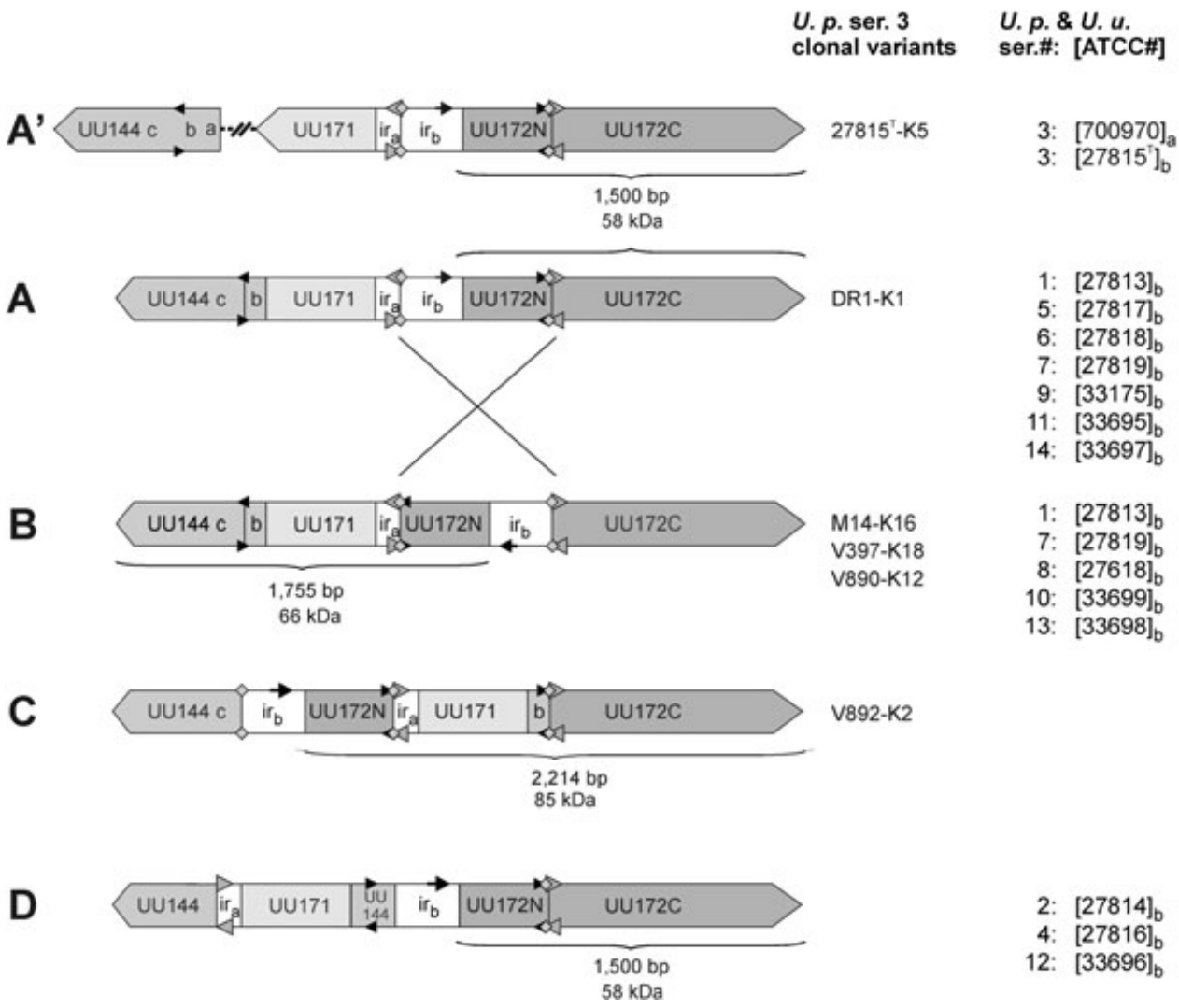

Figure 1.9 Schematic displaying the arrangements of the UU172 element in U. parvum and U. urealyticum. A' displays the UU172 element in U. parvum serovar 3 strain ATCC 700970 and the corresponding locus in clonal variants A-D. The crossing between A and B suggests DNA inversions indicative of phase switching.

Source: Zimmerman *et al.* (2011)[200]

Aside from the evasion of host immune defences, another factor critical in the establishment of infection and colonisation is an organism's ability to adhere to specific tissues, in the case of *Ureaplasma* spp., mucosal surfaces. Numerous reports of *Ureaplasma* spp. attaching to varied types of human cells exist, ranging from spermatozoa,[202] neutrophils,[203] erythrocytes,[204] and human respiratory cells.[205] Yet, the precise adhesin molecules governing *Ureaplasma* spp. cytadherence are not clearly defined. However, cytadhesion inhibition studies carried out on *U. urealyticum* SV8 and HeLa cells showed adherence-inhibition with pre-treatment of *Ureaplasma* cells with HeLa cell extracts, alongside partial inhibition with pre-treatment of either HeLa or *Ureaplasma* cells with N-acetylneuramic acid.[206] Partial adherence-inhibition with N-acetylneuramic acid pre-treatment has been observed for *M. pneumoniae* and several other mycoplasmas, therefore it is assumed that the receptors for *Ureaplasma* spp. adhesins are sialyl residues, or sulphated compounds, or both.[134] As one of the dominant surface-expressed lipoproteins, it has been suggested that MBA is one of the adhesion molecules that mediate the attachment of *Ureaplasma* spp. to HeLa cells and erythrocytes.[196]

Another MBA-related effect of *Ureaplasma* spp. on host tissues, is MBA's role in ureaplasmal pathogenesis. Utilising ovine models, the MBA's effect in intra-amniotic infection with *Ureaplasma* spp. displayed that MBA variation was inversely proportional to the severity of inflammation within the chorioamnion. Infections containing a low number of MBA variants were associated with severe inflammation, whereas animals colonised with many MBA variants showed a relatively insignificant inflammatory response.[207] Thus, indicating that MBA expression patterns *in vivo* play a role in mediating disease in the host. Further investigations utilising *Ureaplasma*-infected ovine models did support the notion that low numbers of MBA size variants were associated with severe histological chorioamnionitis. However, it is worth noting that they did not find a significant difference between chorioamnionitis resulting from avirulent-derived and virulent-derived strains of *Ureaplasma* spp., though this is attributed to the low number of MBA variants generated *in vivo* – 4.2 and 4.6 variants, respectively. Nevertheless, the researchers uncovered a novel finding that maternal humoral

response correlated with the expression of chorioamnion cytokines, highlighting the significance of the host response's role in *Ureaplasma*-related inflammation-mediated adverse pregnancy outcomes.[208] Utilising *Ureaplasma* spp. isolated from human placental tissue, determining serotype/strain and MBA size variation, isolates were correlated alongside the severity of chorioamnionitis and the cytokine profile of blood cytokines. Moreover, *in vitro*, varied sizes of recombinant-MBA proteins were applied to THP-1-derived macrophages and NF-$_k$B activation and cytokine responses were calculated. The study concluded, and agreed with Dando *et al.*, that species or serovars have no impact on virulence, but MBA size variation is inversely related to the incidence of chorioamnionitis, strengthened by the lower levels of cytokines IL-8 and G-CSF in cord blood samples. This hypothesis was further bolstered by the *in vitro* findings; differently size recombinant MBA proteins elicited variable cytokine responses and modulated expression of macrophage NF-kB p65.[209]

1.3.3.2 Phospholipases

Phospholipases are a varied subgroup of lipolytic enzymes that facilitate the hydrolysis of phospholipid ester linkages through phosphodiesterase, coupled with acyl hydrolase, activity.[210] They have been isolated from, and documented to play several roles in, the pathogenesis of a plethora of bacterial pathogens.[211] Similarly in *Ureaplasma* spp., phospholipases have been implicated as virulence factors in ureaplasmal pathogenesis.[212] It is established that human term labour is initiated by chorioamniotic phospholipase A2 through the liberation of arachidonic acid esters from the chorioamnion membrane-phospholipids, causing subsequent production of placental-membrane prostaglandins. This coupled with the fact that the specific activities of bacterial phospholipase A are several times higher than phospholipid A2 of the chorioamnion membrane.[213] Therefore providing a proposed mechanism to account for the association between *Ureaplasma* spp. colonisation and preterm labour.[214] Despite the studies conducted carried out on these ureaplasmal enzymes, the complete published genome data failed to find any putative

orthologous sequences, though this could be owed to sequence divergence compared with other bacteria.[215] Phospholipase derived from non-*Ureaplasma* sources has been shown *in vitro* to inhibit pulmonary surfactant activity.[216] As such, researchers have proposed *Ureaplasma* phospholipase as a contributing factor in the development of acute inflammation and chronic lung disease in neonates colonised with *Ureaplasma* spp.[217]

1.3.3.3 *Ureaplasma* spp. IgA protease

Immunoglobulin A is ubiquitous in human mucosal layers, dominating mucosal immunity, therefore the presence of enzymes that breakdown this immunoglobulin in *Ureaplasma*, indicates they play an integral role their virulence.[218,219] IgA proteases produced by *Ureaplasma* are host specific as they are only able to destroy human IgA 1, and were unable to cleave human IgA 2, murine, porcine or canine IgA. The host-IgA protease specificity was further illuminated as canine *Ureaplasma*s were unable to cleave murine IgA but able to cleave canine IgA, and so on, limiting the specificity of the enzyme to the host from which the isolate was obtained.[220] Following the characterisation of the protease, this specificity was determined to be caused by the mechanism by which IgA is cleaved. Utilising a variety of inhibitory molecules, researchers deduced that the enzyme was a serine proteinase that cleaved the IgA molecule between the proline and threonine residues 235 and 236 in the hinge region of the immunoglobulins heavy chain. This region is absent in human IgA 2, explaining the enzymatic specificity of *Ureaplasma* IgA proteinase.[218] Similarly to the previously described phospholipases, analysis of the whole genome sequence did not reveal any orthologues.[215]

1.3.3.4 Haemolysins

An interesting finding that precipitated from the analysis of the SV3 whole genome sequence was the prediction of two haemolysins, predicted from the two genes – *hlyA* and *hlyC*.[215] Haemolysins are extracellular cytotoxic proteins produced by a variety of bacteria, they damage the plasma

membrane of eukaryotic cells through forming lesions in it.[221] In *Ureaplasma*, it is thought that the *hlyA* gene is the dominant of the two, with *hlyC* being an orthologue of one found in *M. pneumoniae*, where its activity is mediated by H_2O_2.[215] The haemolysin produced from the *hlyA* gene could possibly be a notable virulence factor of *Ureaplasma* spp., this is supported by the identification of *hlyA* orthologues that mediate haemolytic and cytotoxic activities in several pathogenic bacteria. Moreover, mycobacteria lacking this gene are non-pathogenic.[215]

1.3.3.5 *Ureaplasma* spp. Pathogenicity Island

A subgroup of genomic islands — the pathogenicity islands (PAI) — were first conceptualised by Hacker and colleagues in the 1980's following the study of the genetic mechanisms underpinning virulence in uropathogenic *E. coli* (UPEC).[222] Researchers detected genetic linkages between several virulence genes, and though this kind of genetic relationship had been previously described and termed 'virulence blocks', the observation that a single deletion event resulted in the loss of two linked virulence genes, the epithet pathogenicity islands was born.[223] These hypervariable genetic regions located within the genomes of pathogenic bacteria, but absent in non-pathogenic bacteria, are thought to be acquired by horizontal gene transfer. Horizontal gene transfer (HGT) can occur in a few ways: natural transformation, PAI or plasmid conjugation, ICEs and bacteriophage or classic transduction.[224] The majority of PAI are decayed ICEs, lacking the mobilisation elements and transfer genes necessary for HGT.[225] To date, no plasmids have been isolated from ureaplasmas. Momynaliev *et al.* identified that a proportion (93%) of strain-specific genes of *U. parvum* are distributed into 4 zones: UU32-UU33, UU145-UU170, UU440-UU447 and UU527-UU529.[226] Following the discovery of these hypervariable regions, the characterisation of UU145-UU170 suggested this region to be a putative pathogenicity island. This hypothesis was encouraging as genetic elements of this fragment had several characteristics indicative of a pathogenicity island: (1) the fragment is not recoverable from all strains; (2) the putative PAI size of ~20Kb is in the range (10-200Kb) of those in other microorganisms; (3) it is found adjacent to tRNA-ile and tRNA-ala, that are thought to provide anchor

points prerequisite to the acquisition of DNA by horizontal gene transfer; (4) presence of genes encoding mobile genetic elements ripX (UU145) integrase-recombinase and a homolog of a recT phage recombinase (UU154). However, characteristics such as the similarity in G+C content of the region with that of the rest of genome are contradictive of classic PAI. That said, the absence of tetranucleotides GCGC and CGCG within the region, coupled with their higher prevalence elsewhere in the genome, could owe to the fragment's 'foreign' nature. Subsequent comparative genome analysis, following the alignment of 14 ATCC *Ureaplasma* genomes, highlighted two major insertion events.[227] The presence of repeated host sequence on either side of the first identified insertion event was consistent with a transposon insertion, with the integrated fragment found to be 11,822bp (UPA3) and 12,293bp (UPA1). Comprised of 8 genes, coding for 6 putative proteins, one of which contained a subtilase domain and another Type 1 specificity subunit restriction protein. The second insertion was isolated from the majority of sequenced serovars (9/14): UPA3 and 6, UUR4, 5, 7, 10, 11, 12), with the inserted DNA segment ~20Kb in length. This insertion event is likely due to a phage insertion due to the presence of three phage genes in the region. The initial gene in the inserted sequence contains a phage integrase domain (UPA3_0153 [GenBank: YP_001752228]) within a region encoding an integrase-recombinase protein. Further downstream a phage recombination protein Bet (UPA3_0162 [GenBank: YP_001752237] was located, with the final gene in the segment being a phage terminase belonging to the sterile salinex family (UPA3_0176 [GenBank: YP_001752251]. The remainder of the genes, though hypothetical, have transmembrane domains with signal peptides, indicating they play a role in surface interactions of *Ureaplasma*. These findings, together with the similar findings of Momynaliev and colleagues, display the extensive evidence for horizontal gene transfer within *Ureaplasma* spp. and that differential pathogenicity is not determined by specific serovars, but is due to the presence/absence of inserted sequences encoding for various virulence factors.[226,227]

1.3.4 *Ureaplasma* spp. Pathophysiology

Ureaplasma spp. have been shown to play a role in several pulmonary and urogenital pathologies, including, but not limited to: NGU,[228] epididymitis,[229,230] infertility, [231,232] chorioamnionitis,[233,234] cervicitis[235] and premature delivery in women.[187,236] Furthermore, these organisms are frequently transferred from mother to infant *in utero* and have been implicated in several neonatal respiratory complications, such as: pneumonia,[237] persistent pulmonary hypertension,[238] and bronchopulmonary dysplasia (BPD).[203]

1.3.4.1 Male

1.3.4.1.1 *Ureaplasma* spp. and NGU

NGU is a clinical manifestation characterised by clear or clouded urethral discharge, together with urethral pruritus or dysuria. It is confirmed following microscopy analysis of a urethral Gram stain revealing two or more polymorphonuclear leukocytes (PMNs) per oil immersion field, alongside the absence of intracellular diplococci.[239] To treat this condition the CDC STD Treatment Guidelines recommends a single 1g dose of azithromycin or doxycycline 100mg twice a day for 7 days, and to abstain from sexual intercourse until the issue is resolved.[240] NGU is typically characterised into one or two distinct categories—persistent NGU and recurrent NGU, however they are often wrongly used interchangeably in the literature. Persistent NGU is NGU that does no resolve following treatment, whereas recurrent NGU is NGU that reoccurs days to weeks following its apparent resolution. The common STI *Chlamydia trachomatis* has been established as the primary causative agent in ~50% of NGU cases, with the other causative agents being composed of *M. genitalium* (15-25%), *T. vaginalis* (10-20%), *U. urealyticum* (10-20%), with the remainder being comprised of various rare viral induced NGU cases such as Herpes simplex virus and adenovirus.[241] Though *Ureaplasma* are thought to be the causative agent in only between 10-20% of NGU cases, urethritis is the most common symptom male patients present at sexual health clinics.

It should be noted that studies prior to the separation of *Ureaplasma* spp. into two species in 2002, all the studies implicating *Ureaplasma* spp. in NGU were taking a mixture of both *U. urealyticum* and *U. parvum* into account. This could be responsible for the often-conflicting reports on this issue. However, post-2002 research has suggested that *U. urealyticum* is associated with NGU, but *U. parvum* is not. For example, Deguchi and colleagues isolated *U. urealyticum* from a significantly higher percentage (18%) of NGU sufferers in the absence of any other identifiable causative agent, compared with 7.3% in controls. However, *U. parvum* prevalence amongst NGU sufferers and controls was 9.9% and 13.2%, respectively.[242] These findings were supported by Couldwell *et al.* determining that *U. urealyticum* was independently associated with NGU, even after controlling for age and sexual history, factors that were thought to be confounding when determining the significance of *U. urealyticum* in NGU.[228] Furthering this, PCR subtyping of *U. urealyticum* clinical isolates from NGU patients and controls has revealed that only subtype 1 (SV2, 5, 8 and 9) were associated with *M. genitalium*-negative nonchlamydial NGU.[243] This aligns with the reports that urethritis was induced in individuals following intra-urethral inoculation with *U. urealyticum* SV5.[244] Additionally, NGU patients displayed raised serum antibody levels against *U. urealyticum* SV2, one of the serovars categorised under the previously listed subtype 1.[245] There is also an indication that bacterial load of *U. urealyticum* influences the clinical manifestations of NGU, as increased numbers of polymorpholeukocytes in urethral smears was associated with increased *U. urealyticum* bacterial loads.[246]

1.3.4.1.2 Infertility

For men, *U. urealyticum* has been linked to the retardation of the spermatozoa and is associated with increased rates of male infertility. However, the interactions between the host and the bacterium that result in this effect have not been fully elucidated, though a growing body of evidence is compounding the damaging effect *Ureaplasma* can have on fertility. Human *in vitro* fertilisation (IVF) studies comparing the success of the procedure between *Ureaplasma* colonised

individuals and infection-free controls, displayed a significant reduction in pregnancy rate per embryonic transfer.[247] Though it is worth noting that fertilisation rates prior to embryonic transfer were ostensibly unaffected. A possible explanation of how sperm may be able to 'fertilise' eggs that result in unviable pregnancy, which would increase infertility per se, is to disrupt chromatin stability and DNA integrity within the sperm, two critical components of male fertility.[248] Researchers have demonstrated that *U. urealyticum* had a direct, time and dose deleterious effect on sperm nuclear chromatin and DNA.[249] So though sperm activity is seemingly retained post-infection, damaged paternal DNA would impede embryonic development.

1.3.4.2 Female

1.3.4.2.1 Cervicitis

Cervicitis, is an often asymptomatic condition, characterised by the inflammation of the columnar epithelial cells of the endocervix.[250] Though the condition can be asymptomatic, the most common symptoms include vaginal discharge or intermenstrual bleeding.[251] It is a prominent condition presented by between 30-45% of women attending STI clinics as it is typically associated with pathogens that are sexually transmitted.[250] Despite its commonality, cervicitis has potentially severe consequences as it is a prerequisite to PID with the potential for severe reproductive sequelae, regardless of whether the patient is symptomatic or not.[251] Moreover, it is established that cervicitis plays an important role in HIV transmission, as it increases an individual's susceptibility to contracting the virus, alongside this, cervicitis induces increased viral shedding in HIV-positive individuals.[252,253] Interestingly, resolution of chlamydial or gonococcal cervicitis through antibiotic treatment resulted in a > 6-fold decrease in cervical HIV-1 RNA, alongside normalisation of cervical polymorphonuclear counts.[254] Treating *Ureaplasma*-induced cervicitis in the same manner, may have similar effects on cervical HIV-1 RNA in HIV positive patients. Furthermore, cervical inflammation and infection, particularly coinfection with HPV, have been linked to numerous epithelial cancers leading to the suggestion that cervicitis is a plausible cofactor in cervical cancer.[255]

It is analogous to NGU in men, in that, though 50% of cervicitis cases are a direct result of *N. gonorrhoeae* and *C. trachomatis* infection, the aetiological agents causing the remainder of cases are difficult to determine.[256] As such, the term non-specific cervicitis (NSC) is applied to these cases, with *Ureaplasma*s being isolated from ~50% of women with NSC.[257] Even though Schilcht *et al*. isolated *Ureaplasma*s from 54% of women symptomatic for cervicitis compared with a colonisation rate of only 16% for those whom were asymptomatic, there are often conflicting reports as to the role of *Ureaplasma*s in NSC.[258] Consequently, relating ureaplasmal infection with cervicitis remains controversial, but ureaplasmal load may play a significant role in the disease. NSC-positive patients presented ureaplasmal loads of both *U. parvum* and *U. urealyticum* at concentrations 10-fold greater than controls.[235] Ureaplasmal load of $>10^5$ CFU/ml is twice as prevalent in the cervico-vaginal tract of women diagnosed with post-partum endometritis, prompting researchers to suggest an aetiological association between *Ureaplasma* load and post-partum endometritis.[259]

1.3.4.2.2 Preterm Birth

Much like *M. hominis*, *Ureaplasma* are frequently associated with adverse pregnancy outcomes such as preterm labour.[260] Alongside this, *Ureaplasma* are recurrently cited as aetiological agents in post-partum morbidities, neonatal lung injury, for example.

The mechanism by which *Ureaplasma* induced preterm birth is through the stimulation of an inflammatory cascade (described in detail previously for *M. hominis*) by the interaction of the bacteria with host pattern recognition receptors (PRR), toll-like receptors (TLRs). Following TLR stimulation, a subsequent rise in inflammatory cytokines IL-1β, TNF-α, IL-6, and IL-8 followed by leukocyte recruitment, results in increased prostaglandins (PGE2 and PGF2-α) and matrix metalloproteinases (MMP) production.[261] These mediatory compounds have a variety of effects in different tissues, with prostaglandins contributing to increased uterine contractions and MMPs playing a role in placental detachment in the myometrium. Whereas, in the cervix, they have a degradative effect on extracellular matrix, leading to effacement and dilation. Moreover, MMPs

have been shown to reduce the prerequisite pressure for membrane rupture. Throughout pregnancy this cascade is typically suppressed by the immunosuppressive molecules progesterone (P4) and IL-10.[261] Though current literature suggests that disproportionate levels of intrauterine infecting organisms may override said molecules protective effects. Perhaps offering support to the notion that *Ureaplasma* bacterial load plays a role in its pathogenicity. Utilising Rhesus monkeys as infection models, researchers demonstrated that sole intrauterine infection with *U. parvum* resulted in uterine activity, preceded by a rise in amniotic fluid leukocytes, MMP, inflammatory cytokines and PGE2 and PG2Fα.[120] Nevertheless, heat-inactivated *U. parvum* SV1 did not induce a significant increase in inflammatory cytokine or PGE2 in foetal membrane samples collected from term placentas. Yet, *U. urealyticum* SV8 has been shown, in high doses (inoculums $>10^6$ CCU/ml), to illicit an inflammatory response through increased TNF-α, IL-10 and PGE2 production in choriodecidual explants.[262] It has also been documented that following the detection of *Ureaplasma* in the amniotic cavity of women experiencing premature contractions, symptoms can be resolved by clearing the infection through the administering of erythromycin, permitting a full term pregnancy.[263]

1.3.5 *Ureaplasma* spp. antibiotic therapy and resistance

Much like resistance mechanisms previously described for *M. hominis*, as *Mollicutes*¸ *Ureplasma* are inherently resistant to several clinically available antibiotics: the cell wall synthesis inhibitory β-lactams and glycopeptides; RNA polymerase targeting rifampins (owing to the mutated beta-subunit of RNA polymerase);[264] folic acid synthesising sulphonamides and trimethoprim (due to nucleoside scavenger pathways); alongside lincosamides that inhibit protein synthesis.[134] In addition to this, aminoglycosides such as gentamicin, which are often used to treat Gram-negative infections have extremely unreliable activity against *Ureaplasma*. Repeated isolation of *Ureaplasma* from CSF, blood and lower respiratory tract of neonates following >3 days treatment with intravenous gentamicin, has been demonstrated.[265] However, another protein synthesis targeting antibiotic is chloramphenicol, which has relatively good activity against *Ureaplasma*, documented in numerous clinical case-studies and *in vitro* susceptibility testing.[266,267] However, this cannot be used in neonates due to toxicity historically referred to as “grey baby syndrome”.

1.3.5.1 Macrolide resistance

Unlike *M. hominis*, *Ureaplasma* are typically susceptible to 14-membered ring macrolides, such as erythromycin, though resistance mechanisms to these antibiotics have been described. Palu and colleagues outlined the first mechanistic description of such resistance in *Ureaplasma*, following the observation of a significant reduction in radiolabelled erythromycin ribosomal binding, in a highly resistant clinical isolate. A point mutation in the 23S rRNA at the purported macrolide binding site was offered as the most likely explanation for the observed mechanistic resistance. Furthermore, *in vitro* experimentation achieved the production of several macrolide resistant *U. parvum* strains, following serial passage in sub-inhibitory concentrations of antibiotic. The induced macrolide resistance was the consequence of a point mutation of A2058, regardless of additional adjacent bases, occasionally accompanied by additional ribosomal accessory protein mutations in L4 and L22.[268]

1.3.5.2 Tetracycline

The only means of tetracycline resistance described for *Ureaplasma* is attributable to the acquisition of the *tetM* resistance cassette, associated with conjugative transposon elements of the Tn916/Tn1545 family.[269,270] It has been suggested that the original transposition of the *tetM* gene into *Ureaplasma* occurred through horizontal transfer, from Streptococci.[271]

The initial incidence of tetracycline resistance in *Ureaplasma* isolated from men suffering with NGU indicated that 10% of isolates possessed the *tetM* gene.[272] This figure was then later solidified following analysis on isolates collected between 1973–1983, revealing 10% tetracycline resistance.[273] However, it is worth noting that isolates were typically collected from patients following unsuccessful treatment, positively biasing the collection of resistant samples. Another interesting finding of the study was that 39% of tetracycline resistant strains displayed erythromycin resistance, compared with 9% of erythromycin-resistant tetracycline-sensitive isolates. Other studies have provided tetracycline resistance prevalence rates as high as 20%, upon analysis of 65 *Ureaplasma* isolates.[274]

1.3.5.3 Fluoroquinolones

There have been numerous reports of fluoroquinolone resistant *Ureaplasma* isolates, with the mechanism underpinning such resistance being owed to point mutations in the QRDR. Mutations typical of fluoroquinolone resistance in *Ureaplasma* lead to amino acid substitutions in the QRDR such as D82N, Ser83Leu, and E87K in ParC and Q104K in GyrA. The most prevalent mutation leading to fluoroquinolone resistance is the Ser83Leu substitution in the ParC protein.[275] This QRDR mutation is consistent and homologous with the fluoroquinolone-resistance-conferring-substitutions observed in resistant *Staphylococcus aureus* and *S. pneumoniae*.[276]It is well documented that the preferred sites of action of fluoroquinolones is the DNA gyrase of Gram-negative bacteria and the topoisomerase IV of Gram-positive.[277] As *Ureaplasma* are thought to have evolved from Gram-

positive ancestors, the greater incidence of ParC mutations is compatible with their evolutionary history. Though the ParC Ser83Leu is the most prevalent resistance mechanism, fluoroquinolone resistance is observed in a limited number of clinical isolates devoid of this substitution.[278] Consequently, mechanisms observed in other bacteria such as: overexpression of efflux pumps, fluoroquinolone-inactivating enzymes, or the presence of plasmid encoded Qnr proteins could potentially be responsible for resistance in these strains.[279]

1.4 M. hominis and Ureaplasma spp. prevalence

1.4.1 M. hominis

Overall population prevalence for *M. hominis* is often reported to be between 1-20% across the sexes (see Table 1.1). It is thought to be relatively uncommon in males with prevalence rates of between 1-4% typically being reported, though male prevalence as high as 20% has been reported.[280–283] Females routinely possess higher rates of *M. hominis* infection, typically isolated from between 21-53% of sexually mature women.[134,284,285]

1.4.2 Ureaplasma spp.

Compared to *M. hominis*, rates of *Ureaplasma* spp. prevalence are markedly higher (Table 1.2), typically ranging from between 40-80% in females.[134] Whereas for males, though the population prevalence is unclear, researchers estimate it to be between 5-15% in men aged 16-44 years old for *U. urealyticum*.[283,286–289] Whilst the prevalence of *U. parvum* is unknown, Horner *et al.* predict it to be higher than that of *U. urealyticum*, as *U. parvum* is more frequently isolated from male samples from patients without urethritis.[287,290,291] In females it is well-established that *U. parvum* is the dominant *Ureaplasma* spp. infection, with a prevalence range of between 20-89%, compared with 5.2-20% prevalence for *U. urealyticum*.[287,292–295]

Year	Location	Patient Symptoms	Detection Method	Patient Number	*M. hominis* prevalence	Resistance Detection Method	Antibiotic breakpoint applied (µg/mL)	% Resistance	Ref
1983, 1989-2004	Germany	ND	ND	ND	290 (N/a)	Agar, E-test	Ciprofloxacin 4 Clindamycin 4 Azithromycin 8 Tetracycline 16	8% 0% 99% 11%	296
2005-2006	Greece	Vaginitis	IST-2	369	13 (3.64%)	IST-2	Ciprofloxacin 2 Azithromycin 4 Tetracycline 8	80% 100% 0%	297
2008-2011	Hungary	Non-specific urethritis, promiscuous	UMD	4,466	41 (0.9%)	SIRM	Clindamycin 2 Tetracycline 8	5% 12%	298
2009-2013	Italy	Cervicitis and Urethritis	IST-2	2480	99 (4.0%)	IST-2	Ciprofloxacin 2 Azithromycin 4 Tetracycline 8	61% 76% 7%	299
2004-2011	Italy	Urogenital infection or symptoms, pregnancy	IST-2	9,956	204 (10.9%)	IST-2	Ciprofloxacin 2 Azithromycin 4 Tetracycline 8	10% 67% 10%	300
2011	Italy	Symptomatic GUM patients	Gram stain, MFSE3	433	6 (1.4%)	MFSE3	Ciprofloxacin 2 Azithromycin 4	0% 67%	301
2008-2009	Romania	Infertility	RT-PCR	1068	80 (7.5%)	IST-2	Ciprofloxacin 2 Azithromycin 4 Tetracycline 8	77% 39% 17%	302
2016	Serbia	Urogenital infection, infertility, risky behaviour	GS, agar, IF, IST-2	373	6 (1.6%)	IST-2	Ciprofloxacin 2 Azithromycin 4 Tetracycline 8	50% 50% 100%	303
2006-2007	Turkey	Pregnancy with vaginitis or asymptomatic	IST-2	50 50	2 (4.0%) 0 (0.0%)	IST-2	Ciprofloxacin 2 Azithromycin 4 Tetracycline 8	40% 40% 0%	304

2008-2009	Burkina Faso	Abnormal vaginal discharge	MSP	2,000	41 (2.1%)	MSP	Clindamycin 8 Azithromycin 8 Tetracycline 8	30% 66% 40%	305
2009	Gabon	Routine screening	IST-2	1,332	13 (1.1%)	IST-2	Ciprofloxacin 2 Azithromycin 4 Tetracycline 8	8% 54% 46%	306
2003	Guinea-Bissau	Routine family planning screen	IST-2	94	54 (57.4%)	IST	Tetracycline 8	24%	307
2014	China	Infertile men, sperm donators	IST	19,098 3,368	947 (5.0%) 53 (1.6%)	IST	Ciprofloxacin 2 Levofloxacin 2 Azithromycin 4	94% 88% 39%	132
2005-2013	China	Genital symptoms	IST-2	5,025	471 (14.6%)	IST-2	Ciprofloxacin 2 Azithromycin 4 Tetracycline 8	64% 67% 5%ie	308
2007-2011	China	Not specified	IST-2	37,055	4,552 (12.28%)	IST-2	Ciprofloxacin 2 Azithromycin 4 Tetracycline 8	53% 71% 2%	309
2007-2009	China	Urogenital infection	MP Strip	3,306	517 (15.64%)	MP Strip	Levofloxacin 4 Azithromycin 4	35% 86%	310
2009-2013	Cuba	Vaginal Discharge Syndrome	MSP	2354	46 (1.95%)	MSP	Clindamycin 8 Azithromycin 8 Tetracycline 8	14% 92% 58%	29

Table 1.1 – A table tabulating M. hominis prevalence rates from studies conducted at several geographical locations – listed in column 2 – alongside the urogenital pathology (if any) assessed: column 3. Additionally, the method of M. hominis detection employed by the researchers is listed in column 4: ND; non-disclosed, IST-2; Biomerieux, IST; Biomerieux, UMD; Bio-Rad, MSP; sanilabo, MP Strip; Zhuhai Lizhu Bio-Company China. The number of patients screened is listed in column 5, along with the determined prevalence in column 6. Furthermore, the method of AST is listed in column 7, the accompanying breakpoints in column 8 and the resistance prevalence for each antibiotic screened in column 9. References to each study are listed in column 10.

Year	Location	Patient Symptoms	Detection Method	Patient Number	*Ureaplasma* spp. prevalence	Resistance Detection Method	Antibiotic breakpoint applied (µg/mL)	% Resistance	Ref
2005-2006	Greece	Vaginitis	IST-2	369	132 (35.7%)	IST-2	Erythromycin 4 Ciprofloxacin 2 Azithromycin 4 Tetracycline 8	37% 20% 86% 5%	297
2008-2011	Hungary	Non-specific urethritis	UMD	4,466	373 (8.35%)	SIRM	Erythromycin 4 Azithromycin 4 Clindamycin 2 Tetracycline 8	81.23% 9.65% 75.06% 4.02%	298
2004-2011	Italy	Urogenital infection or symptoms, pregnancy	IST-2	9,956	1835 (18.43%)	IST-2	Erythromycin 4 Ciprofloxacin 2 Azithromycin 4 Tetracycline 8	21% 60% 5% 5%	300
2011	Italy	Symptomatic GUM patients	Gram stain, MFSE3	433	152 (35.10%)	MFSE3	Ciprofloxacin 2 Erythromycin 4 Azithromycin 4	66.4% 0% 0%	301
2008-2009	Romania	Infertility	RT-PCR	1068	372 (34.8%)	IST-2	Ciprofloxacin 2 Erythromycin 4 Tetracycline 8	51.72% 16.09% 5.75%	302
2016	Serbia	Urogenital infection, infertility, risky behaviour	GS, agar, IF, IST-2	373	42 (11.26%)	IST-2	Ciprofloxacin 2 Erythromycin 4 Azithromycin 4 Tetracycline 8	83.8% 86.5% 32.4% 86.5%	303
2006-2007	Turkey	Pregnancy with vaginitis or asymptomatic	IST-2	50 50	25 (50%) 2 (4%)	IST-2	Ciprofloxacin 2 Erythromycin 4 Azithromycin 4 Tetracycline 8	92.6% 22.2% 22.2% 0%	304
2008-2009	Burkina Faso	Abnormal vaginal discharge	MSP	2,000	65 (4.23%)	MSP	Clindamycin 8 Erythromycin 4	15% 36%	305

							Azithromycin 8 Tetracycline 8	40% 24%	
2009	Gabon	Routine screening	IST-2	1,332	779 (58.48%)	IST-2	Ciprofloxacin 2 Erythromycin 4 Azithromycin 4 Tetracycline 8	45.4% 34% 29.54% 14%	306
2003	Guinea-Bissau	Routine family planning screen	IST-2	94	37 (39.36%)	IST	Tetracycline 8 Erythromycin 4	24.1% 90.7%	307
2014	China	Infertile men, sperm donators	IST	19,098 3,368	2,294 (12.01%) 139 (4.13%)	IST	Ciprofloxacin 2 Erythromycin 4 Levofloxacin 2 Azithromycin 4	93.6% 46.2% 87.6% 39.2%	132
2005-2013	China	Genital symptoms	IST-2	5,025	1,942 (38.6%)	IST-2	Erythromycin 4 Ciprofloxacin 2 Azithromycin 4 Tetracycline 8	7.25% 83% 3.3% 2.3%	308
2007-2011	China	Not specified	IST-2	37,055	19,679 (53.11%)	IST-2	Ciprofloxacin 2 Erythromycin 4 Azithromycin 4 Tetracycline 8	75.28% 0.49% 0.08% 3.22%	309
2007-2009	China	Urogenital infection	MP Strip	3,306	1,998 (60.44%)	MP Strip	Levofloxacin 4 Azithromycin 4	20.09% 15.21%	310
2009-2013	Cuba	Vaginal Discharge Syndrome	MSP	2354	459 (19.49%)	MSP	Ciprofloxacin 2 Erythromycin 4 Tetracycline 8	77% 27% 9%	29

Table 1.2 – A table tabulating Ureaplasma spp. prevalence rates from studies conducted at several geographical locations – listed in column 2 – alongside the urogenital pathology (if any) assessed: column 3. Additionally, the method Ureaplasma spp. detection employed by the researchers is listed in column 4: ND; non-disclosed, IST-2; Biomerieux, IST; Biomerieux, UMD; Bio-Rad, MSP; sanilabo, MP Strip; Zhuhai Lizhu Bio-Company China. The number of patients screened is listed in column 5, along with the determined prevalence in column 6. Furthermore, the method of AST is listed in column 7, the accompanying breakpoints in column 8 and the resistance prevalence for each antibiotic screened in column 9. References to each study are listed in column 10.

1.5 Current Detection Methods

1.5.1 Culture

Ureaplasma spp. and *M. hominis* are notoriously fastidious organisms, with stringent growth requirements. The bedrock of *Ureaplasma* culture mechanisms rests on their ability to produce ammonia through urea hydrolysis. The arginine-dihydolase pathway is similarly used for the broth culture of *M. hominis*. These metabolic pathways can be coupled with 'genital mycoplasmas' preference for an acidic culture medium, which conveniently retards the growth of other bacteria. The subsequent addition of a pH indicator (typically phenol red) permits the visualisation of a colour shift in the media due to ureaplasmal or mycoplasmal growth, from yellow-orange to cerise red, as the pH increases from around 6.5-6.8 to >7.4. This is critical as *Ureaplasma* cultures are unable to grow to turbidity. The shift in media pH, reduction in urea concentration, and toxic metabolites they expel inhibit growth, culminating in a rapid death phase. Liquid cultures are typically incubated at 37^{o}C in atmospheric conditions with colour changes taking between 24-48 hours to develop, in the presence of ureaplasmal or mycoplasmal growth. Culturing *Ureaplasma* on solid media requires atmospheric CO_2 concentrations of 5%, with growth taking between 1 and 5 days. As *Ureaplasma* cannot form a confluent bacterial lawn on the surface of the agar, a microscope or hand lens is required to visualise the colonies. Unlike *M. hominis*, *Ureaplasma* do not form the signature 'fried-egg' colony morphology typical of mycoplasmas, but instead produce brown zones due to urease activity and conversion of manganous sulphate to manganese dioxide by the presence of ammonia.[311] To quantify the ureaplasmal or *M. hominis* load in each sample, serial 10-fold dilutions using the broth culture methodology can provide a result as colour changing units (CCU). The CCU is determined following the cessation of colour change in the media, the last well in which a colour change is observed is determined to be 1 CCU. Subsequent multiplication of the dilution factor allows the determination of the original sample's concentration.

1.5.2 Molecular Detection Methods

PCR has long been an important and sensitive tool in the detection of fastidious bacterial pathogens, with *Ureaplasma* and *M. hominis* being no exception. Traditionally, PCR methods focussed on the detection of the 16S rRNA gene.[312] However, for *M. hominis* numerous PCR protocols have been developed with varying gene targets, such as the *gap*, *fstY*, and *yidC* genes.[313–315] Though upon examining the heterogeneity of the *gap* gene, it was observed that its variation was greater than that of the 16S rRNA gene.[313] Furthermore, the pitfalls of utilising the *gap* gene as a target for *M. hominis* detection have been demonstrated, whereby *gap* negative PCR samples have been successfully cultured and determined to be *M. hominis* positive. Ferandon *et al*. real-time PCR assay for *M. hominis* detection, targeting the *yidC* gene, displayed 100% specificity and a sensitivity of 7 gene copies per reaction.[314] The *yidC* gene encodes for a putative inner membrane translocase component.

Whereas for *Ureaplasma*, the primary gene targets for real-time PCR methodologies have been the *mba* gene and urease gene. For example, Mallard and colleagues designed two Taqman primer and probe combinations, targeting the urease gene (*ureB*).[316] The assay showed a sensitivity of 5 gene copies per reaction and was specific to both *U. parvum* and *U. urealyticum* when tested against 16 reference strains, commonly encountered in a clinical setting. Though this assay was not tested against actual clinical specimens. Moreover, the UAB Diagnostic Mycoplasma laboratory developed and runs a real-time PCR assay targeting UU063 (NP_077893) a gene encoding a hypothetical protein conserved between all four *U. parvum* serovars. For the detection *U. urealyticum*, the assay targeted UUR10_0680 (NC_011374.1), a 15,072-bp open reading frame conserved throughout the 10 serovars.[317] Additionally, this real-time methodology displayed greater sensitivity for *Ureaplasma* detection when compared with traditional PCR assay based on the urease gene following intralaboratory method assessments. This real-time assay demonstrated an impressive sensitivity level of <1 CFU for both *U. parvum* and *U. urealyticum*.

Throughout Europe there has emerged commercially available molecular-based assays that incorporate the detection of genital mycoplasmas. Products such as the STD6 and STD6B ACE from Seegene, Inc. detect *T. vaginalis*, *M. hominis*, *M. genitalium*, *C. trachomatis*, *N. gonorrhoeae* and *Ureaplasma* spp. in endocervical samples. The STD6 assay incorporates a dual-priming oligonucleotide system encompassing two distinct priming regions, for *Ureaplasma* spp. the target is a 130-bp region of the *ureD* gene. However, the format is not available in real-time and post-PCR manual or automated gel electrophoresis is required.[318]

1.5.3 PCR vs Culture: advantages and disadvantages of genital mycoplasma detection methods

Both PCR and culture methodologies have their advantages and disadvantages, with regards to *Ureaplasma* or *M. hominis* detection. PCR is often found to be the more sensitive methodology for diagnostic purposes. The Birmingham Diagnostic *Mycoplasma* Laboratory, at the University of Alabama published data displaying real-time qPCR detected ureaplasmal DNA in 39.4% of specimens, compared with 24.2% detected through culture.[317]

Though the consensus is that molecular-based methodologies such as real-time qPCR are more sensitive for the detection of mycoplasmas relative to culture-based approaches, they remain uneconomical and impractical for mycoplasmal detection in laboratories with low to moderate sample volumes. Moreover, culture-based approaches, particularly the commercially available cultured-based detection kits require little specialist training or equipment. A further advantage of culture-based approaches for mycoplasma detection is that they provide a viable isolate, on which antimicrobial sensitivity testing (AST) can be performed. This advantage is often used in conjunction with the previously cited advantages in commercially available diagnostic assays, for the simultaneous detection and AST of organisms such as *Ureaplasma* spp. and *M. hominis*.

1.6 Antimicrobial Susceptibility Testing: AST for *Ureaplasma spp.* and *M. hominis*

Historically, various methodologies for AST of mycoplasmas have been cited, the first of which was published in the 1967.[319] Despite a multitude of mycoplasma AST publications in the subsequent years reporting the efficacy of various antimicrobial agents against these organisms, no universally accepted AST methodology predominated in the literature. This inability for researchers to reach a consensus on the AST protocol applied to human mycoplasmas has resulted in much confusion surrounding the efficacy of several drugs against these organisms. In response to this, in 2011, an international group of mycoplasmologists, headed by Ken Waites, formally published a body of work that had taken over a decade to complete as the Clinical Laboratory Standards Institute (CLSI) guidance document M43-A to harmonise and standardise the AST methodology for human mycoplasmas and establish breakpoint concentrations for antibiotics deemed effective against these organisms.[320] The subcommittee's publication set the of the internationally recognised standard for AST testing for human mycoplasmas, which superceeded the previous document referred to as Cumitech 34 published in 2004.

Nevertheless, the publication of a standardised AST methodology for human mycoplasmas did not result in routine AST for these organisms. This is possibly due to the complex nature of the test procedures, when compared with the simpler disk-diffusion AST methodologies applied to the majority of bacterium that can grow as a confluent lawn solid agar. Moreover, *Ureaplasma* spp. and *M. hominis* are incapable of growing to visual turbidity in liquid media due to small cell size and the self-toxic properties of their metabolites, meaning the use of McFarland standards to estimate and prepare a defined inoculum is not permitted. The CLSI guidelines require and inoculum of between 10^4 and 10^5 CFU/mL for reliable and accurate AST. Therefore, a predetermination of the mycoplasmal concentration prior to testing is required, which likely involves freezing the initial inoculum, followed by possibly diluting the sample accordingly. This is a time-consuming and laborious process that

cannot be feasibly incorporated into the routine screening practices of most clinical laboratories. It is this niche that commercial diagnostic assays aim to populate.

1.7 Commercial Kits: a review of the currently available commercial *in vitro* diagnostics for the detection and AST of *Ureaplasma* spp. and *M. hominis*

A brief review of the leading commercially available assays for the detection of *Ureaplasma* spp. and *M. hominis* in urogenital samples has been undertaken. All the assays reviewed rely on proprietary selective culture media, typically lyophilised within detection wells, alongside wells containing the dehydrated media supplemented with breakpoint concentrations of antibiotic. The culture media and antibiotic become liberated into solution following inoculation. In addition to these wells, these kits possess a range of semi-quantitative wells that approximate the mycoplasmal concentration of the inoculum transferred into them e.g $<10^4$ CCU or $\geq10^5$ CCU. How the manufacturers estimate bacterial load, without the inclusion of an additional dilution step is undisclosed, though it is likely through the addition of empirically determined threshold concentrations of exclusive inhibitory agents.

The MYCOFAST revolutioN (ELiTech Diagnostic, France) is a 20-well assay, 7 wells are dedicated to the identification and enumeration of *Ureaplasma* spp. and *M. hominis*. Comprised of 3 wells for *Ureaplasma* enumeration (10^3, 10^4 and $\geq10^5$ CCU/mL), 1 well for *M. hominis* enumeration ($\geq10^4$ CCU/mL), and 3 'indentibiotic' wells that utilise each species' intrinsic resistance to specific antibiotics to further confirm the identification. The remaining 13 wells are wells supplemented with various concentrations of antibiotic; Levofloxacin 1µg/mL, 2µg/mL and 4µg/mL; Moxifloxacin 0.25µg/mL, 2µg/mL; Erythromycin 8 µg/mL,16 µg/mL; Clindamycin 0.25µg/mL, 0.5µg/mL; Tetracycline 1µg/mL, 2µg/mL, 4µg/mL,8 µg/mL. Compared with a PCR methodolgy the MYCOFAST revolution revealed an overall sensitivity and specificity for urogenital mycoplasmas of 77.3% and 80%, respectively.[321] A smaller 6-well kit is offered in the Mycoplasma Duo (Biorad; Watford, U.K), this rapid diagnostic assay offers the identification of both *Ureaplasma* spp. and *M. hominis* alongside 1 semi-quantitative well per species to display when an inoculum is determined to be

'clinically significant' (≥10^4 CCU/mL). Though the Mycoplasma Duo does not offer AST, Biorad produce the SIR Mycoplasma, a 16-well complementary kit that involves preparing a standardised inoculum of *Ureaplasma* spp. or *M. hominis* following a positive identification in the Mycoplasma Duo. The SIR Mycoplasma screens against 8 different antibiotics: doxycycline (4µg/mL & 8µg/mL), tetracycline (4µg/mL & 8µg/mL), azithromycin (2µg/mL & 4µg/mL), josamycin (2µg/mL & 8µg/mL), erythromycin (1µg/mL & 4µg/mL), clindamycin (2µg/mL), pristinamycin (2µg/mL) and ofloxacin (1µg/mL & 4µg/mL). Researchers compared the results of PCR and Mycoplasma Duo for the detection of *Ureaplasma* spp. in the endotracheal aspirates of 60 premature neonates, with an overall agreement between the two tests of 96%.[322] No published data on the sensitivity of Mycoplasma Duo, compared with PCR, for the detection of *M. hominis* could be found. The *Mycoplasma* system plus (Liofilchem, Italy) is a 24-well option for the detection and partial enumeration of *Ureaplasma* spp. and *M. hominis*, alongside AST. The system offers semi-quantitative wells as follows: >10^4 CCU/mL, <10^5 CCU/mL and >10^5 CCU/mL for both *M. hominis* and *Ureaplasma* spp. The antibiotics screened against for the *Mycoplasma* system plus are tetracycline, (4 µg/mL & 8µg/mL), pefloxacin (8µg/mL & 16µg/mL), ofloxacin (1µg/mL & 4 µg/mL), doxycycline (4µg/mL & 8 µg/mL), erythromycin (1µg/mL & 4µg/mL), clarithromycin (8µg/mL & 16 µg/mL), minocycline (4µg/mL & 8 µg/mL), clindamycin (4µg/mL & 8µg/mL) and azithromycin (4µg/mL & 8µg/mL). Though a few publications utilise the *Mycoplasma* system plus as a method for urogenital mycoplasma identification, no studies determining the sensitivity or specificity of the assay against other identification methodologies are available.[29] Finally, the most popular commercially available assay for the identification of genital mycoplasmas is seemingly the Mycoplasma IST2 (Biomerieux, France). It is a 22-well assay, with 2 identification wells (1 x *Ureaplasma* spp. & 1 x *M. hominis*) alongside 2 semi-quantitative wells (1 x each species) determining whether the inoculum is ≥10^4 CCU/mL. The antibiogram consists of doxycycline (4µg/mL & 8µg/mL), josamycin (2µg/mL & 8µg/mL), ofloxacin (1µg/mL & 4µg/mL), erythromycin (1µg/mL & 4µg/mL), tetracycline (4µg/mL & 8µg/mL), ciprofloxacin (1µg/mL & 2µg/mL), azithromycin (0.12µg/mL & 4µg/mL), clarithromycin

(1µg/mL & 4µg/mL) and pristinamycin (2µg/mL). Again, many publications have utilised the Mycoplasma IST2 for both detection, enumeration and resistance reporting.[309,323–326] However, with regards to the sensitivity and specificity of the assay, only one study comparing the IST2 with real-time qPCR is available. The IST2 was compared against the Seeplex and the Anyplex 2 PCR assays, 897 samples were processed for the detection of *Ureaplasma* spp. and *M. hominis*, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were determined. For *Ureaplasma* spp. these were determined to be 44.9%, 87.9%, 29.0% and 93.5%, respectively. For *M. hominis*, these values were calculated as 44.7%, 99.6%, 87.5% and 97.0%, respectively.[327]

From this review there are a few consistencies between the commercially available assays; utilisation of a yellow-cerise red colour changing specific growth media; incubation of the assay at 37°C for 24-48hours. However, much like the previously described AST for these organisms prior to the publication of the CLSI guidelines, there are still many inconsistencies. For example, the quantitative wells differ hugely between kits and interpreting them is seemingly unclear. The Mycoplasma IST2 quantitation well indicates when the inoculum is $\geq 10^4$ CCU/mL (the minimum concentration stipulated by CLSI) but does not indicate if the inoculum exceeds the CLSI limit of $>10^5$ CCU/mL, meaning the inoculum could far exceed that which is required for CLSI compliant AST. It has been well documented that inocula of $>10^5$ CCU/mL consistently produces false-positives in the AST of *Ureaplasma* spp. by simply overwhelming the small amount of antibiotic present.[328] Furthermore, many of the antibiograms do not differentiate between *Ureaplasma* spp. and *M. hominis* in mixed cultures. As described previously, *M. hominis* has an intrinsic resistance to 14- and 15-membered ring macrolides, such as erythromycin. This can lead to reporting falsely inflated levels of macrolide-resistant *Ureaplasma* spp. within samples of concomitant *Ureaplasma* spp. and *M. hominis*, as the investigators do not purify the culture and confirm the resistance through CLSI compliant AST.[323] Consequently, this leads to huge variability in the resistance rates reported within the same country. Macrolide resistance, erythromycin particularly, was reported at 54% for *Ureaplasma* spp. obtained from 1951 patients in Xiangya, China.[323] A figure that is dramatically undermined by resistance rates

reported for *Ureaplasma* spp. by Song *et al.* and Ye *et al.*, whom examined 1513 and 15,594 patients 800km away in Hangzhou, with respective erythromycin resistance of 11% and 1%.[308,309] Finally, and ultimately the most important inconsistency between commercial urogenital mycoplasma assays, is the utilisation of breakpoint concentrations of antibiotic that differ from those published within the CLSI AST guidelines for human mycoplasmas.[320] The Mycoplasma IST2 for instance, and the *Mycoplasma* system plus, include erythromycin at concentrations of 1µg/mL and 4µg/mL, whereas the CLSI guidelines stipulate ≥16µg/mL as a resistant breakpoint. Again, this could potentially lead to the over-reporting of macrolide resistance among *Ureaplasma*s. On the contrary, the IST2 incorporates tetracycline at concentrations of 4µg/mL and 8µg/mL, which, though appropriate concentrations for determining *M. hominis* is not compliant with CLSI guidelines for determining tetracycline resistance in *Ureaplasma* spp. Though this could, in theory, lead to the under-reporting of tetracycline resistance among *Ureaplasma*s, Tet(M)-mediated resistance typically leads to MIC values of ≥32µg/mL. However, there are several documented cases of *tet(M)* positive mycoplasmas that are phenotypically susceptible to concentrations of antibiotic lower than that used to determine tetracycline resistance, yet culture in sub-inhibitory concentrations of tetracycline induces resistance in these organisms.[168]

1.7.1 MYCO WELL D-ONE: a CLSI-compliant alternative

In response to many of the issues highlighted in the review of commercially available assays for the detection of genital mycoplasmas, CPM SAS (Rome, Italy) developed the MYCO WELL D-ONE. It is the first commercially available assay that offers CLSI compliant antibiotic breakpoints for the screening of both *Ureaplasma* spp. and *M. hominis* on a single assay. Furthermore, unlike the previously described kits, whereby the antibiogram is shared between both species, the MYCO WELL D-ONE has a dedicated antibiogram for both *Ureaplasma* spp. and for *M. hominis*. Alongside this, it also contains a semi-quantitative well for the partial enumeration of *Ureaplasma* spp. to ensure the inoculum tested is of the correct concentration to permit an accurate AST. An overview of the assay is displayed.

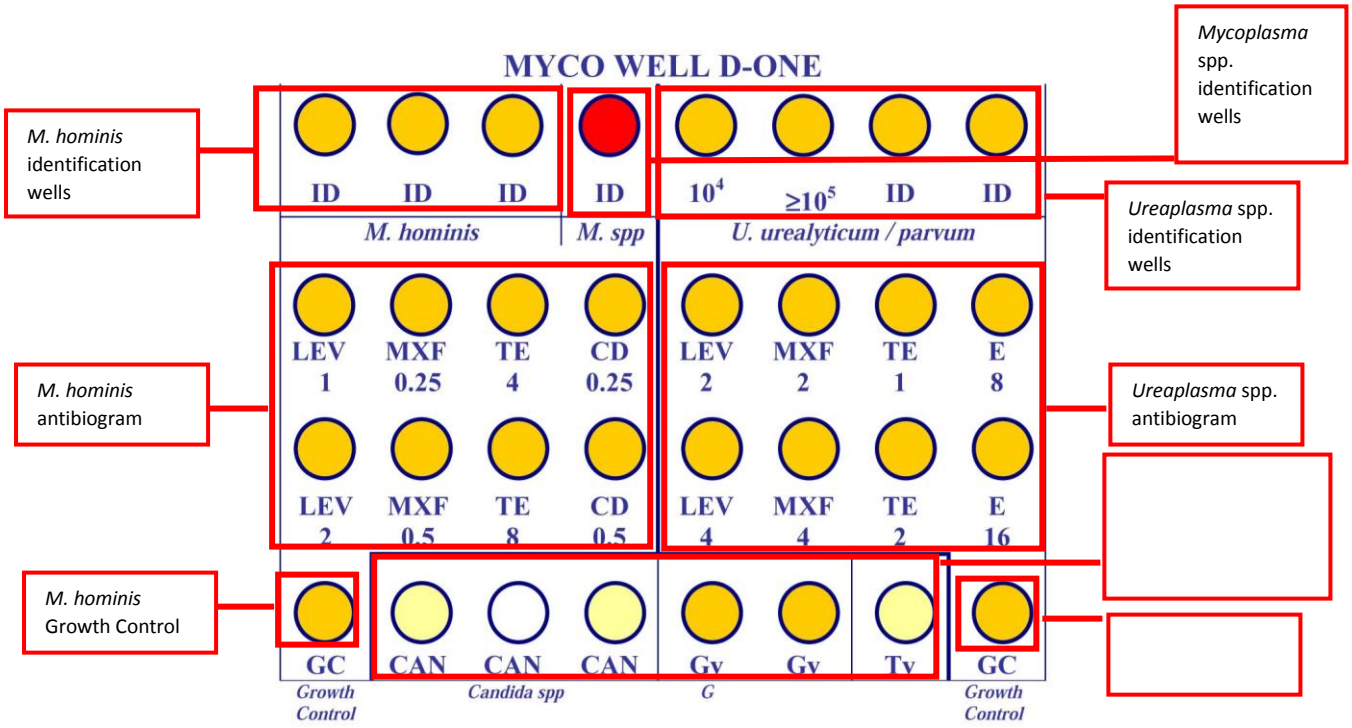

Figure 1.10 A graphic representation of the MYCO WELL D-ONE assay plate. The top four wells on the right-hand side are for the identification and load estimation of Ureaplasma spp. The Ureaplasma antibiogram has 2 x wells for each antibiotic to determine intermediate and resistant isolates; levofloxacin 2 & 4µg/mL, moxifloxacin 2 & 4µg/mL, tetracycline 1 & 2µg/mL and erythromycin 8 & 16µg/mL. The 3 x ID wells at the top right are used to confirm the presence/absence of M. hominis infection. The M. hominis Ureaplasma antibiogram has 2 x wells for each antibiotic to determine intermediate and resistant isolates; levofloxacin 1 & 2µg/mL, moxifloxacin 0.25 & 0.5µg/mL, tetracycline 4 & 8µg/mL and clindamycin 0.25 & 0.5µg/mL. Growth controls for both Ureaplasma spp. and M. hominis are present at the bottom corner of the antibiogram. The remaining wells are for the identification of Candida spp. (3 x CAN wells), G. vaginalis (2 x Gv) and T. vaginalis (1 x Tv well)

1.8 Aims

The aims of the study were as follows:

- To determine the sensitivity, specificity, PPV, NPV and accuracy of the MYCO WELL D-ONE when compared with the 'gold-standard' detection methodology: real-time qPCR methodology.
- To assess the accuracy of the breakpoint antimicrobial susceptibility testing offered by the MYCO WELL D-ONE for both *Ureaplasma* spp. and *M. hominis* against a CLSI compliant AST.
- Utilise whole genome sequencing to determine the underlying mechanisms mediating resistance in confirmed resistant isolates.
- Calculate the prevalence rates of *Ureaplasma* spp. and *M. hominis* for the population of GUM clinic attendees throughout the Valleys convergence area.
- Assess the species composition of *Ureaplasma* spp. colonisation among GUM clinic attendees throughout the South Wales Valleys convergence area.

CHAPTER 2
MATERIALS AND METHODS

2 Materials and Methods

A schematic/flow-diagram outlining the steps following sample collection is displayed in figure 2.2.

2.5 Patient Recruitment and Sample Collection

Ethics for the recruitment of individuals undergoing routine sexual health screenings at walk-in and appointment sexual health clinics within Cwm Taf University Health Board were obtained as ethics numbers: sample collection under IRAS 230693 "MYCO WELL D-ONE: A new rapid test to detect *Ureaplasma* and *Mycoplasma* in clinical samples" and correlation of patient data to *Mollicutes* results under IRAS 253889 "Do genitourinary *Mycoplasma* species cause disease. Informed consent was obtained from patients for the analysis of the urine and/or swab (endocervical or endourethral) sample they provided as part of their screening, by the methods described herein. A total of 983 samples were collected from 856 patients; 526 female patients, 122 of which provided both urine and endocervical swabs; 335 males, 5 of which provided both urine and endourethral swabs.

2.6 Sample Processing

Upon receipt of a sample, they were processed as follows:

Urine: Samples were required to have a minimum volume of approximately 2.5ml, those which did not were excluded from the study and not recorded. Following the receipt of an appropriate sample, 200µl of urine was inoculated into 10ml of sterile saline solution (included in CPM SAS kit) for both MYCO WELL D-ONE and culture titration inoculation. 2ml of the urine sample was transferred to a 2ml centrifuge tube and appropriately labelled using an indelible marker, for later DNA extraction. Samples were anonymised using a prefix, dependant on hospital and patient gender: DF/DM (Dewi Sant Female/Male), KF/KM (Keir Hardie Female/Male), YF/YM (Yysbyty Cwm Cynon Female/Male), followed by a sequential sample number i.e DF001, DF002 etc. All plates, tubes and assays used this unified anonymization system and the research nurses (who consented the patients) kept a log of

the research numbers relative to the sexual health file number for post-study analysis (IRAS ethics 253889).

Swab: Upon receipt of an endocervical or endourethral swab direct inoculation into 10ml sterile saline solution was performed. 2ml of inoculated sterile saline was transferred into a sterile 2ml centrifuge tube and labelled with research anonymization code as described above.

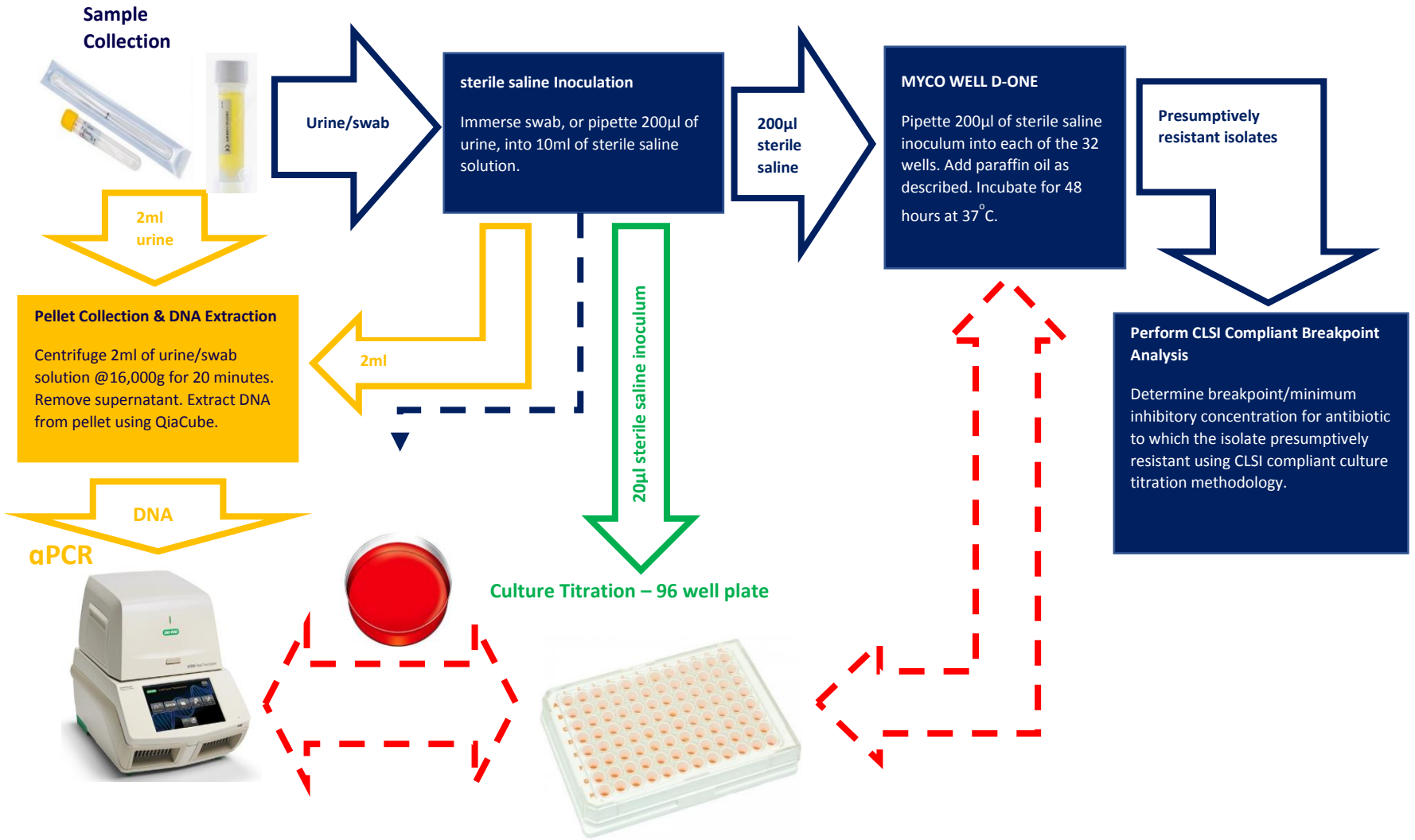

Figure 2.2 A schematic outlining the process steps involved in the analysis of a sample from the point at which it is collected.

2.7 MYCO WELL D-ONE

150µl of inoculated sterile saline was pipetted into each of the 32 wells on the plate. 2 drops of paraffin oil were applied to the *Mycoplasma* and *Ureaplasma* wells to generate an anaerobic environment within each well. Inoculated assays were labelled with sample number, date and time of saline addition. Samples were incubated at 37°C, in a humidified tissue incubator at ambient CO_2, and read at various intervals up to 72 hours post-incubation.

2.8 Broth Culture Titration (comparative gold standard culture method)

Culture titration plates were set up as follows, using 96-well flat-bottomed microtitre plates: 90µl of *Ureaplasma* specific medium (USM) purchased from Mycoplasma Experience Ltd. (Surrey, United Kingdom) was pipetted into wells 1A–1H, 4A–4H, 7A–7H and 10A–10H; 90µl of *M. hominis* specific medium (MHSM), kindly supplied by CPM SAS (Rome, Italy), was pipetted into wells 2A–2H, 5A–5H, 8A–8H, 11A–11H. Thus, allowing four separate samples to be processed on one prepared 96-well microtitre plate. Samples were processed by pipetting 10µl of sample-inoculated sterile saline into well A of 90µl of USM, a further 10µl of inoculated sterile saline was pipetted into well A of 90 µl of MHSM. Subsequently, each initial 1:10 inoculum of sample-USM/MHSM in well 1, is serially diluted (1:10 dilution) to a final 10^{-7}dilution, wells A–G. Well H remained uninoculated and acted as a negative control. Following the processing of four samples on one 96-well plate, the plate was sealed with adhesive sealing tape (Elkay: Basingstoke, United Kingdom), and were incubated at 37°C, in a humidified tissue incubator at ambient CO_2, and read at various intervals up to 72 hours post-incubation.

2.8.1 Undiluted Urine Culture – Inoculation of USM

Alongside the culture titration methodology 20µl of undiluted urine was pipetted into 180µl and incubated at 37°C, in a humidified tissue incubator at ambient CO_2, and read at various intervals up to 72 hours post-incubation. This was to avoid false negatives from low titre samples as 10 microlitres of 1:500 diluted urine in sterile saline was 15-fold less than the amount inoculated into the MYCO WELL D-ONE.

2.8.2 US1 Agar

5µl of inoculated sterile saline solution was pipetted directly onto the surface of US1 agar (Mycoplasma Experience) and incubated at 37°C, in a humidified tissue incubator at ambient CO_2, and read at various intervals up to 72 hours post-incubation. Positives were determined through the presence of a classic 'fried-egg' morphology under light microscopy for M. hominis, and red pH colour change in the media with tiny ureaplasma colonies.

2.9 Preparation of Antibiotic Testing Media

2.9.1 Antibiotic Stocks

Antibiotics were purchased from Sigma-Aldrich (Dorset, UK) and reconstituted into the concentrations displayed in Table 2.1—based on the CLSI published guidelines.[320] Erythromycin was supplied as a 1mg/ml stock solution in water. Josamycin was prepared as a 1mg/ml stock solution in water. Moxifloxacin hydrochloride was prepared as a 1mg/ml stock solution in water. Tetracycline hydrochloride was prepared as 1mg/ml solution in water. Levofloxacin hydrochloride was prepared as a 1mg/ml solution in water. Clindamycin was prepared as a 1mg/ml solution in water. Working stocks were then prepared for use with USM/MHSM and added to the growth media to result in the concentrations listed overleaf.

Antibiotic	Solvent	Stock Concentration	USM Concentration	MHSM Concentration
Erythromycin	Water	1mg/ml	16µg/ml	N/A
Josamycin	Water	1mg/ml	8µg/ml	8µg/ml
Moxifloxacin	Water	1mg/ml	4µg/ml	2µg/ml
Levofloxacin	Water	1mg/ml	4µg/ml	2µg/ml
Tetracycline	Water	1mg/ml	2µg/ml	4µg/ml
Clindamycin	Water	1mg/ml	N/A	0.5µg/ml

Table 2.1 Table detailing the antibiotics used to screen for both Ureaplasma spp. and M. hominis isolates for resistance. Concentrations shown for both stock and concentrations of USM and MHSM supplemented with antibiotic.

2.10 Break Point Screening of Isolates

Analysis of putative resistant isolates was conducted in accordance with a previously published methodology for breakpoint analysis for *Ureaplasma* spp.[328] The method was modified to include additional antibiotics, alongside utilising MHSM for the breakpoint screening of *M. hominis* isolates. Isolates were screened against the CLSI guideline concentrations of antibiotic. Isolates were only screened against antibiotics if they had shown putative resistance to that antibiotic on the MYCO WELL D-ONE assay.

In a 96-well flat-bottomed plate, 180µl of antibiotic supplemented USM/MHSM (at CLSI compliant concentration) is pipetted across the 8 well row (A1-H1). If the isolate is subject to testing against more than one antibiotic, additional antibiotic supplemented USM/MHSM, at the required concentration, is pipetted into the subsequent rows e.g A2-H2, A3-H3 and so on. One row contains one antibiotic. An additional row, directly beneath the last antibiotic to be tested, contained an antibiotic free 180µl of USM/MHSM (figure 2.1) as a growth control. Multiple isolates were screened on one plate for direct comparison, the number examined was dependent on the number of antibiotics the isolates needed to be confirmed against from MYCO WELL D-ONE screening results.

Upon completion of the plate set-up 20µl of *Ureaplasma* or *M. hominis* culture was added to wells in the A row, containing 180µl USM/MHSM for all antibiotics the isolate was to be screened against, plus the antibiotic free well. Subsequently, serial 10-fold dilutions are performed for each row. No positive control for resistant strains were used as no characterised strains were available. Isolates that produced a positive colour change in antibiotic wells which corresponded to 10^4 CCU in the antibiotic-free positive control row were subjected to further investigation. Those isolates that did not grow in an antibiotic well corresponding to 10^4 CCU were determined to be not resistant (false MYCO WELL D-ONE resistance result) to the antibiotic. Antibiotic resistant strains were subject to whole genome sequencing to determine the underlying mechanisms, which conferred their resistance.

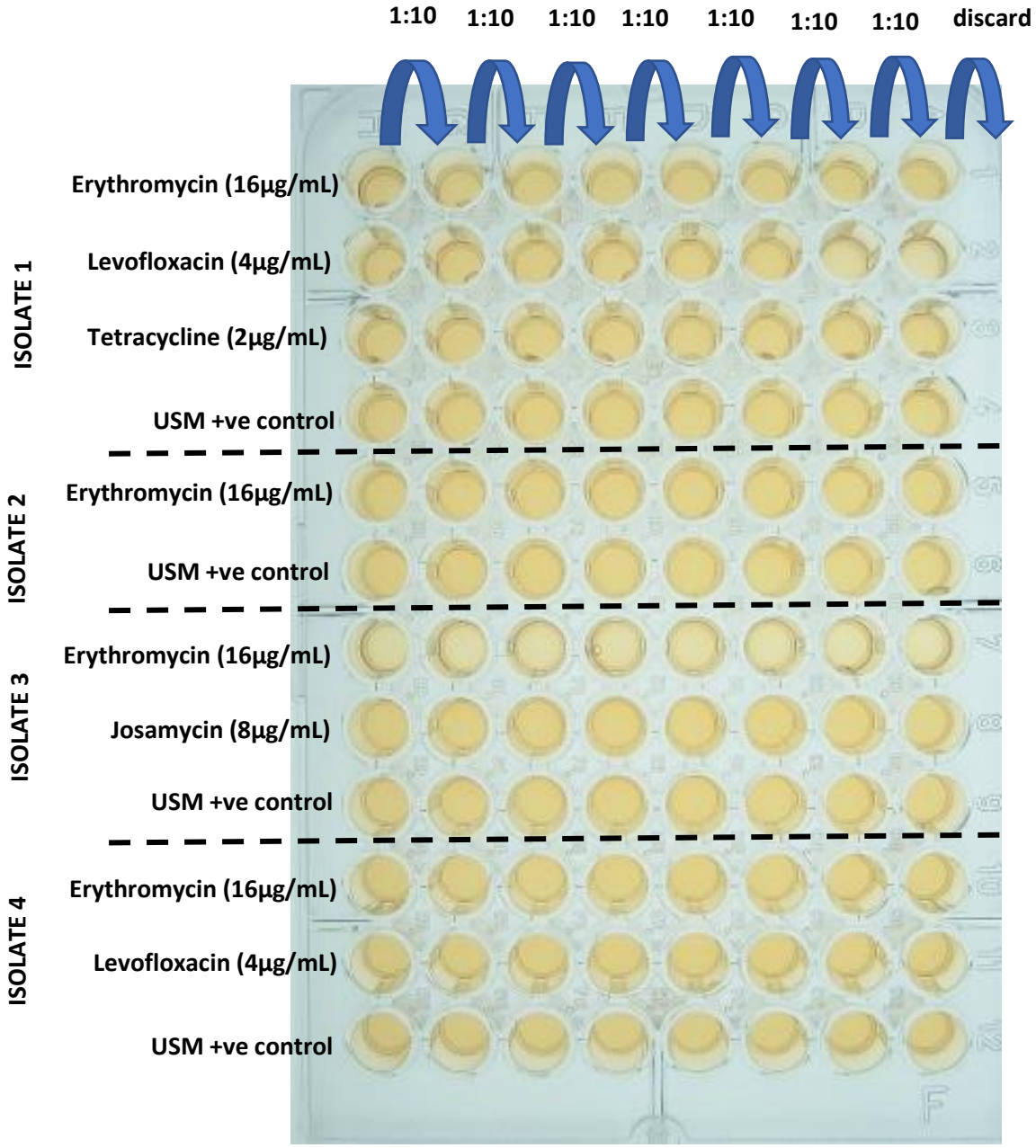

Figure 2.1 Image outlining the screening of multiple Ureaplasma spp. isolates against different antibiotics. An isolate, of unknown concentration is cultured in USM, before being titrated across the wells containing antibiotic-supplemented USM or USM as a control, as indicated by the arrows. Growth, indicated by a colour-change, in an antibiotic-supplemented well coupled with a CLSI compliant organism concentration (determined by serial dilution) is indicative of antibiotic resistance.

2.11 DNA Extraction

2.11.1 Sample Processing

The 2ml urine sample or 2ml swab-inoculated saline sample taken from each patient was pelleted through centrifugation at 20,000*g* for 20 minutes. Following centrifugation, the supernatant was discarded, and the generated pellet is subjected to a further wash step. The wash step consisted of the addition of 1ml urine-inoculated saline or 1ml swab-inoculated saline (dependant on sample type) left over from the MYCO WELL D-ONE preparation to the generated pellet. Followed by a further spin of 20,000*g* for 20 minutes, supernatant was removed and discarded. Labelled tubes containing these pellets were transferred from clinical site to -80^{o}C until DNA extraction by QiaCube at the earliest convenience.

2.11.2 QIAcube DNA Extraction

DNA was extracted from the pellet derived from urine and swab samples using the Qiagen (Manchester, United Kingdom) QIAamp extraction kit. The extraction method was automated utilising the QIAcube (Qiagen; Manchester, United Kingdom), a robotic DNA extraction system set to standard program for Gram-negative organisms from pellet and elution in 100 microlitres of sterile water. In brief, the methodology is a lyse-bind-wash-elute extraction process for the elution of high-quality genomic DNA. The sample is initially lysed in a proprietary lysing buffer alongside 20mg/mL proteinase K. Following lysis incubation at 56^{o}C followed by a brief RNAase (20 mg/ml) addition and incubation, the robot transfers the solution into a QIAamp spin column, whereby the DNA binds to the silica membrane of the column. A subsequent 2-stage wash removes contaminants and inhibitors, before eluting the DNA into 100µl of molecular grade H_2O. Extracted DNA samples were transferred immediately following extraction and stored at -80^{o}C until qPCR analysis.

2.12 Multiplex qPCR

2.12.1 Real-Time qPCR Assay

The BioRad Laboratories (Watford, UK) CFX96 Touch Real-Time PCR thermocycler slaved to a Microsoft computer running Maestro software was used to run the assays. SsoAdvanced Universal Probes Supermix for hydrolysis probes (BioRad Laboratories) and 96-well plates and optical clear adhesive seals (BioRad Laboratories) were used. Ten microliters of 2x mastermix, made up to fifteen microliters with the addition of 2 microliters of molecular water and 1 microliter of each of the following specific fluorescent probe and primers: (0.25 pmol/µL *Ureaplasma parvum* forward primer, 0.25 pmol/µL *Ureaplasma parvum* reverse primer, 0.01 pmol/µl *Ureaplasma parvum* probe; 0.25 pmol/µL *Ureaplasma urealyticum* forward primer, 0.25 pmol/µL *Ureaplasma urealyticum* reverse primer, 0.01 pmol/µl *Ureaplasma urealyticum* probe; 0.25 pmol/µL *Mycoplasma hominis* forward primer, 0.25 pmol/µL *Mycoplasma hominis* reverse primer, 0.01 pmol/µl *Mycoplasma hominis* probe) and molecular grade H_2O were added to each well, along with 5 microlitres of sample to be analysed – total reaction volume: 25µL. Genomic copy equivalents were determined against a 6-point standard curve (10^6-10^1 copies) of diluted plasmid containing concatamers of the primer and probe targets separated by 30 bp intervening sequences (synthesized by GenScript) and measured by Life Technologies Qubit fluorometer DNA quantification system. Primers and Taqman hydrolysis probes for *Ureaplasma parvum* and *Ureaplasma urealyticum* were previously clinically validated[329] as were primers and Taqman hydrolysis probes for *Mycoplasma* hominis.[314] Biorad CFX96 cycling conditions were as follows: initial activation and denaturation 95°C for 5 minutes, next 45 cycles of 95°C for 15 seconds and 60°C for 15 seconds. Readings were acquired in between cycles on channels Green, Yellow, Red and Crimson. Data was analysed on Biorad CFX Maestro software. Presence of amplification inhibitors were assessed using Qiagen Quantinova as per manufacturer's instructions.

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	

* Calculated using EurofinMWG oligonucleotide quality tool

2.13 Whole Genome Sequencing

All samples that proved to be truly resistant to antibiotics were sub-cultured to concentrations suitable for whole genome sequencing. Cultures were pelleted in 2mL centrifuge tubes and DNA extracted using an automated Qiacube using Qiagen reagents for Gram-negative protocol described above. Following extraction of DNA, Qubit 3.0 (Life Technologies) fluorometric analysis verified a sequenceable amount of DNA (1-8 ng/microliter) had been generated. Extractions were kindly sequenced by Edward Portal and assembled by Dr. Kirsty Sands, both members of our Department of Medical Microbiology. Briefly, whole genome sequencing was performed using a Nextera XT library preparation and sequenced with a V3 chemistry on an Illumina MiSeq platform. Cardiff University have a HPC cluster system and access to CLIMB to allow high throughput whole genome assembly & analysis. The bioinformatics pipeline is comprised of 3 main areas; (1) QC pipeline to validate & trim the raw sequence reads: FastQC and Trimgalore (for quality control of raw sequence reads); (2) whole genome assembly and mapping: Flash (merge paired-end reads; reduces contig miss-assembly), SPAdes (generates single-cell assemblies), BWA (maps low-divergent sequences to reference genome), pilon (improvement of draft assemblies and detect inter-strain variation) and quast (genome assembly evaluation); (3) Whole genome annotation and profiling of genetic determinants using a combination of available software (using both fastq and *de novo* assembled reads): prokka (annotation of prokaryotic genomes), NCBI BLAST (aligns and determines regions of similarity to previously published genomes), kmerfinder (bacterial identification in whole genome sequence data), CARD (AMR gene detection), srst2 (reports sequence types from MLST database), and VFDB (virulence factor identification). Assembled contigs were further analysed utilising Geneious sequence analysis software (BioMatters ltd. New Zealand) and aligned and assessed against reference sequences for the identification of mobile genetic elements or point mutations.

CHAPTER 3
RESULTS

3 Results

3.1 Patient Recruitment

A total of 983 samples were collected from 856 patients; 526 female patients, 122 of which provided both urine and endocervical swabs; 335 males, 5 of which provided both urine and endourethral swabs.

3.2 MYCO WELL D-ONE

The multiplex qPCR developed and used by Public Health England (PHE) for the detection of *Ureaplasma* spp. and *M. hominis* infections was utilised as the 'gold standard' reference methodology to determine the sensitivity and specificity of the MYCO WELL D-ONE assay, in detecting these organisms (Table 3.1 and 3.2). For the detection of *Ureaplasma spp.*, overall (983 samples), a total of 413 true positive samples, 515 true negative samples, 19 false positive samples and 26 false negative samples were identified, when comparing MYCO WELL D-ONE data with that of qPCR. Utilising these data, the sensitivity and specificity for the detection of *Ureaplasma* infection for the MYCO WELL D-ONE is calculated to be 91.98% and 96.44%, respectively. The positive predictive value (PPV) and negative predictive value (NPV) of the MYCO WELL D-ONE to determine *Ureaplasma* infection was 95.6% and 93.47%, respectively, with an accuracy of 94.4%.

This specificity and sensitivity data can be further sub-categorised, by both sample type and gender. For instance, swab samples (297 total samples) across both male and females displayed a sensitivity and specificity of 92.05% and 93.39% for *Ureaplasma* spp. detection, respectively. The PPV and NPV for swab samples overall, was respectively calculated as 98.29% and 88.98%. Whereas for *Ureaplasma* detection in urine samples (686 total samples), sensitivity and specificity were calculated to be 91.94% and 99.27%, respectively. The MYCO WELL D-ONE has a PPV and NPV of 98.82% and 94.91%, respectively, for the detection of *Ureaplasma* infections in urine samples. Analysis of MYCO WELL D-ONE results for female patients (648 samples), against the qPCR results for

their respective DNA sample, revealed a sensitivity, specificity, PPV and NPV of 93.14%, 93.31%, 95.15% and 90.61%, respectively, for *Ureaplasma* infections. Whereas for male patients, sensitivity, specificity, PPV and NPV were calculated to be 85.71%, 99.62%, 98.36% and 96.35%, respectively, for *Ureaplasma* infections.

For the detection of *M. hominis* overall (983 samples), a total of 97 true positive samples, 849 true negative samples, 10 false positive samples and 27 false negative samples were identified, when comparing MYCO WELL D-ONE data with that of qPCR. Utilising these data, the sensitivity and specificity for the detection of *M. hominis* infection for the MYCO WELL D-ONE is calculated to be 78.23% and 98.84%, respectively. The positive predictive value (PPV) and negative predictive value (NPV) of the MYCO WELL D-ONE to determine *M. hominis* infection was 90.65% and 96.92%, respectively, with an accuracy of 96.24% (Table 3.2).

For clarification:

- Sensitivity: probability that a test result will be positive when the organism is present (true positive rate); true positives divided by the sum of true positives and false negatives.
- Specificity: probability that a test result will be negative when the organism is not present (true negative rate); true negatives divided by the sum of true negatives and false positives.
- Positive Predictive Value (PPV): probability that disease is present when the test is positive; true positives divided by the sum of true positives and false positives.
- Negative Predictive Value (NPV): probability that disease is not present when the test is negative; true negatives divided by the sum of true negatives and false negatives.

	Total Sample Number	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	Accuracy
Overall	983	91.98%	96.44%	95.60%	93.47%	94.40%
Swab	297	92.05%	93.39%	98.29%	88.98%	92.59%
Urine	686	91.94%	99.27%	98.82%	94.91%	96.36%
Male	335	85.71%	99.62%	98.36%	96.35%	96.72%
Female	648	93.14%	93.31%	95.15%	90.61%	93.21%

Table 3.1 A table displaying the sensitivity, specificity, positive predictive value, negative predictive value and accuracy for the MYCO WELL D-ONE, utilising PHE's qPCR as the gold-standard, for the detection of Ureplasma spp. infection across sample types and sexes. The MYWO WELL D-ONE was most sensitive for the detection of Ureaplasma spp. infections in females and swab samples. Specificity and accuracy were highest for male and urine samples.

	Total Sample Number	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	Accuracy
Overall	983	78.23%	98.84%	90.65%	96.92%	96.24%
Swab	297	78.57%	97.93%	89.80%	95.16%	94.28%
Urine	686	77.46%	99.35%	93.22%	97.45%	97.09%
Male	335	92.86%	99.69%	92.86%	99.69%	99.40%
Female	648	76.36%	98.33%	90.32%	95.32%	94.60%

Table 3.2 A table displaying the sensitivity, specificity, positive predictive value, negative predictive value and accuracy for the MYCO WELL D-ONE, utilising PHE's qPCR as the gold-standard, for the detection of M. hominis infection across sample types and sexes. The MYWO WELL D-ONE was most sensitive for the detection of Ureaplasma spp. infections in male samples. Specificity and accuracy were highest for male and urine samples.

3.3 Accuracy of MYCO WELL D-ONE Semi-Quantitative *Ureaplasma* spp. wells

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MYCO WELL D-ONE semi-quantitative wells provide an estimation of the concentration of the inoculum transferred into the plate wells. A positive *Ureaplasma* spp. identification is accompanied by a concentration of either 10^4 or $\geq 10^5$. The accuracy of these wells was determined against the culture titration methodology, that serially diluted the inoculum to provide an accurate determination of the ureaplasmal concentration of swab/urine inoculated sterile saline. Overall, 424 samples were culture positive in both MYCO WELL D-ONE and culture titration, permitting a direct comparison of inoculum quantitation. Against the concentrations calculated through the culture titration methodology for each sample, the MYCO WELL D-ONE results agreed with the culture titration results in only 43.6% of cases. Out of 424 samples the MYCO WELL D-ONE accurately determined the concentration to be $\leq 10^4$ CCU/mL in 17.9% of cases (76/424), it correctly determined the concentration to be $\geq 10^5$ CCU/mL in 23.6% of cases (109/424). Conversely, the MYCO WELL D-ONE inaccurately determined the inoculum concentration to be $\geq 10^5$ CCU/mL, when it was determined to be $\leq 10^4$ CCU/mL by culture titration, in 55.9% of cases (237/424). Alongside inaccurately determining the inoculum concentration to be $\leq 10^4$ CCU/mL, when it was determined to be $\geq 10^5$ CCU/mL by culture titration in 0.9% of cases (2/424). Additionally, the accuracy of the MYCO WELL D-ONE semi-quantitative well was determined for the following sub-categories: female patients, male patients, urine samples and swab samples. The MYCO WELL D-ONE was most accurate in determining the concentration of saline inoculated with swab samples, with an agreeability of 62.9%, with culture titration. For urine samples, the rapid assay was least accurate in estimating the inoculum titre, correctly determining the concentration in only 30.7% of samples. The remaining 69.3% of incorrectly determined titres were over-estimations, providing a $\geq 10^5$ CCU/mL value for sample determined to be $\leq 10^4$ CCU/mL by culture titration.

OVERALL	Culture Titration	
MYCO WELL	**≤10^4**	**≥10^5**
10^4	76	2
≥10^5	237	109

Table 3.3 displaying the overall agreement between USM culture titration and MYCO WELL D-ONE in determining the Ureaplasma spp. concentration of both swab- and urine-inoculated saline samples. The overall accuracy for the MYCO WELL D-ONE was 43.63%.

SWAB	Culture Titration	
MYCO WELL	**≤10^4**	**≥10^5**
10^4	13	2
≥10^5	61	94

Table 3.4 displaying the agreement between USM culture titration and MYCO WELL D-ONE in determining the Ureaplasma spp. concentration of both swab--inoculated saline samples. The overall accuracy for the MYCO WELL D-ONE was 62.94%.

FEMALE	Culture Titration	
MYCO WELL	**≤10^4**	**≥10^5**
10^4	55	2
≥10^5	198	108

Table 3.5 displaying the overall agreement between USM culture titration and MYCO WELL D-ONE in determining the Ureaplasma spp. concentration of female positive samples. The overall accuracy for the MYCO WELL D-ONE was 44.09%.

MALE	Culture Titration	
MYCO WELL	**≤10^4**	**≥10^5**
10^4	21	0
≥10^5	39	1

Table 3.6 displaying the overall agreement between USM culture titration and MYCO WELL D-ONE in determining the Ureaplasma spp. concentration of male positive samples. The overall accuracy for the MYCO WELL D-ONE was 36.07%.

URINE	Culture Titration	
MYCO WELL	**≤10^4**	**≥10^5**
10^4	63	0
≥10^5	176	15

Table 3.7 displaying the overall agreement between USM culture titration and MYCO WELL D-ONE in determining the Ureaplasma spp. concentration of urine positive samples. The overall accuracy for the MYCO WELL D-ONE was 30.71%.

Key	
	Agreement
	Over reported
	Under reported

3.4 MYCO WELL D-ONE Detection Time

The detection time of the MYCO WELL D-ONE, alongside the culture titration methodology, was also recorded following incubation. Plates were inoculated immediately following receipt of the sample at the Hospital in which they were collected: Dewi Sant, Keir Hardie or Ysbyty Cwm Cynon. Following transport to the Heath hospital laboratory, samples were incubated overnight and read at the earliest convenience the next day, and periodically over the following days. Every assay was checked at each read and if a positive result was noted on either assay the detection time was recorded. For the culture titration assay the mean detection time for *Ureaplasma* spp., in female patient samples, was 28.14 hours post-incubation. Whereas for the MYCO WELL D-ONE the mean time to detection for *Ureaplasma* spp. in female patients was 27.57 hours post-incubation. Statistical analysis revealed no significant difference between the detection time of both assays (p=0.4265; t-test), for the detection of *Ureaplasma* spp. in female patient samples. The mean detection time for *Ureaplasma* spp. in male patient samples was 34.09 hours for both assays. Statistical analysis revealed no significant difference between the detection time of both assays (p=1.00; t-test), for the detection of *Ureaplasma* spp. in male patient samples. Furthermore, the mean detection time for *M. hominis* in female patient samples was 43.26 and 43 hours for the MYCO WELL D-ONE and culture titration assay, respectively. Statistical analysis revealed no significant difference between the detection time of both assays (p=0.8131; t-test), for the detection of *M. hominis* in female patient samples. Whereas for the detection of *M. hominis* the mean time to a positive result was 46.54 hours for both assays. Statistical analysis revealed no significant difference between the detection time of both assays (p=1.00; t-test), for the detection of *M. hominis* in male patient samples.

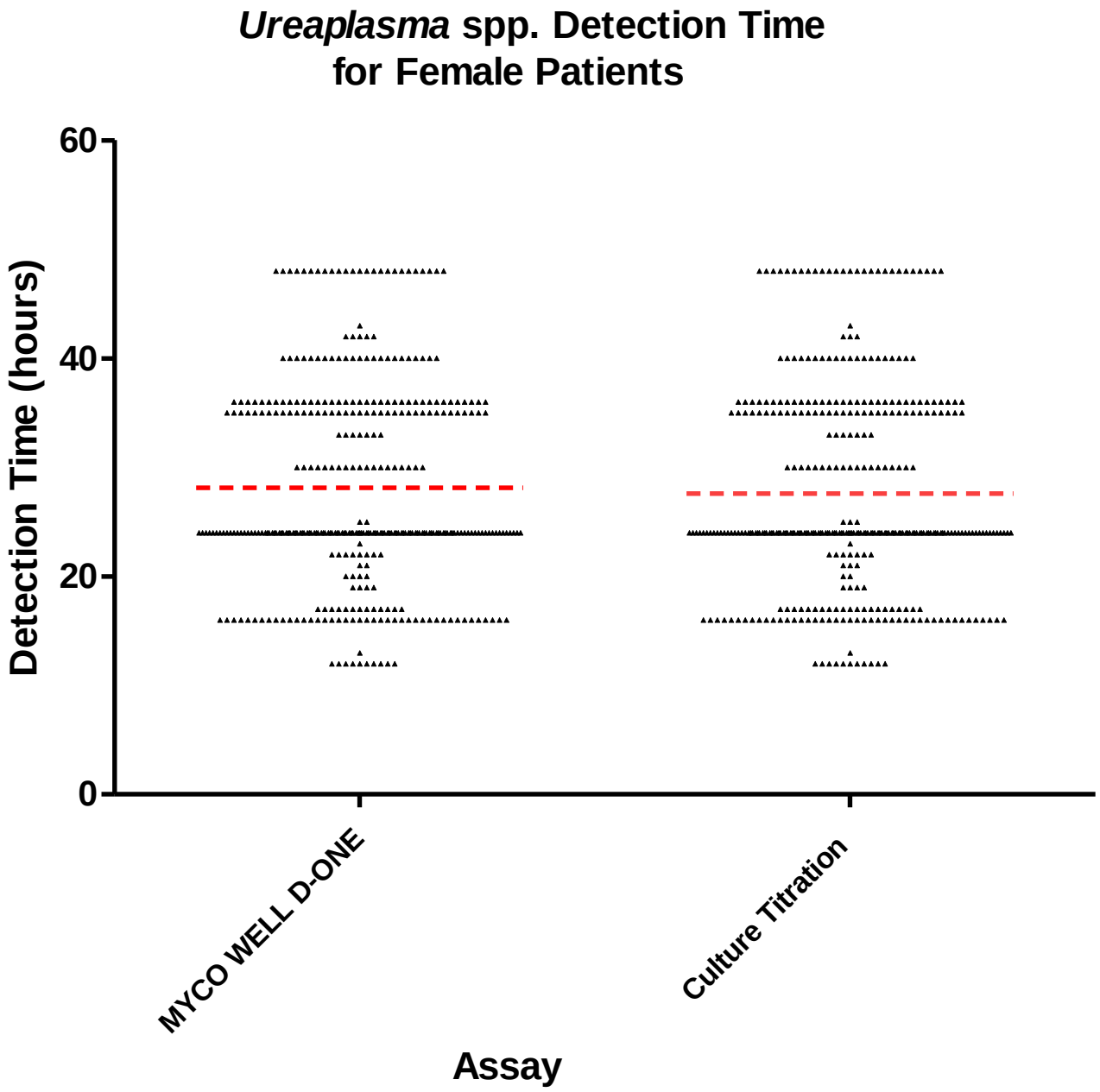


Figure 3.1 A graph comparing the detection time of the culture titration assay alongside the MYCO WELL D-ONE for Ureaplasma-positive female patients. The mean detection time for the culture titration assay was 28.14 hours, whereas the mean time to detection for MYCO WELL D-ONE assay was 27.57 hours. There was no significant difference between the detection times of the both assay in identifying female patient samples as Ureaplasma-positive.

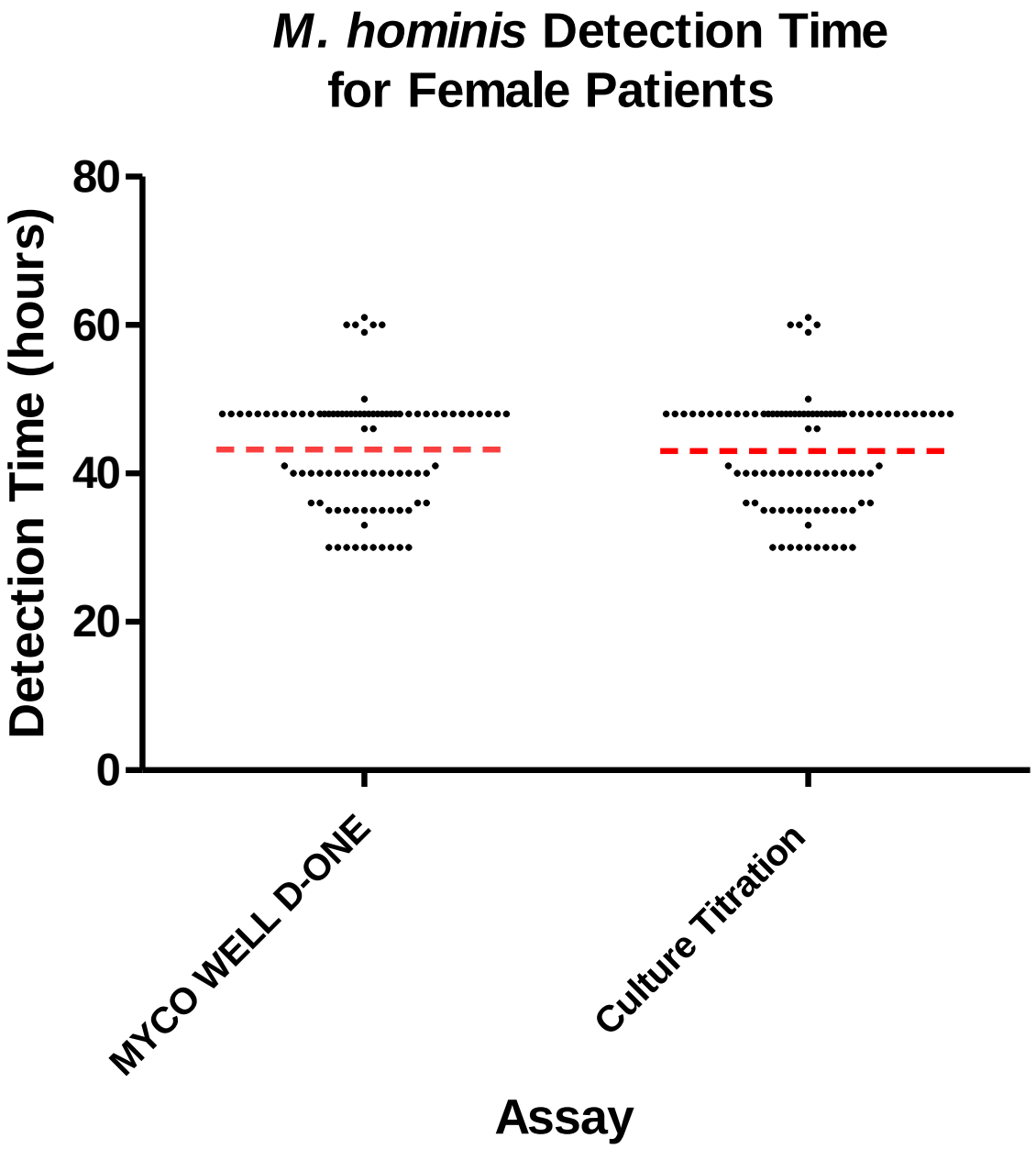


Figure 3.2 A graph comparing the detection time of the culture titration assay alongside the MYCO WELL D-ONE for M. hominis-positive female patients. The mean detection time for the culture titration assay was 43.26 hours, whereas the mean time to detection for MYCO WELL D-ONE assay was 43 hours. There was no significant difference between the detection times of the both assay in identifying female patient samples as M. hominis-positive.

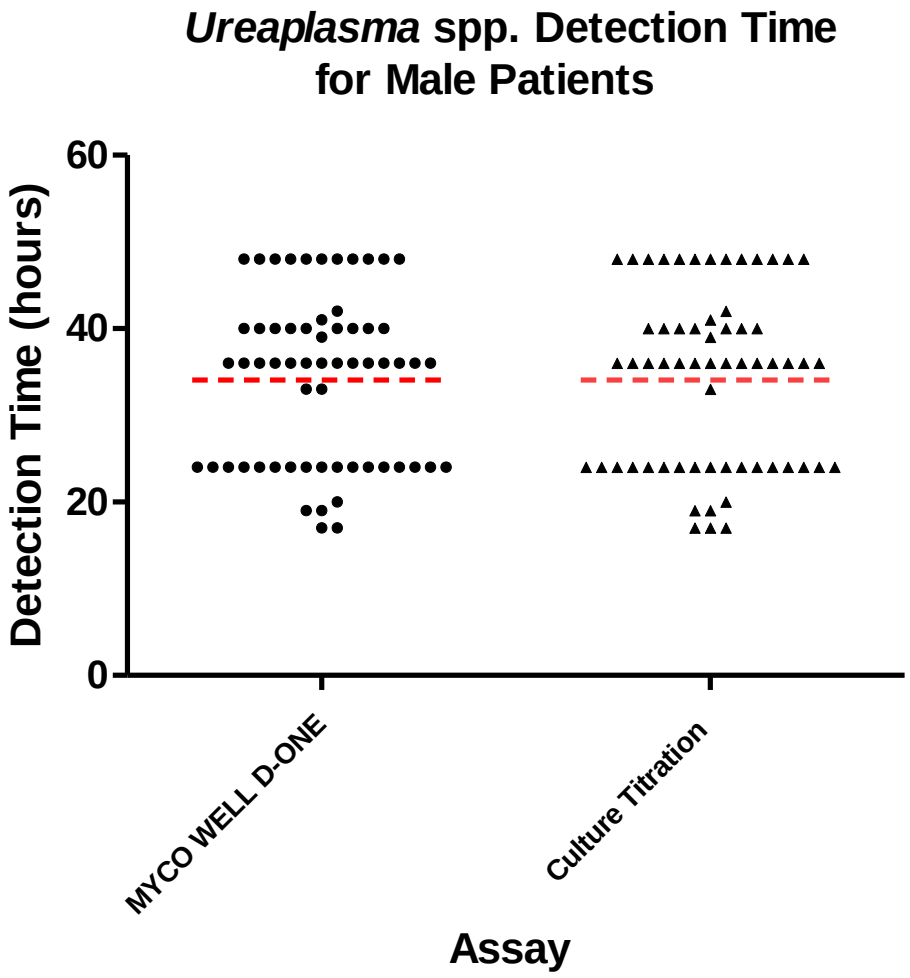


Figure 3.3 Graph displaying the mean detection time for Ureaplasma spp. in male patient samples. The mean detection time was 34.09 hours for both assays. Statistical analysis revealed no significant difference between the detection time of both assays (p=1.00; t-test), for the detection of Ureaplasma spp. in male patient samples

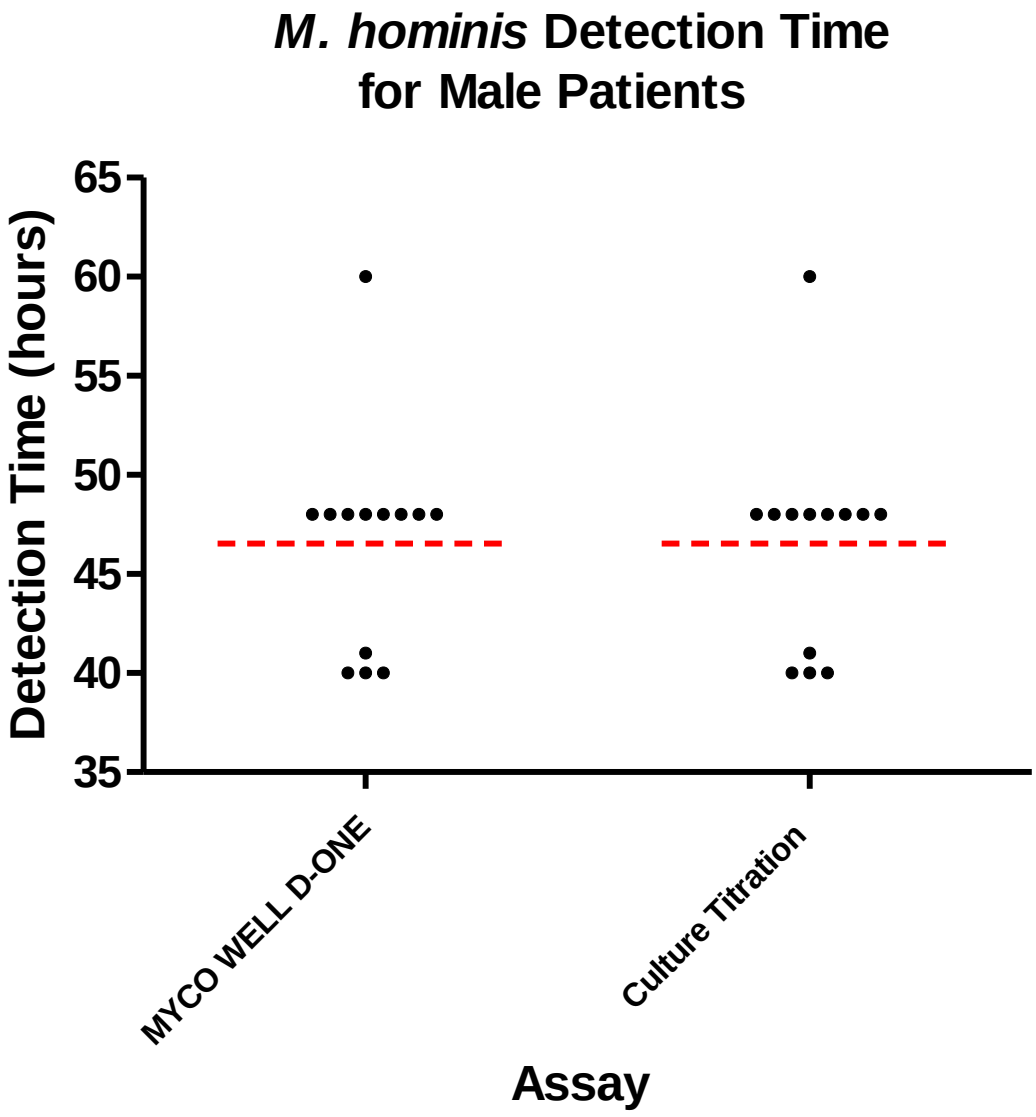


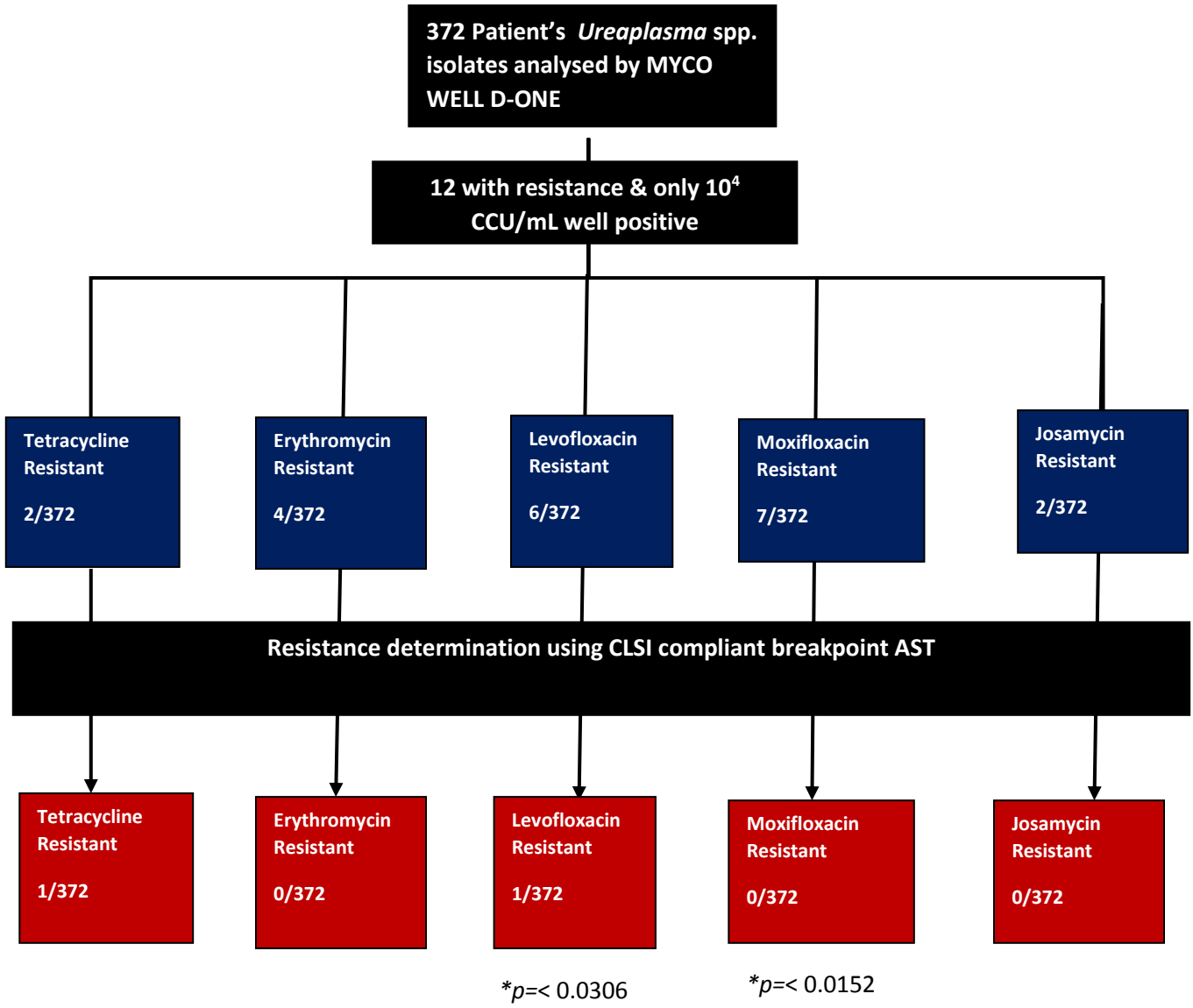
Figure 3.4 Graph comparing the mean detection times for the identification of M. hominis-positive males between the culture titration and MYCO WELL D-ONE assays. the mean time to a positive result was 46.54 hours for both assays. Statistical analysis revealed no significant difference between the detection time of both assays (p=1.00; t-test), for the detection of M. hominis in male patient samples.

3.5 MYCO WELL D-ONE Antibiotic Susceptibility Testing

All samples displaying a positive result in the highest concentration of antibiotic (resistance determining concentration) were screened for antimicrobial resistance utilising the methodology outlined in Beeton *et al*.[328] However, the instruction of the MYCO WELL D-ONE assay stipulates that if the $\geq 10^5$ well is positive any positivity in resistance determining wells is invalid and is to be confirmed by other means. In total 106 *Ureaplasma* spp. isolates were pure-cultured through serial dilution in specialist media and screened for antibiotic resistance against one or more antibiotic, due to positivity displayed in AST wells of the MYCO WELL D-ONE; 69 against erythromycin; 25 against josamycin; 30 against tetracycline (supposed doxycycline resistance tested against tetracycline); 32 against levofloxacin; 23 against moxifloxacin. Of these tested, 94 had a positive $\geq 10^5$ MYCO WELL D-ONE well so would not have been classified as truly resistant by the MYCO WELL D-ONE, nevertheless they were still screened for possible resistance. The 12 remaining isolates possessed a positive 10^4 well and various combinations of positive resistance wells. Isolates were screened against one or more antibiotic; 4 against erythromycin; 2 against josamycin; 2 against tetracycline; 6 against levofloxacin; 7 against moxifloxacin. Of these 12 isolates determined to be resistant, 2 (1 levofloxacin-resistant and 1 tetracycline-resistant) were determined to be truly resistant when subjected to CLSI compliant AST. A further 2 resistant isolates (1 levofloxacin-resistant and 1 tetracycline-resistant) were discovered following screening of isolates that possessed a positive $>10^5$ CCU/mL well, indicating resistance confirmation should be determined by other means. In total 2 levofloxacin-resistant strains (DF99 and KF86) and 2 tetracycline-resistant strains (DF145 and DF28) were identified to be truly resistant, when subjected to CLSI compliant AST testing. These isolates were further sub-cultured for whole genome sequencing.

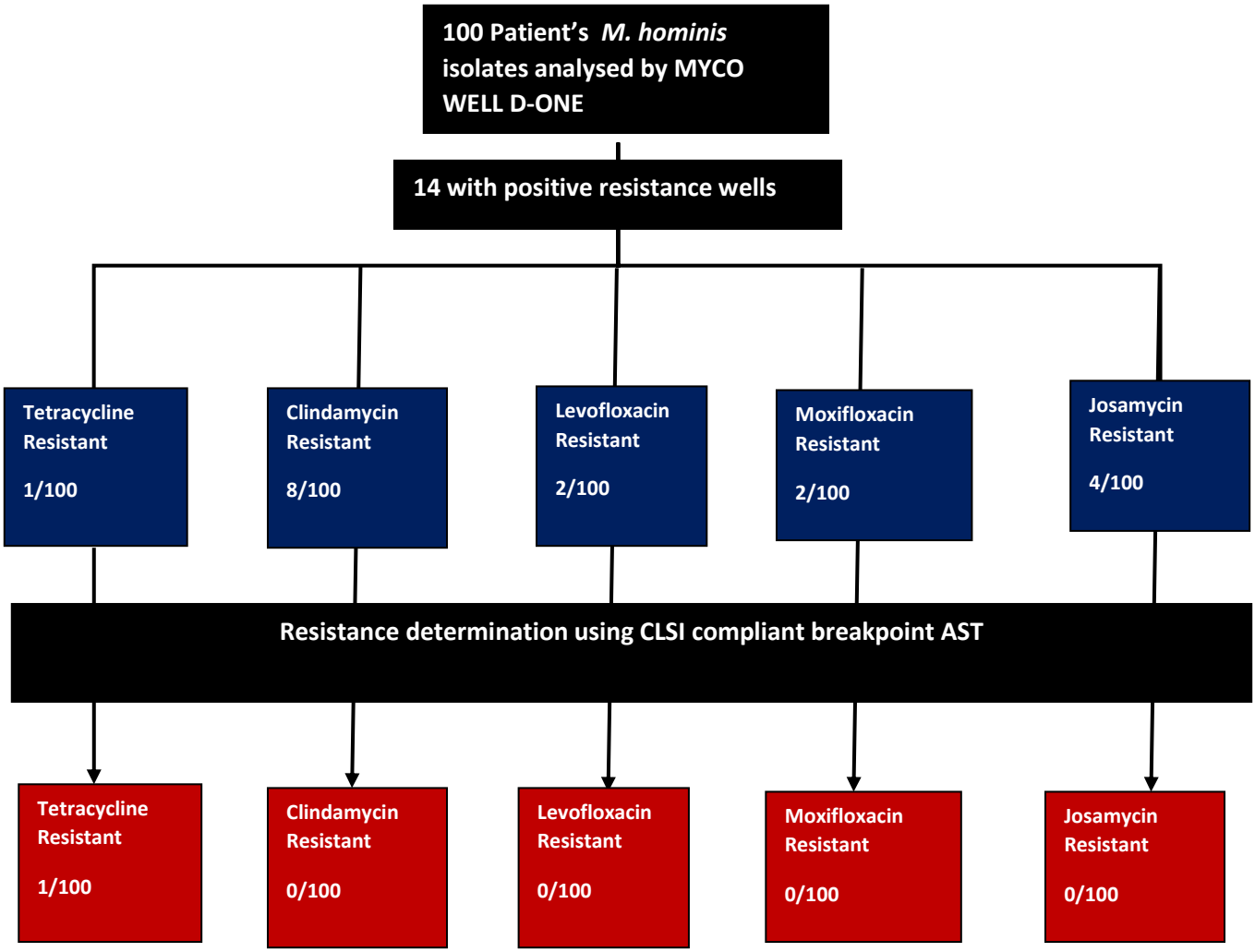
For *M. hominis*, 14 isolates were identified as resistant on the MYCO WELL D-ONE assay. These isolates were pure cultured and screened against one or more antibiotics; 8 against clindamycin; 4

against josamycin; 1 against tetracycline; 2 against levofloxacin; 2 against moxifloxacin. A single *M. hominis* isolate was found to be tetracycline resistant (DF28).



*Fisher's exact test highlighting significant overreporting of MYCO WELL D-ONE

Figure 3.5 A Chart displaying the AST screening of all putative antibiotic resistant Ureaplasma identified by the MYCO WELL D-ONE by a CLSI compliant methodology.



* Fisher's exact test highlighting significant overreporting of MYCO WELL D-ONE

Figure 3.6 Chart displaying the AST screening of all putative antibiotic resistant M. hominis identified by the MYCO WELL D-ONE by a CLSI compliant methodology.

3.5.1 Whole Genome Sequencing

Whole genome sequencing of the isolates determined to be truly resistant through CLSI compliant AST, revealing the mobile genetic elements and single nucleotide polymorphisms conferring the antibiotic resistance to the isolated organism. Both *Ureaplasma* spp. isolated determined to be levofloxacin resistant possessed amino acid substitution Ser83Leu (serine to leucine substitution) in the *parC* gene of the QRDR. Moreover, the tetracycline resistant *Ureaplasma* spp. isolated both possessed the Tn916 *tet(M)* mobile genetic element. Likewise, the *M. hominis* isolate harboured the Tn916 *tet(M)* mobile genetic element, conferring tetracycline resistance.

Sample No.	Antibiotic	Breakpoint (µg/mL)	Resistance Mechanism	Species
DF99	Levofloxacin	4	Ser83Leu mutation	*U. parvum*
KF86	Levofloxacin	4	Ser83Leu mutation	*U. parvum*
DF28	Tetracycline	2	Tn916 *tet(M)*	*U. parvum*
DF145	Tetracycline	2	Tn916 *tet(M)*	*U. parvum*

Table 3.8 Table displaying the Ureaplasma spp. sample/isolate numbers that were identified and confirmed as resistant, alongside the mechanism that conferred such resistance, as identified through whole genome sequencing.

3.6 Ureaplasma spp. and M. hominis Prevalence

Analysis of the multiplex qPCR data was utilised to determine the prevalence rates of *Ureaplasma* spp. and *M. hominis*. For females, a total of 526 patients were tested with 301 positives for *Ureaplasma* spp. displaying a prevalence rate of 57.2%. Additionally, out of the 526 female patients, 86 were positive for *M. hominis* resulting in a prevalence rate of 16.3%. A total of 329 males were tested for the presence of *Ureaplasma* spp. and *M. hominis* in their genitourinary tract, 71 male patients were positive for *Ureaplasma* spp. and 14 positives for *M. hominis*, prevalence rates are 21.6% and 4.3%, respectively. Female patients had significantly higher prevalence rates of *Ureaplasma* spp. colonisation, when compared to males (p=< 0.0001; Fisher's exact test). Similarly, female patients had significantly higher rates of *M. hominis* colonisation (p=0.0081).

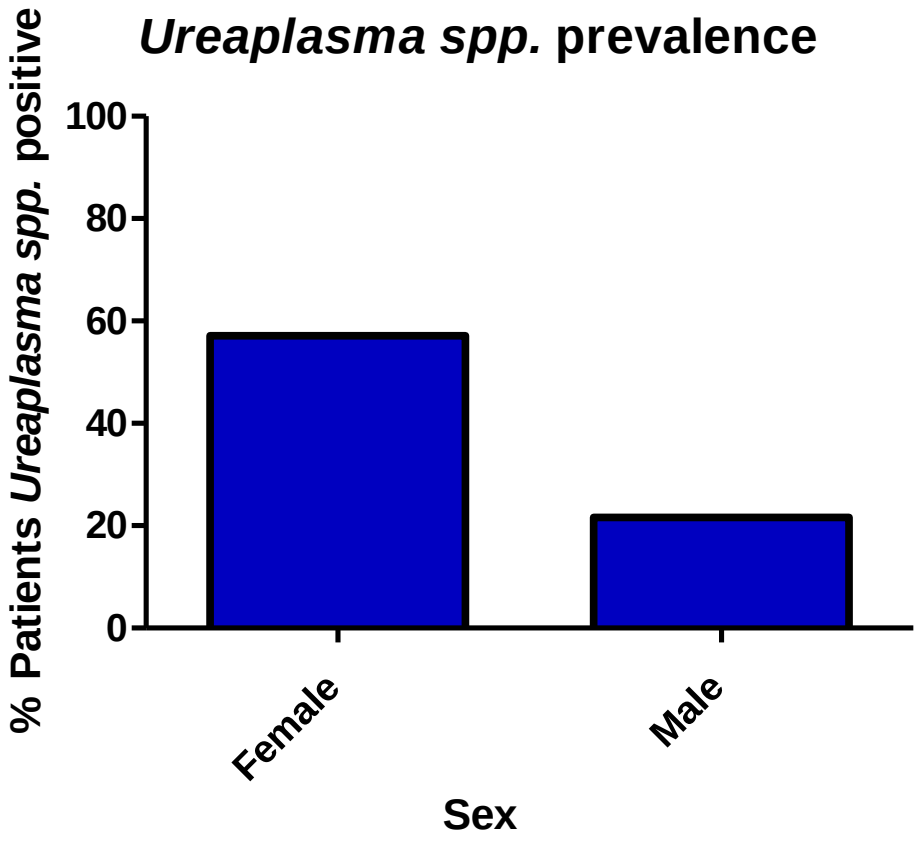


Figure 3.7 Graph displaying Ureaplasma spp. prevalence for both female and male patients. Female patients had a prevalence rate of 57.2%, with males being Ureaplasma-positive in 21.6% of cases. Females possessed significantly higher prevalence rates than males (p=<0.0001; Fisher's exact test).

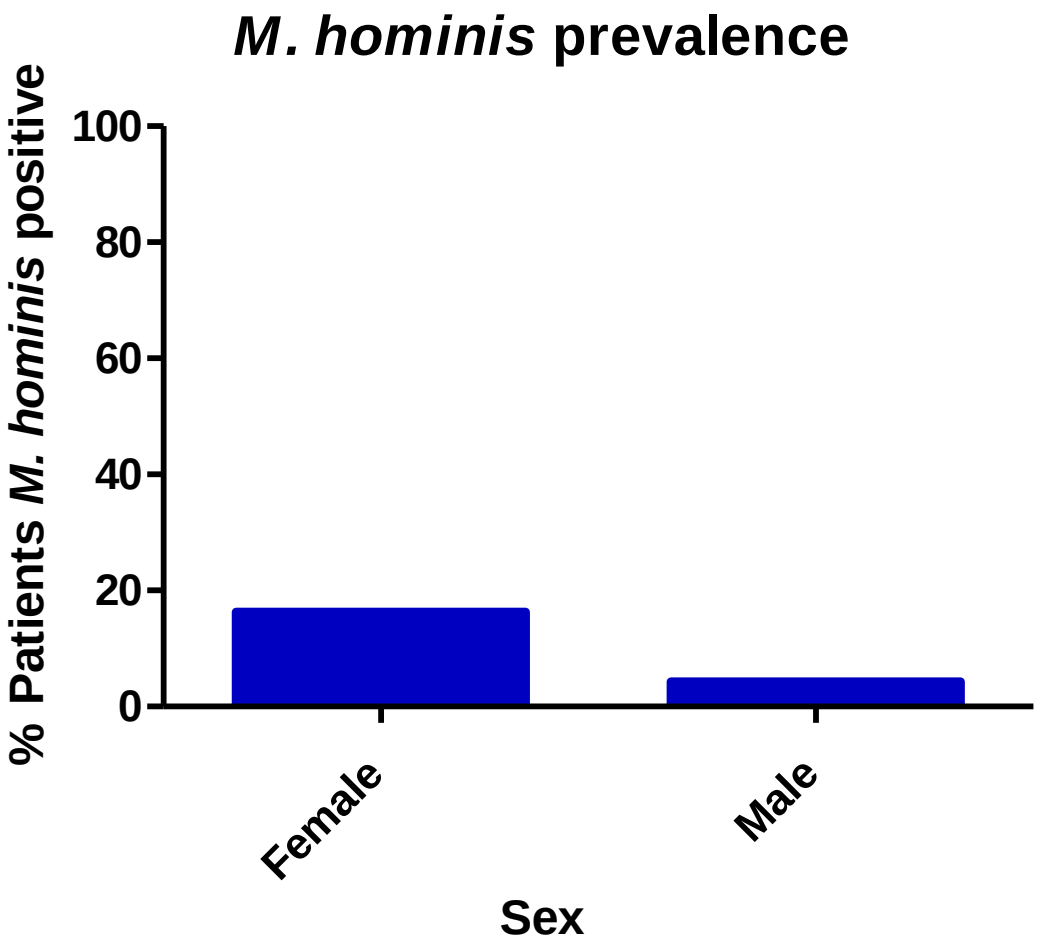


Figure 3.8 Graph displaying M. hominis prevalence for both female and male patients. Female patients had a prevalence rate of 16.3, with males being M. hominis-positive in 4.3% of cases. Females possessed significantly higher prevalence rates than males (p=<0.0081; Fisher's exact test).

3.7 Concomitant Ureaplasma spp. and M. hominis

Of the 86 females positive for *M. hominis*, 91.9% (79/86) patients were also positive for *Ureaplasma* spp. colonisation, the remaining 8.1% (7/86) patients were positive only for *M. hominis*. For males, of the 14 patients positive for *M. hominis*, 71.4% (10/14) were also positive for *Ureaplasma* spp., whereas the remaining 28.6% were only positive for *M. hominis*. Females had significantly higher rates of concomitant *M. hominis* and *Ureaplasma* spp. infection (p=0.0454; Fisher's exact test).

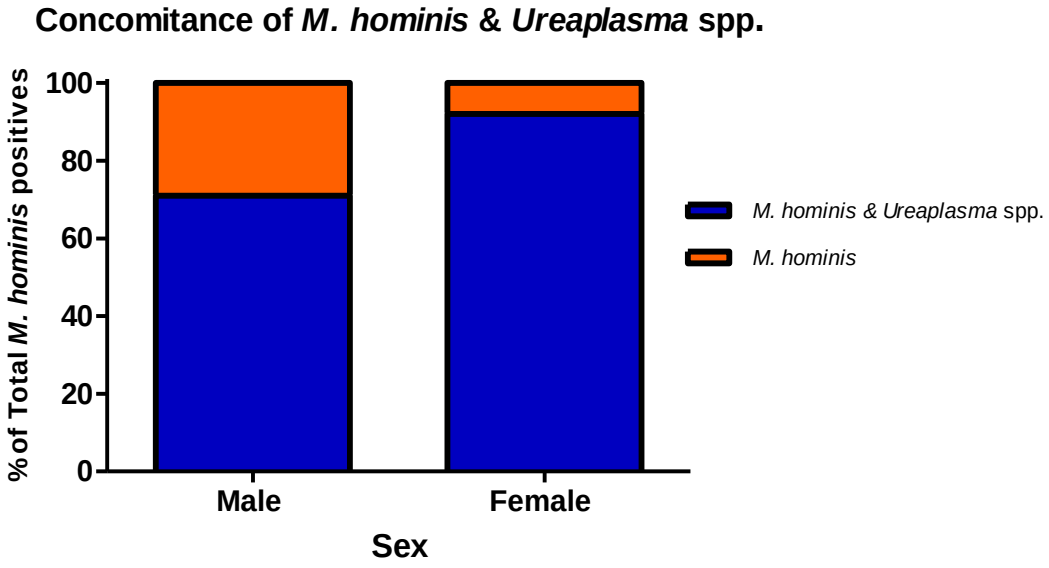


Figure 3.9 Graph displaying concomitant M. hominis and Ureaplasma spp. infection, alongside M. hominis monoinfections, as a % of total M. hominis-positive for both male and female patients. 91.9% of females positive for M. hominis were also positive for Ureaplasma spp., compared with only 71.4% of males. Females had significantly higher rates of concomitant M. hominis and Ureaplasma spp. infection (p=0.0454; Fisher's exact test).

To further sub-categorise the concomitance rates of *M. hominis* and *Ureaplasma* spp., the type of *Ureaplasma* infection that was present alongside *M. hominis* infection was also determined. Overall, out of the 89 concomitant *M. hominis*- and *Ureaplasma*-positive patients, 60.7% (54/89) were positive for *M. hominis* and *U. parvum*, 21.3% (19/89) were positive for *M. hominis* and *U. urealyticum*, and 18% (16/89) were positive for *M. hominis, U. parvum* and *U. urealyticum*. Furthermore, female concomitance was comprised of 63.3% (50/79) *M. hominis* and *U. parvum*,

17.7% (14/79) *M. hominis* and *U. urealyticum*, and 19% (16/79) *M. hominis*, *U. parvum* and *U. urealyticum*. Whereas, male concomitance *M.hominis-Ureaplasma* spp. was composed of 40% (4/10) *M. hominis* and *U. parvum*, 50% (5/10) *M. hominis* and *U. urealyticum*, and 10% (1/10) *M. hominis*, *U. parvum* and *U. urealyticum*. Furthermore, a Fisher's exact test analysis of the number of *M. hominis* only, *M. hominis* and *U. parvum*, *M. hominis* and *U. urealyticum* and *M. hominis*, *U. parvum* and *U. urealyticum* infections, revealed a statistically significant distribution in the composition of *M. hominis* infection type between sexes (p=0.0212; Fisher's exact test).

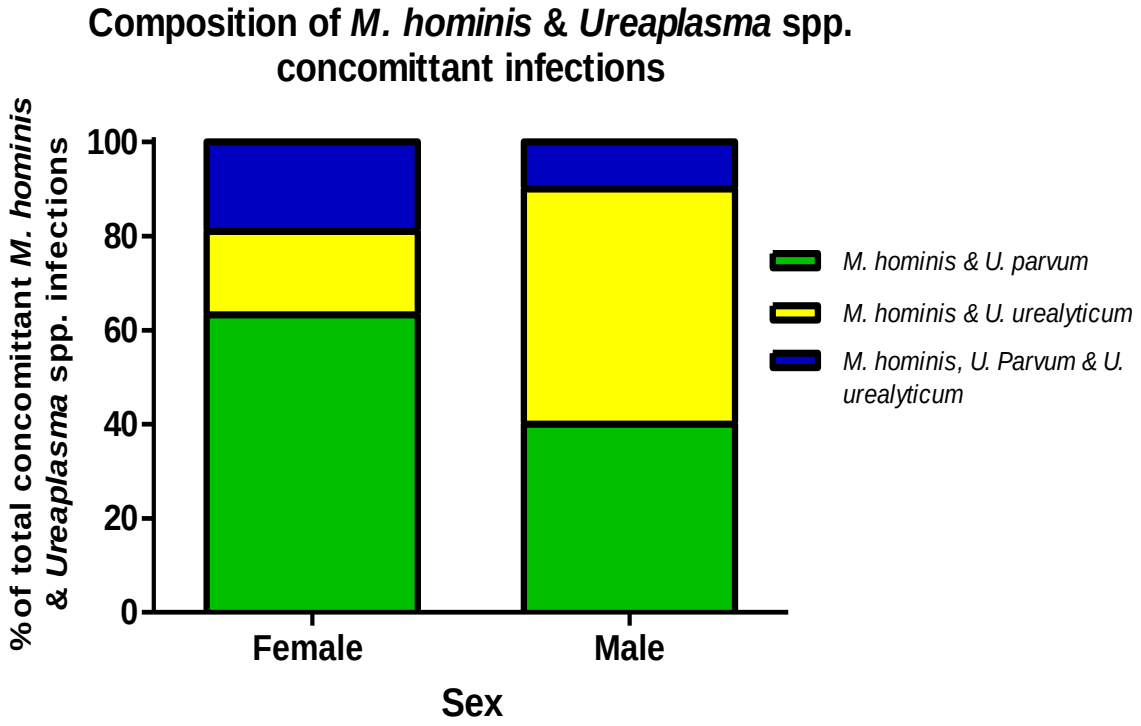


Figure 3.10 Graph displaying concomitant M. hominis and Ureaplasma spp. comprised of infection type. For females the most prevalent concomitant infection was M. hominis and U. parvum, accounting for 63.3% of total concomitant M. hominis-Ureaplasma spp. infections. Followed by 17.7 % M. hominis and U. urealyticum infections, alongside 19% M. hominis, U. parvum and U. urealyticum infections. For male concomitant infections, M. hominis and U. urealyticum infections were most common, at 50%. Furthermore, 40% of male concomitant infections were M. hominis and U. parvum and 10% M. hominis, U. parvum and U. urealyticum. a Fisher's exact test analysis revealed a statistically significant distribution in the composition of M. hominis infection type between sexes (p=0.0212).

3.8 *Ureaplasma* spp. Speciation

Utilising multiplex qPCR data, the prevalence of each type of *Ureaplasma* infection was determined for both male and female patient groups. Female patients had a colonisation rate of 40.5% *U. parvum* (213/526), 10.8% *U. urealyticum* (57/526) and 5.89% concomitant *U. parvum* & *U. urealyticum* (31/526). The prevalence of *Ureaplasma* infection type for male patients was determined to be 6.9% *U. parvum* (23/329), 14.3% *U. urealyticum* (47/329) and 0.3% concomitant *U. parvum* & *U. urealyticum* (1/329).

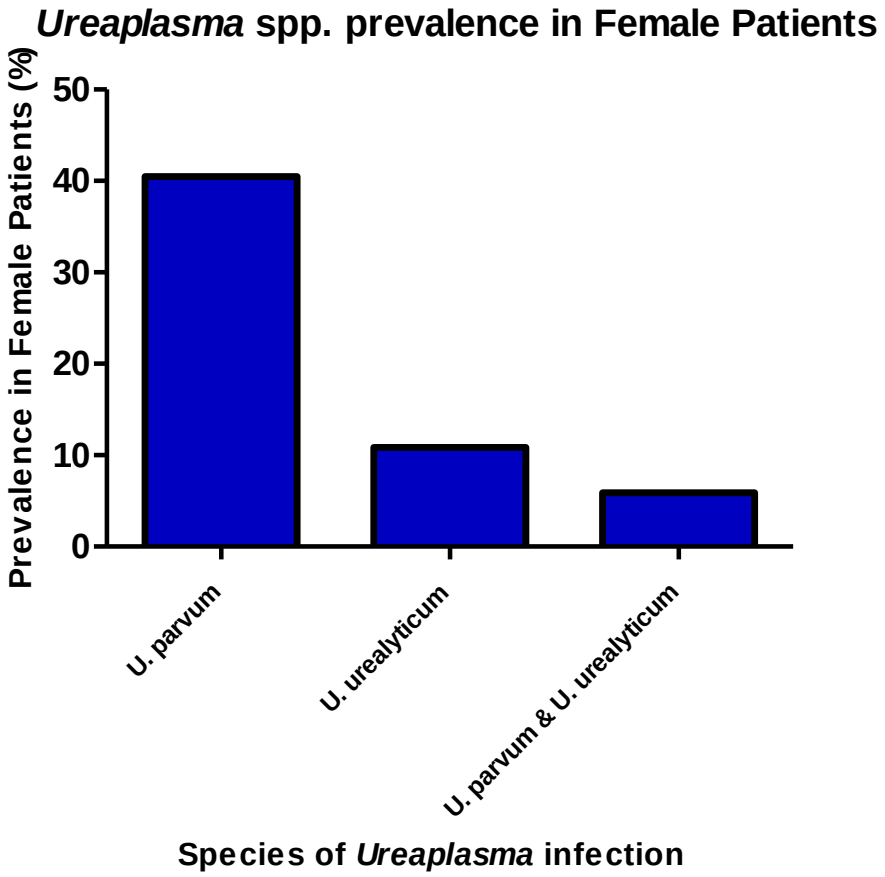


Figure 3.11 Graph displaying rates of Ureaplasma spp. infection type for female patients. The dominant Ureaplasma spp. infection type was U. parvum, with an overall prevalence rate of 40.5%. Followed by U. urealyticum with a prevalence rate of 10.8%, with dual U. parvum and U, urealyticum infection having a prevalence rate of 5.9%.

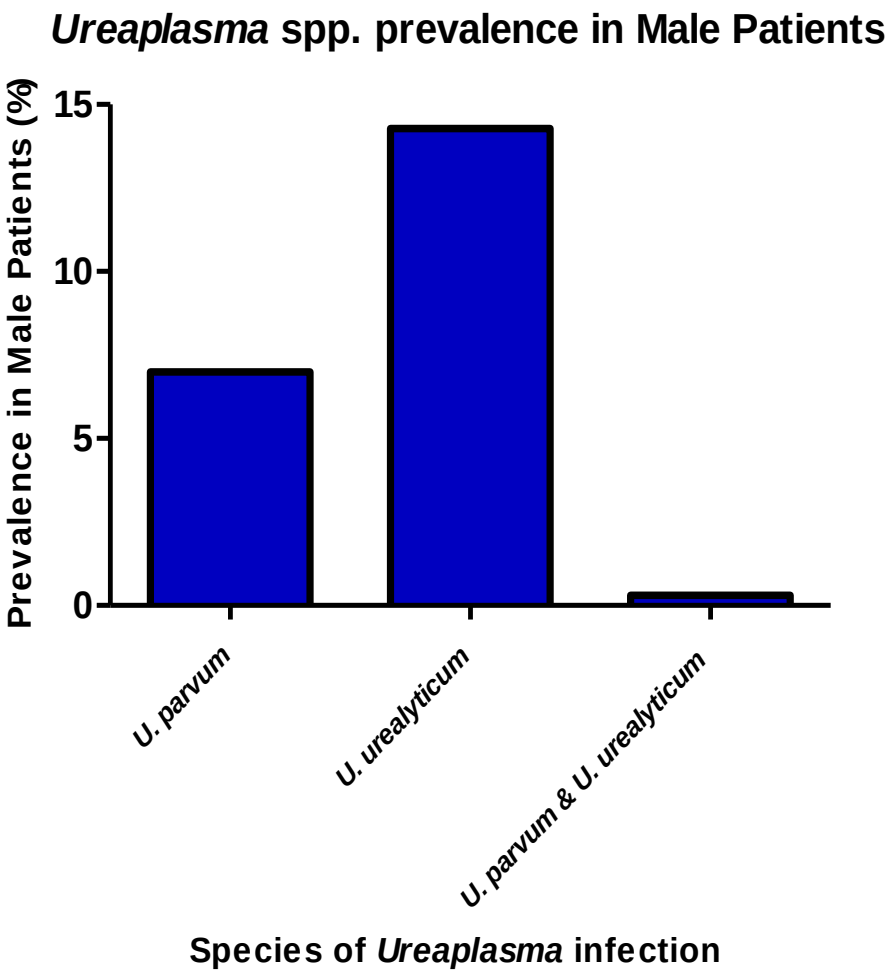


Figure 3.12 Graph displaying rates of Ureaplasma spp. infection type for male patients. The dominant Ureaplasma spp. infection type was U. urealyticum, with an overall prevalence rate of 14.3%. Followed by U. parvum with a prevalence rate of 6.9%, with dual U. parvum and U, urealyticum infection having a prevalence rate of 0.3%.

In addition to calculating the prevalence of each type of *Ureaplasma* spp. infection amongst male and female patient groups, the percentage of *Ureaplasma* infection type against the total number of *Ureaplasma*-positive patients was determined. Overall the composition of *Ureaplasma* spp. positives was comprised of the following: 63.4% *U. parvum* (236/372), 28% *U. urealyticum* (104/372) and 8.6% concomitant *U. parvum* & *U. urealyticum* (32/372). For females, the species of composition of total *Ureaplasma* spp. infections was 70.8% *U. parvum* (213/301), 18.9% *U. urealyticum* (57/301) and 10.3% concomitant *U. parvum* & *U. urealyticum* (31/301). Whereas for males, the species composition was as follows: 32.4% *U. parvum* (23/71), 66.2% *U. urealyticum* (47/71) and 1.4% concomitant *U. parvum* & *U. urealyticum* (1/71).

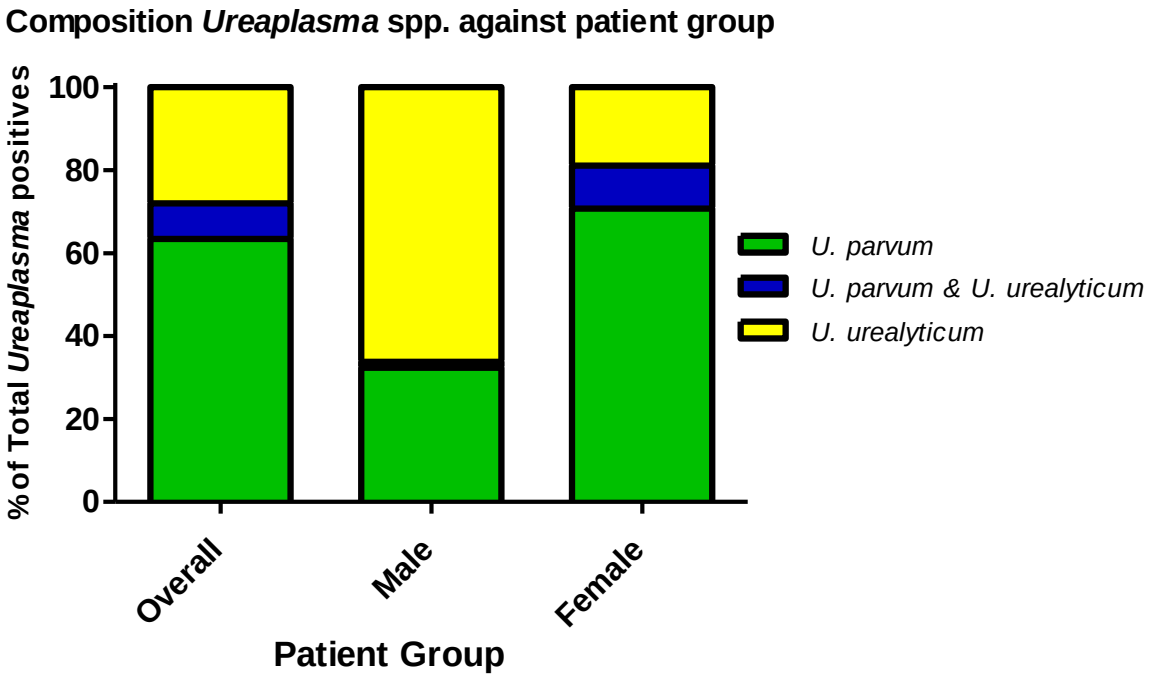


Figure 3.13 Graph displaying composition of Ureaplasma spp. infection across patient groups. Overall the composition of Ureaplasma spp. positives was comprised of the following: 63.4% U. parvum, 28% U. urealyticum and 8.6% concomitant U. parvum & U. urealyticum. For females: 70.8% U. parvum, 18.9% U. urealyticum and 10.3% concomitant U. parvum & U. urealyticum. For males: 32.4% U. parvum, 66.2% U. urealyticum and 1.4% concomitant U. parvum & U. urealyticum.

3.9 Bacterial Load and Sample Type

Utilising the serial dilution culture titration methodology, bacterial loads for each positive *Ureaplasma*-positive and *M. hominis*-positive sample were determined through the multiplication of the number of positive wells by the dilution factor. The limit of detection for the culture titration methodology was determined to be 10^3 CCU/mL, for the inoculated sterile saline solution. Additional positive samples i.e those that were *Ureaplasma* culture-positive for undiluted urine, grew *M hominis* colonies on US1 agar, or those samples which were subsequently sub-cultured from positive MYCO WELL D-ONE identification wells, were determined to have a bacterial load value <1000 CCU/mL. A total of 422 samples were culture-positive for *Ureaplasma* spp., consisting of 168 swab samples and 254 urine samples. For *M. hominis*, a total of 91 samples were culture-positive, including 48 swab samples and 43 urine samples. The primary concern with regards to bacterial load and assays such as the MYCO WELL D-ONE, particularly in relation to antibiotic susceptibility testing, is whether the sample is $>10^5$ CCU/mL or $<10^5$ CCU/mL, as a bacterial load of $<10^5$ CCU/mL is required to test against the CLSI breakpoint concentrations of antibiotic contained within the AST wells. Therefore, positive samples were divided into those greater, and those less, than 10^5 CCU/mL and further sub-categorised by sample type. For *Ureaplasma*-positive urine samples, 5.9% (15/254) were $>10^5$ CCU/mL, with 94.1% of samples having a *Ureaplasma* load of $<10^5$ CCU/mL. While for swab samples, 55.9% of *Ureaplasma* positives had a bacterial load of $>10^5$ CCU/mL, with the remainder of positives (44.1%) having a *Ureaplasma* load of $<10^5$ CCU/mL. The prevalence of a *Ureaplasma* load of $>10^5$ CCU/mL captured on swab samples is significantly higher than that of urine samples, for *Ureaplasma* positive patients (p= < 0.0001; Fisher's exact test). Moreover, analysis of *M.* hominis-positive samples similarly revealed swabs having generally greater bacterial loads than *M. hominis*-positive urine samples. *M. hominis*-positive swab samples with a bacterial load of $>10^5$ CCU/mL accounted for 40% of total swab-positives (19/48), 60% of swab-positives had a *M. hominis* load of $<10^5$ CCU/mL (29/48). Whereas, 93% of *M. hominis*-positive urine samples had a bacterial

load <10^5 CCU/mL (40/43) with 7% of positive urines having a *M. hominis* load of >10^5 CCU/mL. A Fisher's exact test revealed that the *M. hominis* positive swab samples have a greater incidence of bacterial loads exceeding 10^5 CCU/mL, when compared with *M. hominis* loads of urine-positive samples (*p*=0.0004).

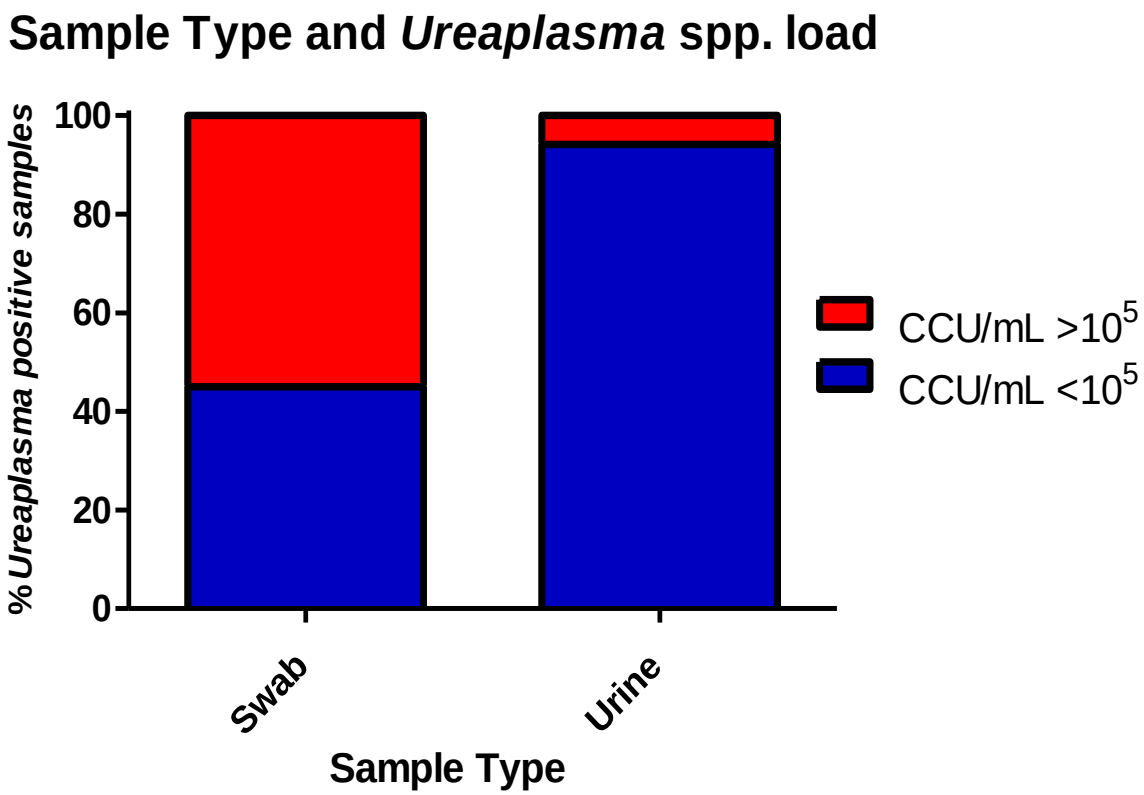


Figure 3.14 Composition of bacterial loads for Ureplasma spp. positive samples against sample type: swab or urine. For Ureaplasma-positive urine samples 5.9% possessed a load >10^5 CCU/mL. Whereas for Ureaplasma-positive swab samples 55.9% possessed a load >10^5 CCU/mL. The prevalence of a Ureaplasma load of >10^5 CCU/mL captured on swab samples is significantly higher than that of urine samples, for Ureaplasma positive patients (p= < 0.0001; Fisher's exact test).

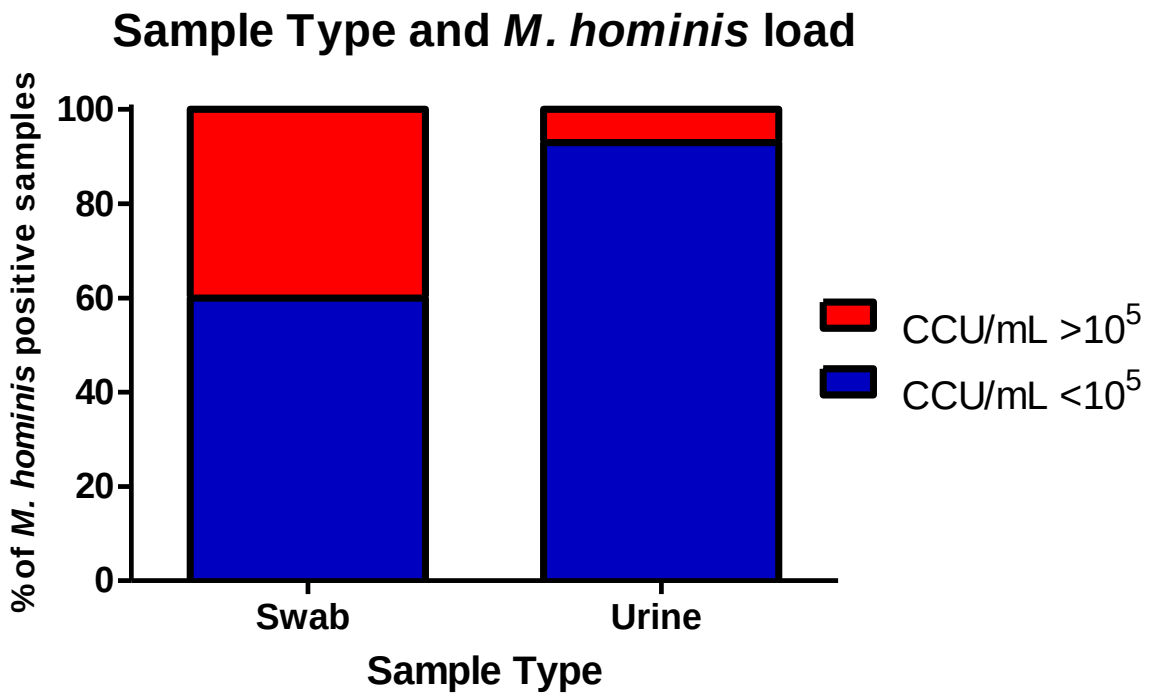


Figure 3.15 Composition of bacterial loads for M. hominis positive samples against sample type: swab or urine. For M. hominis-positive urine samples 7% possessed a load >10^5 CCU/mL. Whereas for M. hominis-positive swab samples 40% possessed a load >10^5 CCU/mL. The prevalence of a M. hominis load of >10^5 CCU/mL captured on swab samples is significantly higher than that of urine samples, for Ureaplasma positive patients (p=0.0004; Fisher's exact test).

3.10 Paired Swab and Urine Samples

In total 127 patients provided paired swab and urine samples, comprised of the following: 122 endocervical swab-urine pairings from female patients and 5 endourethral swab-urine pairings from male patients. Out of which, 71 patients were positive for *Ureaplasma* spp. and 21 patients *M. hominis*-positive by culture titration. The limit for detection for the culture titration method was 10^3 CCU/mL. Therefore, to permit a direct comparison between both urine and swab samples a bacterial load of 10^2 CCU/mL was applied to those samples of patients that fell below the limit of detection for the culture titration but were *Ureaplasma*-positive in direct urine culture, *M. hominis* colony positive on US1 agar and/or positively sub-cultured from MYCO WELL D-ONE identification wells. This was the highest possible bacterial load these samples could possess without a positive reaction in well 1 of the culture titrations.

For 100% of paired *Ureaplasma*-positive urine and swab samples, swab loads were equal to or greater than their urine sample counterpart. In 38% of cases swabs had bacterial loads equal to or 10 times greater than their urine counterparts. Whereas bacterial loads between 100-1000 times higher in swab samples, compared with their respective urines, were observed in 43.7% of *Ureaplasma*-positive samples. Additionally, swabs with *Ureaplasma* loads between 10,000-100,000 times and 1,000,000-10,000,000 times greater than their urine counterpart samples were observed in 15.5% and 2.8% of *Ureaplasma*-positive patients, respectively. A paired t-test between paired urine and swab samples displays swabs have significantly higher bacterial loads than their respective urine samples (p=0.0255).

M. hominis-positive paired patient samples, 76.2% (16/21) possessed swab-positives cultures with bacterial loads equal to, or greater than, their respective urine samples. For 9.5% (2/21) of *M. hominis*-positive patients with paired samples, *M. hominis* could only be recovered from urine samples and was undetectable in the complementary swab sample. Conversely, for a further 9.5% (2/21) of *M. hominis*-positive patients with paired samples, *M. hominis* was undetectable in the

urine samples, but was recovered from the complementary swab samples. The remaining sample (1/21), accounting for 4.75% of total *M. hominis*-positive paired samples, possessed a urine sample with a greater bacterial load than its swab counterpart. Swab samples had *M. hominis* loads equal to, or 10 times higher, in 42.9% of cases (9/21). Swab samples with 100-1000 times higher, and swab samples with 10,000-100,000 times higher *M. hominis* loads accounted for 23.8% and 14.3% of paired samples, respectively. Additionally, 9.5% of paired swab samples had bacterial loads 1,000,000-100,000,000 times higher, than their respective urine samples. However, in two instances, both comprising 4.5% each of the total *M. hominis*-positive paired samples, urine samples had a higher bacterial load than their swab counterparts, one with a bacterial load 100 times greater and the other with a load 1,000,000 times higher. Finally, though swab samples more frequently had greater bacterial loads than their respective urine samples, a paired t-test of the listed samples did not determine the difference to be statistically significant (p=0.0939).

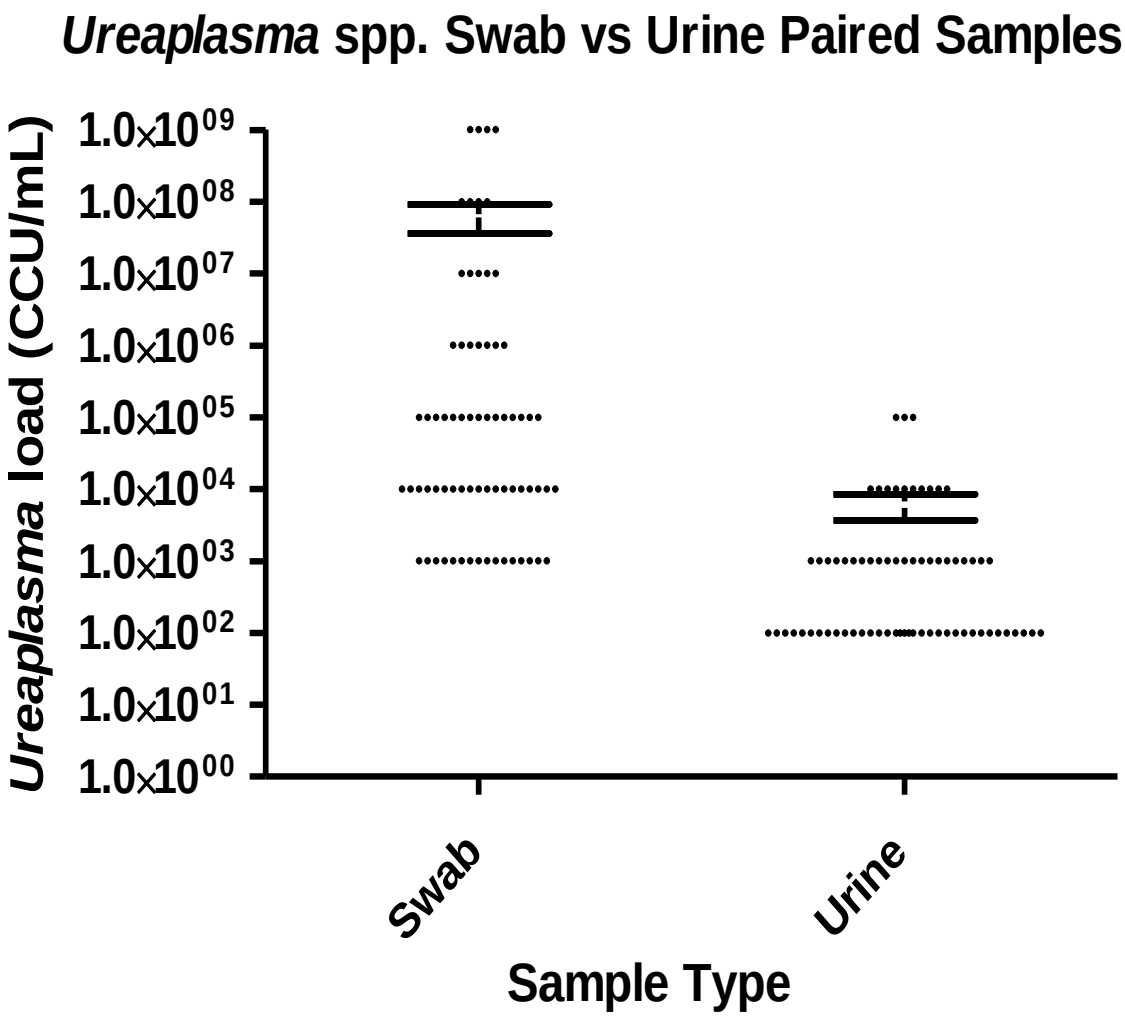


Figure 3.16 Graph plotting the Ureaplasma loads isolated from the 71 Ureaplasma-positive paired samples. Ureaplasma load in swab samples was equal to, or greater than, its urine counterpart in 100% of cases. In most cases (43.7%) the swab sample had a bacterial load 100-1000x greater than its urine counterpart. A t-test between paired urine and swab samples displays swabs have significantly higher bacterial loads than their respective urine samples (p=0.0255).

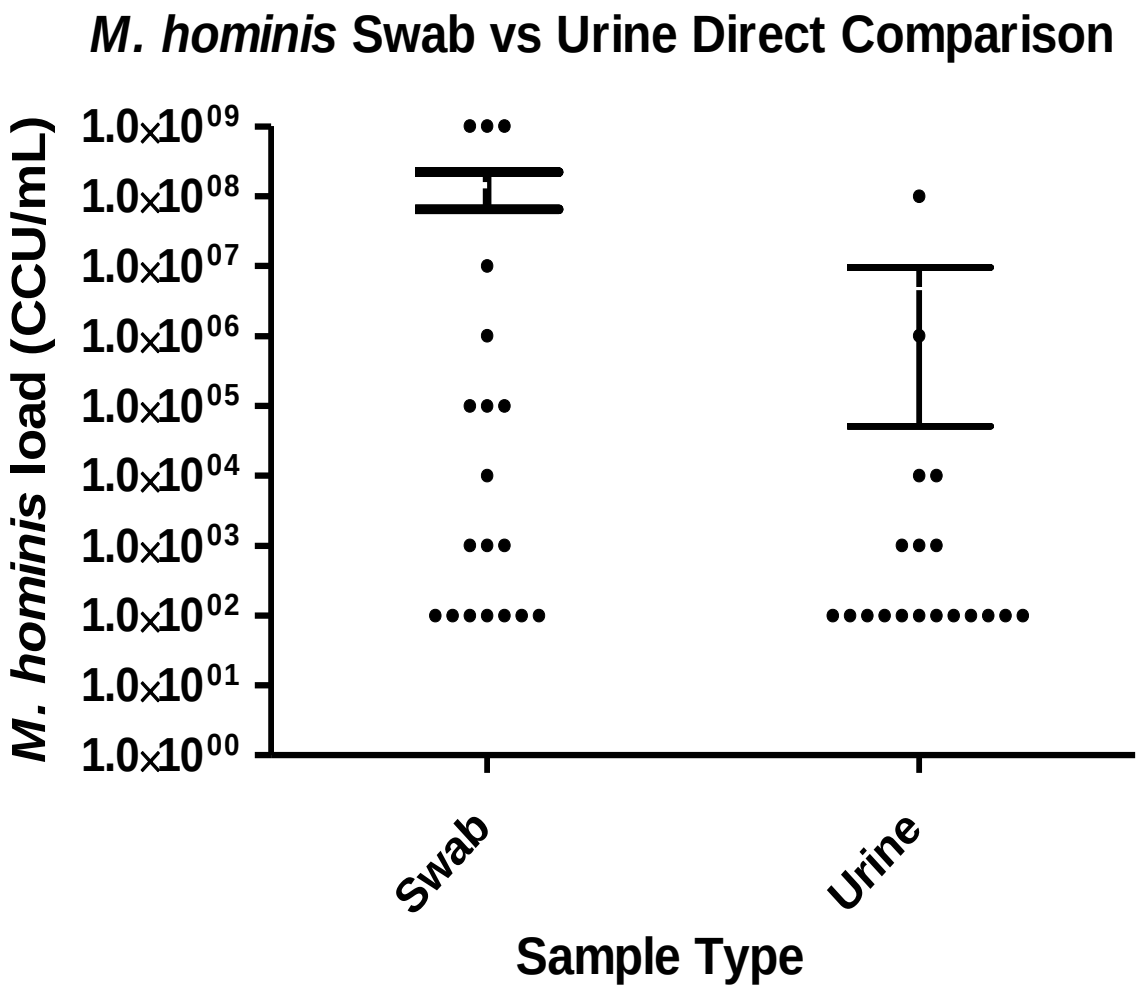


Figure 3.17 Graph plotting the 21 M. hominis-positive paired samples, 76.2% of swab-positive cultures had bacterial loads greater than, or equal to, their respective urine samples. In 42.9% of cases swab samples had a bacterial load 10 x greater than their urine counterparts. though swab samples more frequently had greater bacterial loads than their respective urine samples, a t-test of the listed samples did not determine the difference to be statistically significant (p=0.0939).

3.11 Age and *Ureaplasma* spp. Prevalence

Furthering the analysis from overall prevalence patients were grouped by age, and the prevalence of *Ureaplasma* positive patients determined for each age group utilising multiplex qPCR data. Patients were grouped into the following age categories, with their age determined from the date of birth (DOB) accompanying their samples: 16-20, 21-30, 31-40, 41-50 and >50. The prevalence of *Ureaplasma* spp. decreased with age group, with 70% (69/99) of 16-20-year-olds, 60% (146/245) of 21-30-year-olds, 53% (59/111) of 31-40-year-olds, 38% (14/37) of 41-50-year-olds and 39% (9/23) of >50-year olds, positive for *Ureaplasma* spp. On average, the difference in prevalence between the first 3 categories (16-40) saw a 10.62% decrease in prevalence between age groups, before plateauing beyond the age of 41 with a 1.3% difference in prevalence rates between age groups 41-50 and >50. Chi-squared analysis of *Ureaplasma* prevalence in females between age groups displayed that the trend of decreasing colonisation with increasing age, is statistically significant (p=0.0026). For male patients, the same age categories were applied however the trend was slightly different. For age categories, 16-20 & 21-30 there was only a 0.95% difference in prevalence rates between category, 27.50% (11/40) and 26.55% (47/177) respectively. Between the 21-30 and 31-40 there was a 60% decrease in male *Ureaplasma* prevalence to 10.53% (6/57). Beyond the age ranges of 31-40 through >50, there was little variation in colonisation rate, with a prevalence of 10% (3/30) and 12% (3/25) for age groups 41-50 and >50, respectively. Though the trend of decreasing prevalence with increasing age is less pronounced for males, when compared with females, Chi-squared analysis of the distribution of male *Ureaplasma* prevalence rates between age groups indicates it is statistically significant (p=0.0228).

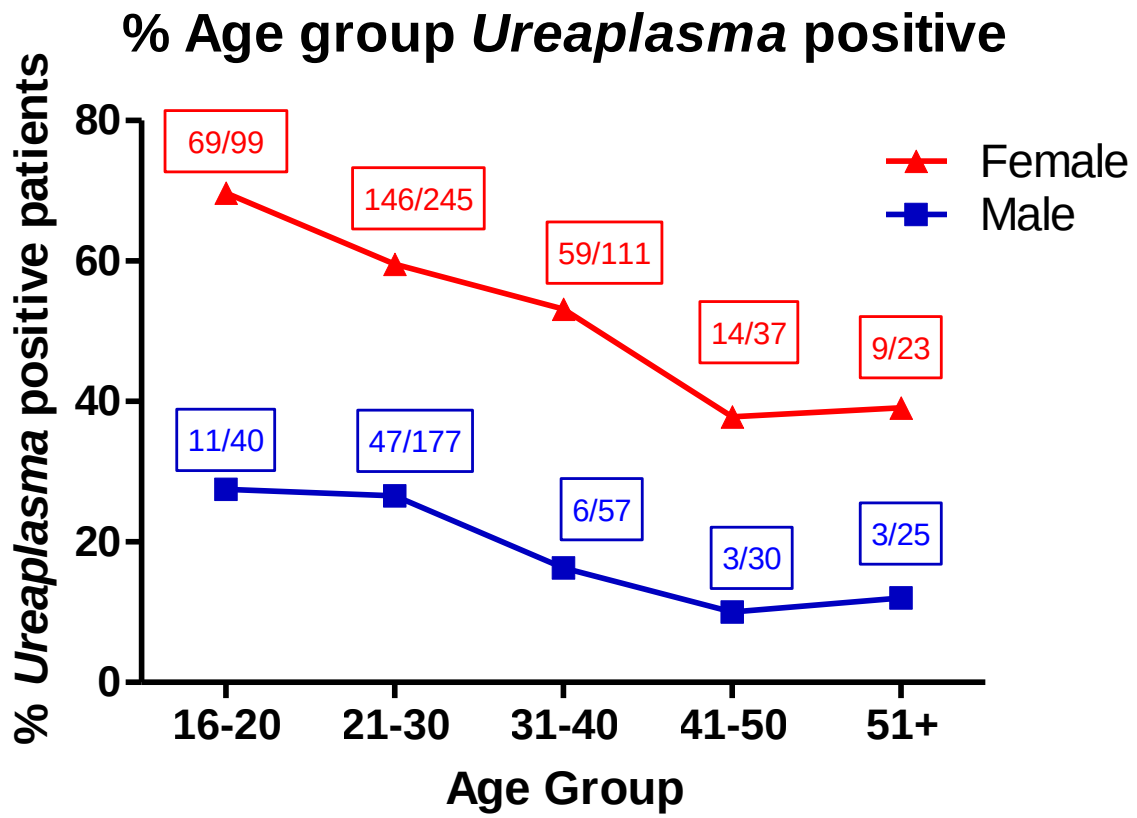


Figure 3.18 Line graph plotting Ureaplasma spp. prevalence across age groups for both male and female patients. The prevalence of Ureaplasma spp. decreased with age group, with 70% of 16-20-year-olds, 60% of 21-30-year-olds, 53% of 31-40-year-olds, 38% of 41-50-year-olds and 39% of >50-year olds, positive for Ureaplasma spp. Chi-squared analysis of Ureaplasma prevalence in females between age groups displayed that the trend of decreasing colonisation with increasing age, is statistically significant (p=0.0026). For males: 27.5% at age 16-20, 26.55% at age 21-30, 10.53% aged 31-40, 10% aged 41-50 and 12% for males aged ≥51. Chi-squared analysis of the distribution of male Ureaplasma prevalence rates between age groups indicates it is statistically significant (p=0.0228).

3.12 Age and *M. hominis* Prevalence

Similarly, and in line with *Ureaplasma* spp. colonisation of females, prevalence rates of *M. hominis* decreases alongside an increase in age. The same age groups were applied, to both male and female patients, as were used in the analysis of age and *Ureaplasma* spp. prevalence. The first age group (16-20) had a female *M. hominis* prevalence rate of 28.28% (28/99), dropping by 11.95% to 16.33% (40/245) for 21-30-year-olds. The decrease in *M. hominis* prevalence continues between 21-30-year-olds and 31-40-year-olds, declining by a further 4.62% to a prevalence rate of 11.71% (13/111). Followed by a subsequent 3.6% decline in prevalence to 8.11% (3/37) in 41-50-year-olds, where it plateaus (slight 0.58% increase) at a prevalence of 8.69% (2/23) for >50-year-olds. A Chi-squared test applied to the prevalence rates between age groups showed the distribution to be statistically significant (p=0.0057). For male patients, a clear trend was not observed, 16-20-year-olds had a *M. hominis* prevalence rate of 2.5% (1/40), followed by a 4.84% increase in colonisation rate to 7.34% (13/177) in 21-30-year-olds. Subsequently, male *M. hominis* prevalence dropped to 0% for the remaining age groups, 41->50. Statistical significance was not determined for the distribution of male *M. hominis* prevalence between age groups (p=0.0513).

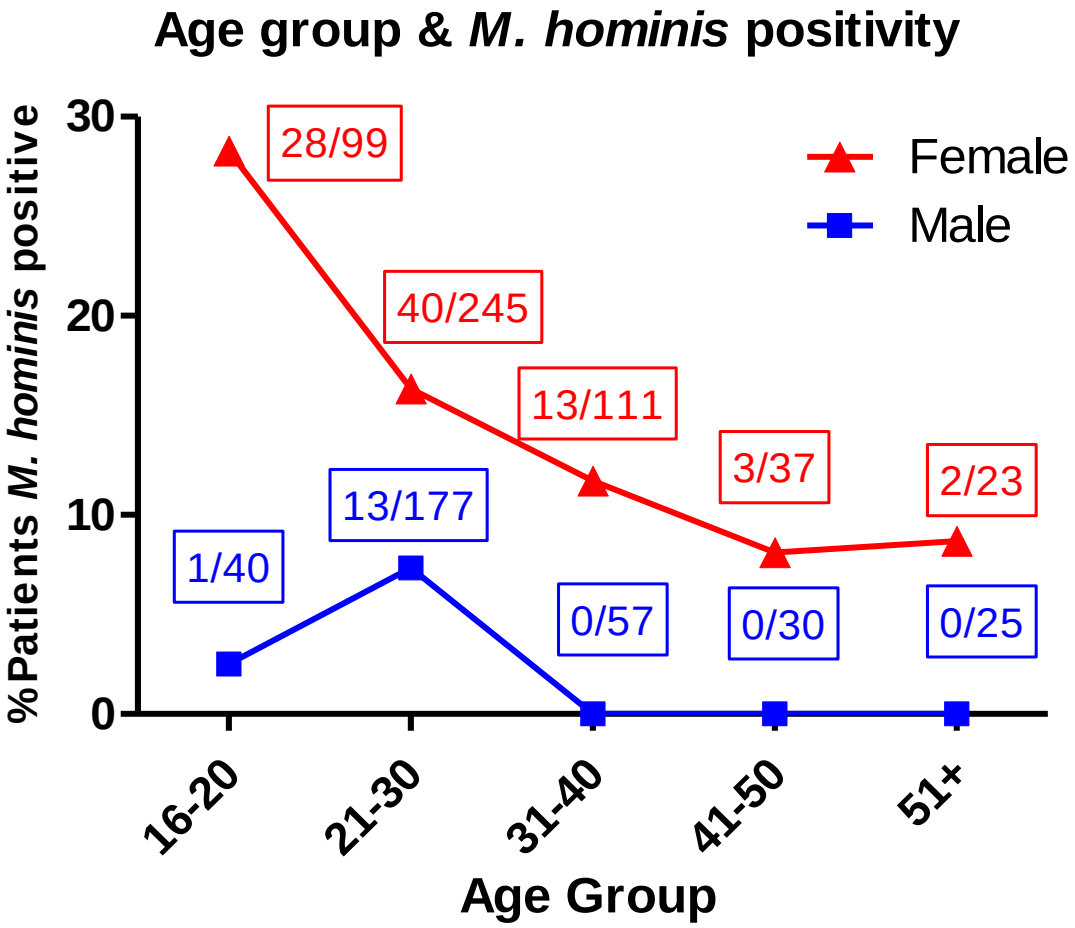


Figure 3.19 Line graph plotting M. hominis prevalence across age groups for both male and female patients. The prevalence of Ureaplasma spp. decreased with age group, with 28.28% of 16-20-year-olds, 16.33% of 21-30-year-olds, 11.71% of 31-40-year-olds, 8.11% of 41-50-year-olds and 8.69% of >50-year olds, positive for Ureaplasma spp. Chi-squared analysis of Ureaplasma prevalence in females between age groups displayed that the trend of decreasing colonisation with increasing age, is statistically significant (p=0.0057). For males: 2.5% at age 16-20, 7.34% at age 21-30, dropping to 0% beyond the age of 31. Statistical significance was not determined for the distribution of male M. hominis prevalence between age groups (p=0.0513).

3.13 Age and Concomitant *Ureaplasma* spp. and *M. hominis* prevalence

3.13.1 Female Patients

The prevalence of female patients positive for both *M. hominis* and *Ureaplasma* spp. concomitantly was also analysed. For females aged 16-20, concomitance *M. hominis* and *Ureaplasma* spp. was present in 27.3% of patients (27/99). Like *M. hominis* prevalence, rates of *M. hominis* and *Ureaplasma* spp. concomitance subsequently decrease as age increases;13.9% (40/245) of 21-30-year-old females; 10.8% (13/111) of 31-40-year-old females. Before plateauing at 8.1% (3/37) and 8.7% (2/23), for 41-50-year-olds and >51-year-olds, respectively. In addition to this, the prevalence of ureaplasmal infection type concomitantly colonising *M. hominis*-positive female patients were also determined. Female patients aged between 16 and 20, positive for *M. hominis* and *U. parvum* infection accounted for 13.1% (13/99) of the populous screened, with *M. hominis* and *U. urealyticum*, and *M. hominis* alongside both *U. parvum* and *U. urealyticum*, accounting for 8.1% (8/99) and 6.1% (6/99), respectively. Within the 21-30-year-old female patient group, *M. hominis* concomitant with *U. parvum*, again accounted for most concomitant infections with a prevalence rate of 9.4% (23/245). In contrast to the 16-20-year-old group, *M. hominis* concomitant with both *U. parvum* and *U. urealyticum* was narrowly more prevalent with 2.9% (7/245) of positive female patients, when compared to concomitant *M. hominis* and *U. urealyticum* with a prevalence of 1.6% (2/245), for the 21-30-year-old female patient group. The 31-40 female age group had similar concomitant *M. hominis* and *Ureaplasma* spp. composition; 9% (12/111) *M. hominis* and *U. parvum*; 0.9% (1/111) *M. hominis* and *U. urealyticum*; 0.9% (1/111) *M. hominis*, *U. parvum* and *U. urealyticum*. Whereas for the 41-50-year-old female patient group, only *M. hominis* and *U. parvum* concomitant infections were detected, with a prevalence rate of 8.1% (3/37). Prevalence rates of concomitant *M. hominis* and *U. parvum* 4.3% (1/23), were the same as *M. hominis* and *U.*

urealyticum 4.3% (1/23), for the 51+ female patient group. No concomitant *M. hominis*, *U. parvum* and *U. urealyticum* was found in the 51+ female patient group.

3.13.2 Male Patients

Like overall *M. hominis* prevalence for male patients, concomitant *M. hominis* and *Ureaplasma* spp. follows a lower, but similar, trend. For males between the age of 16-20, *M. hominis* concomitant with *Ureaplasma* spp. had a prevalence rate of 2.5% (1/40). With the single case of concomitant infection being composed of *M. hominis* and *U. urealyticum*. The only other age group displaying *M. hominis* concomitant with *Ureaplasma* spp. was the 21-30-year-olds, with a prevalence rate of 5.1% (9/177); 2.25% (4/177) *M. hominis* and *U. parvum*, 2.25% (4/177) *M. hominis* and *U. urealyticum*, and 0.56% (1/177) *M. hominis*, *U. parvum* and *U. urealyticum*.

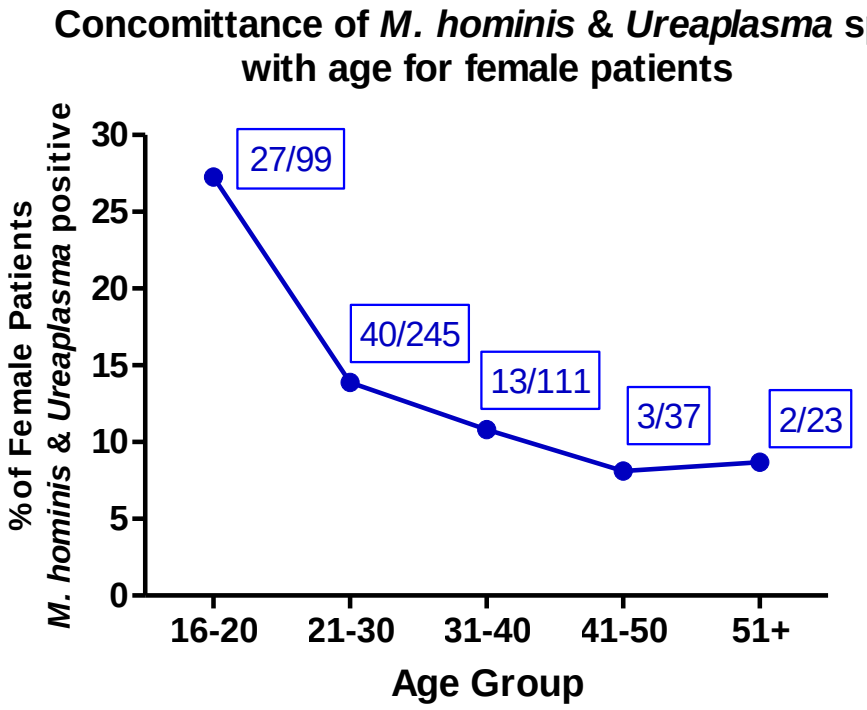


Figure 3.20 Line graph plotting concomitant M. hominis and Ureaplasma spp. prevalence across age groups for female patients. The prevalence of concomitant M. hominis and Ureaplasma spp. decreased with age group, with 27.3% of 16-20-year-olds, 13.9% of 21-30-year-olds, 10.8% of 31-40-year-olds, 8.1% of 41-50-year-olds and 8.7% of >50-year olds. Chi-squared analysis of concomitant M. hominis and Ureaplasma prevalence in females between age groups displayed that the trend of decreasing colonisation with increasing age, is statistically significant (p=0.0108).

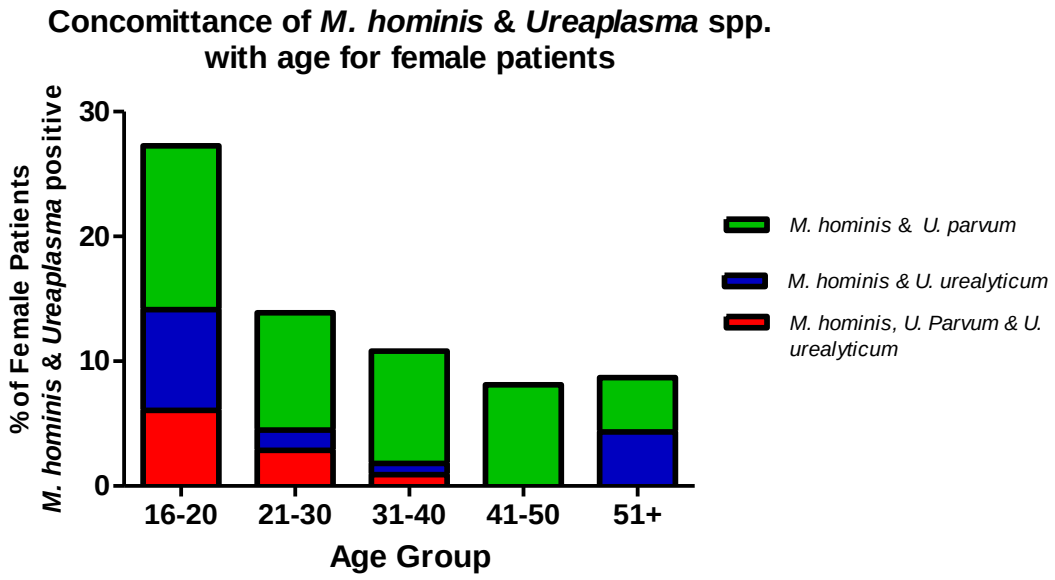


Figure 3.21 Bar graph displaying the prevalence of concomitant M. hominis and Ureaplasma across age groups for female patients alongside the speciation of the Ureaplasma spp. infection. Females aged 16-20: 13.1% M. hominis and U. parvum; 8.1% M. hominis, U. parvum and U. urealyticum; 6.1% M. hominis and U. urealyticum. Females aged 21-30: 9.4% M. hominis and U. parvum; M. hominis, U. urealyticum and U. urealyticum 2.9%; M. hominis and U. urealyticum 1.6%. Females aged 31-40: 9% M. hominis and U. parvum; 0.9% M. hominis and U. urealyticum; 0.9% M. hominis, U. parvum and U. urealyticum. Females aged 41-50: 8.1% M hominis and U. parvum. Females aged ≥51: 4.3% M. hominis and U. parvum; 4.3% M. hominis and U. urealyticum.

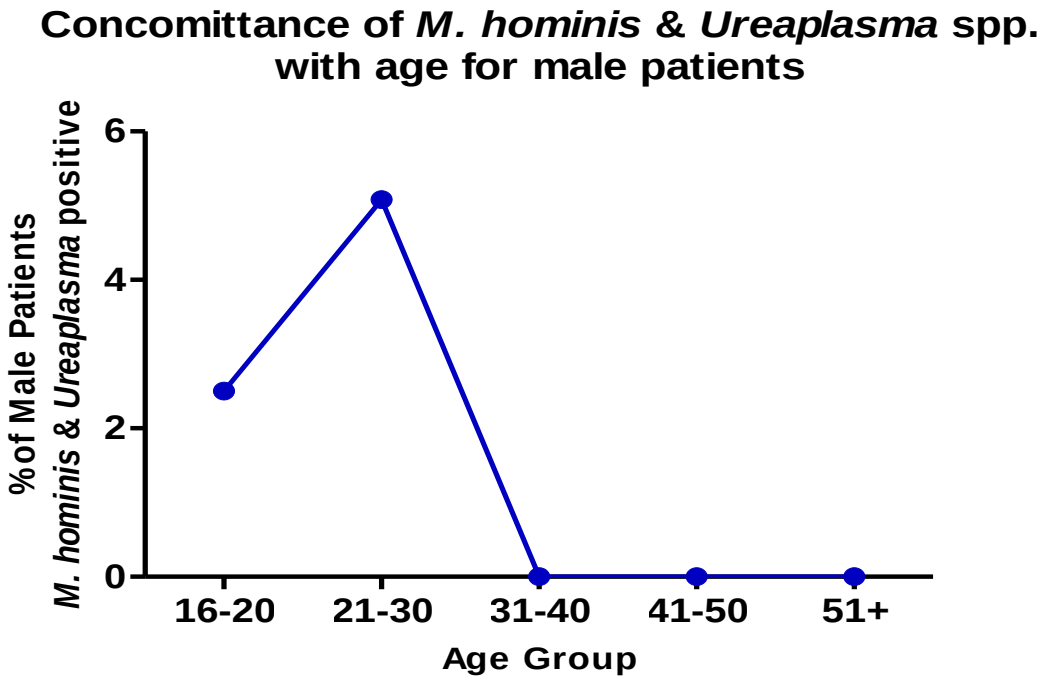


Figure 3.22 Males between the age of 16-20: M. hominis concomitant with Ureaplasma spp. had a prevalence rate of 2.5%. The only other age group displaying M. hominis concomitant with Ureaplasma spp. was the 21-30-year-olds, with a prevalence rate of 5.1%. Chi-squared analysis of concomitant M. hominis and Ureaplasma prevalence in males between age groups displayed that the trend of decreasing colonisation with increasing age, is not statistically significant (p= 0.2133).

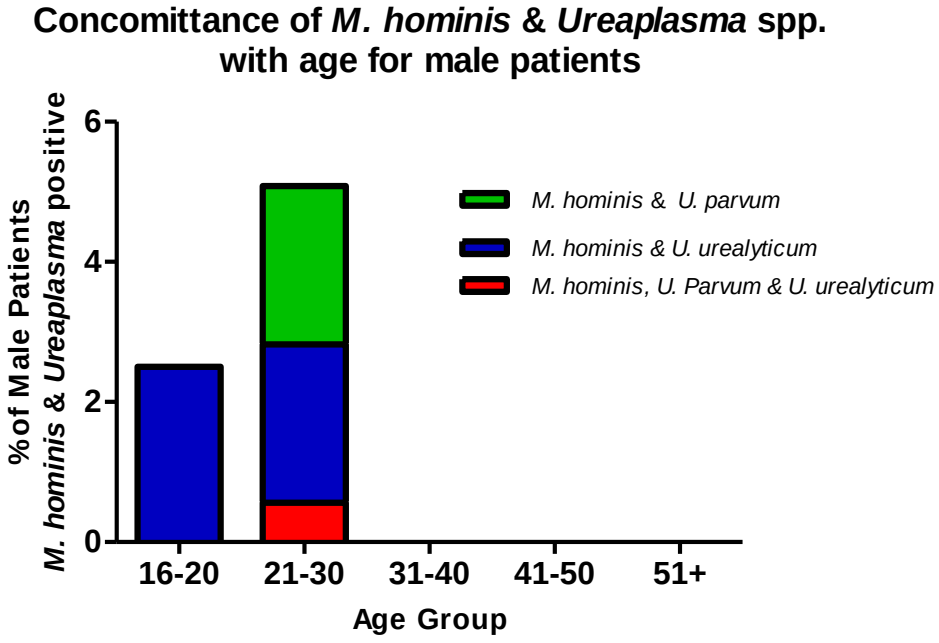


Figure 3.23 The single case of concomitant M. hominis and U. urealyticum accounts for the 2.5% prevalence observed for males aged 16-20 years old. For males 21-30-year-olds, with a prevalence rate of 5.1%; 2.25% M. hominis and U. parvum, 2.25% M. hominis and U. urealyticum and 0.56% M. hominis, U. parvum and U. urealyticum.

3.14 Bacterial Load

3.14.1 Ureaplasma spp. Load

Further age-group specific analysis was undertaken to determine the variation in the frequency of ureaplasmal loads isolated between age groups. For female swab samples, between the ages of 16-30, 39.02% (48/123) of *Ureaplasma*-positive samples have a bacterial load of $\leq 10^4$ CCU/mL. Whereas 60.98% (75/123) of *Ureaplasma*-positive 16-30-year-old swab samples have a bacterial load $\geq 10^5$ CCU/mL. Conversely, for swab samples taken from female patients aged 31+, 60.98% (25/41) had a *Ureaplasma* load of $\leq 10^4$ CCU/mL with 39.02% (16/41) having a load of $\geq 10^5$ CCU/mL. Whereas for female urine samples, samples with $\geq 10^5$ CCU/mL where only discovered in patients between the ages of 16 and 40; no female urine *Ureaplasma*-loads $\geq 10^5$ CCU/mL were observed for patients aged ≥41. Those female urine ureaplasmal loads $\geq 10^5$ CCU/mL for patients ≤40-years-old comprised a minority of the overall urine-positives. For example, urine loads $\geq 10^5$ CCU/mL were discovered in only 2% of 16-20-year-old samples. Analysis of the 21-30-year-old female urine samples displayed a similar composition; urine *Ureaplasma* loads of 10^5 CCU/mL accounted for 8.6% of samples; *Ureaplasma* loads of 10^6, 10^7 and 10^9 CCU/mL each accounted for 1.07% of the total urine-positive samples, respectively. The urine-positive samples of the 31-40-year-old female patient group displayed a $\geq 10^5$ CCU/mL *Ureaplasma* load in 7% of samples; 10^5 CCU/mL in 2.3% of urine-positive female samples; 10^6 CCU/mL in 4.7% of urine-positive female samples.

Ureaplasma-positive male urine samples, analysed by age group for ureaplasmal load revealed that only the 21-30-year-old patients had urine samples with titrations of 10^4 CCU/mL, accounting for 9.3% of total positive urines. For urine-positive patients ≥51, 100% of samples had a bacterial load of $<10^3$ CCU/mL. Whereas for the remaining age groups urine positive samples were divided between those with a bacterial load of $<10^3$ CCU/mL and those with a bacterial load of 10^3 CCU/mL. The 16-20-year-old urine-positive male patients with a 60%/40% split between $<10^3$ CCU/mL and 10^3 CCU/mL *Ureaplasma* loads. For male urine-positives in the 31-40-year-old age group, the $<10^3$:10^3

CCU/mL split was 71.43% and 28.57%, respectively. With the $<10^3$:10^3 CCU/mL split for the 41-50-year-old age group, for *Ureaplasma*-positive urine samples being 66.67%:33.33%.

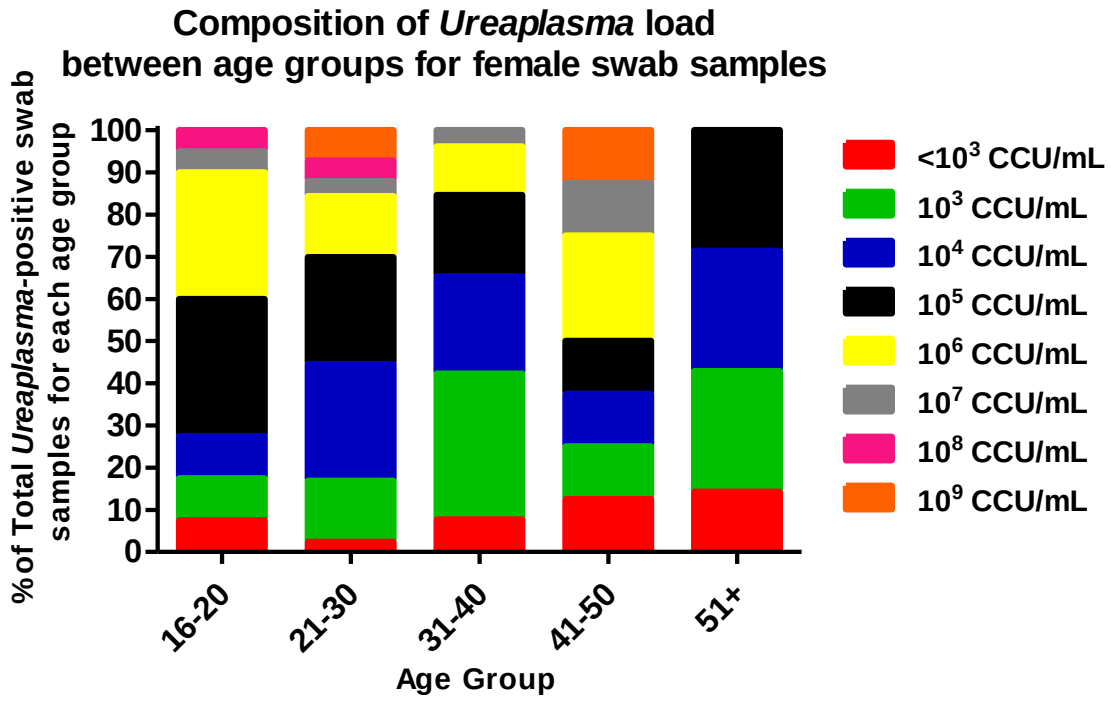


Figure 3.24 Bar graph displaying the composition of Ureaplasma-positive swab samples alongside bacterial load across age groups. 39.02% of female swab samples aged 16-30 have a bacterial load of ≤104 CCU/mL, with the remaining 60.98% having a Ureaplasma spp. load ≥105 CCU/mL. Whereas for female swab samples aged ≥31 39.02% of samples have Ureaplasma loads ≥105 CCU/mL, with the remaining having a load of ≤104 CCU/mL. Fisher exact analysis revealed females aged ≤30 swab samples have significantly higher levels of Ureaplasma loads ≥105 CCU/mL (p=0.0183).

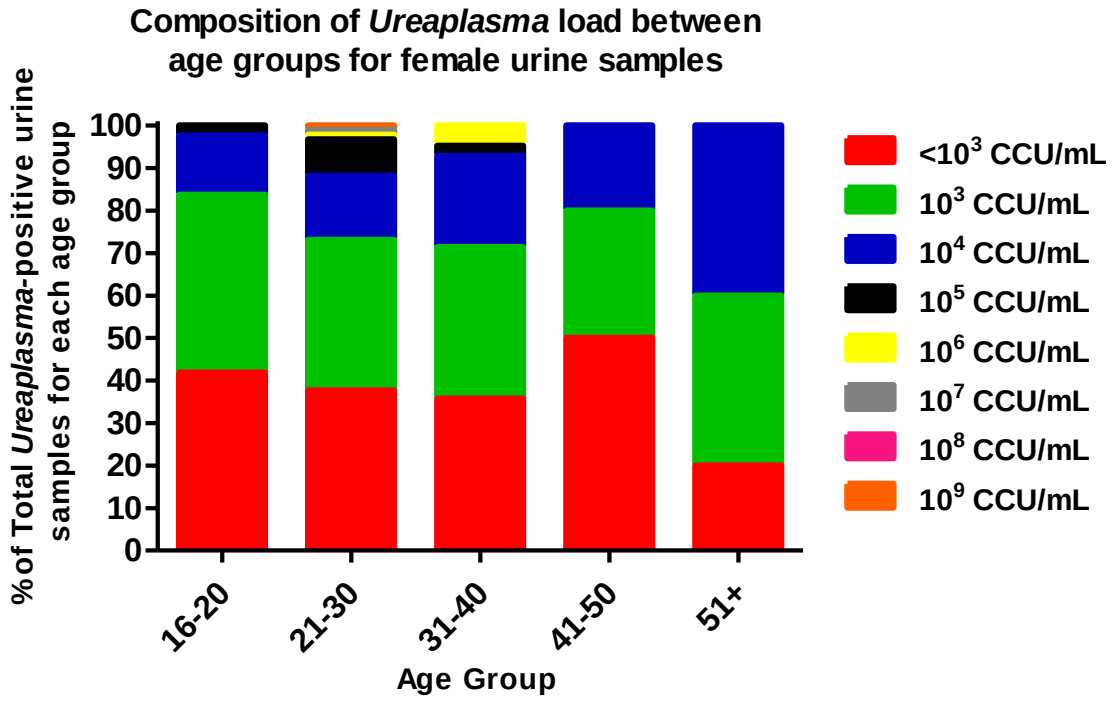


Figure 3.25 Bar graph displaying the composition of Ureaplasma-positive swab samples alongside bacterial load across age groups for female patients.

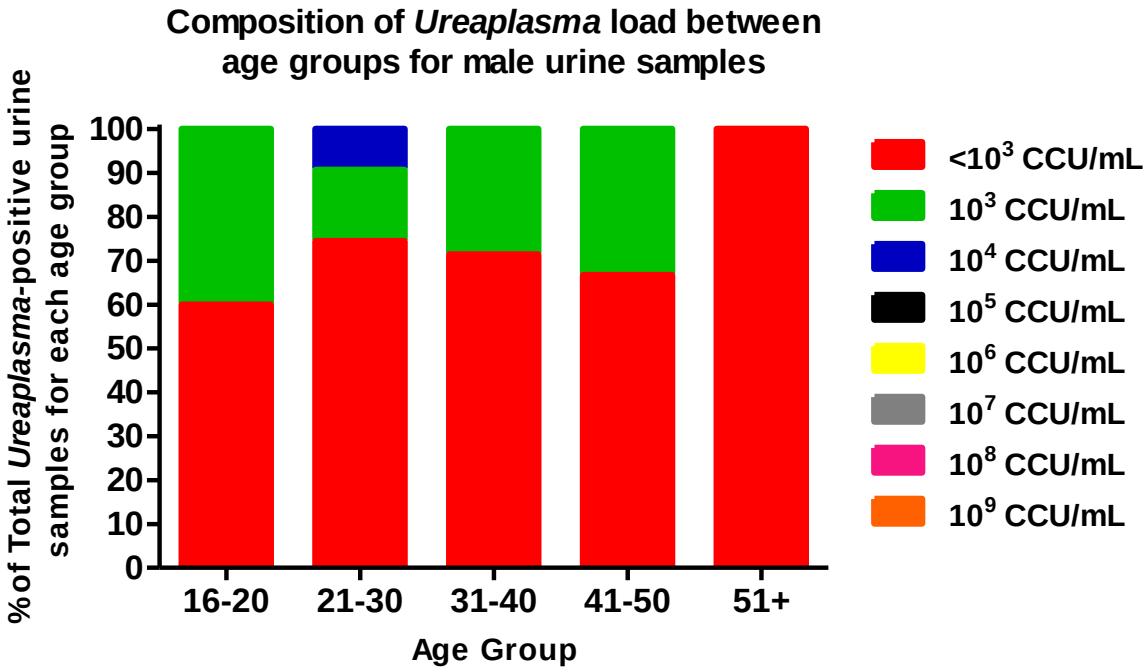


Figure 3.26 Bar graph displaying the composition of Ureaplasma-positive swab samples alongside bacterial load across age groups for female patients.

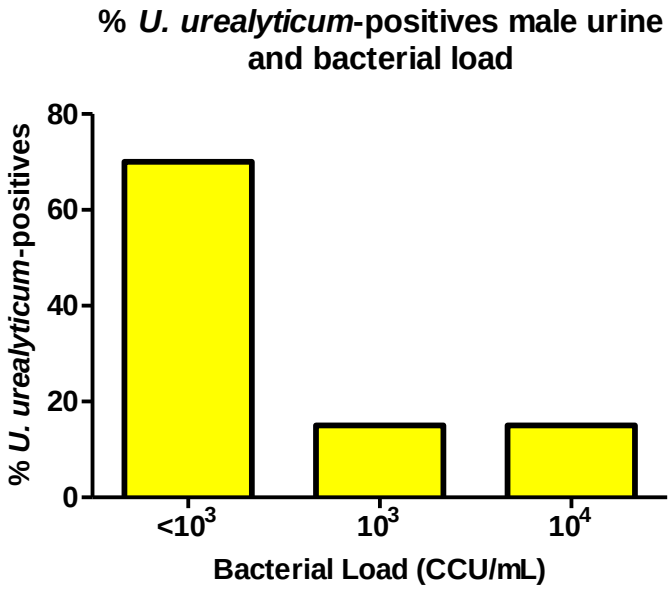


Figure 3.27 Bar graph displaying the composition of Ureaplasma-positive urine samples alongside bacterial load more male patients. 70.73% of U. urealyticum positive males have a load of <10^3 CCU/mL, with the remaining 29.27% of positive samples having a bacterial load ≥10^3 CCU/mL.

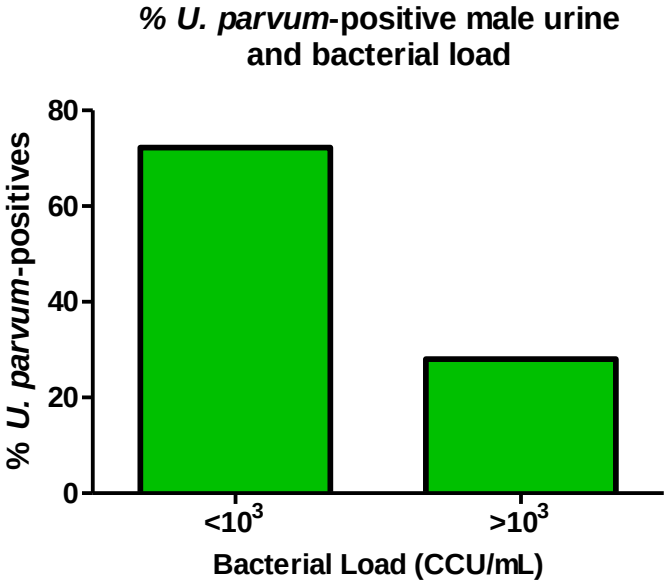


Figure 3.28 Bar graph displaying the composition of Ureaplasma-positive urine samples alongside bacterial load more male patients. 72.22% of U. parvum positive males have a load of <10^3 CCU/mL, with the remaining 27.78% of positive samples having a bacterial load ≥10^3 CCU/mL.

3.14.2 *M. hominis* load

Analysis of female patient age groups swab-positive for *M. hominis* revealed that for patients 16-20 and 21-30 loads of $\geq 10^5$ CCU/mL were observed in 38.89% (7/18) and 38.09% (8/21) of samples respectively. This increased for swab-positives in the 31-40-year-old female patient group, with 75% (3/4) of samples having a *M. hominis* load of $\geq 10^5$ CCU/mL. Of the two *M. hominis* swab-positives present in each of the remaining age groups (41-50 and 51+) the composition of loads was as follows; 41-50-year-olds with 50% (1/2) 10^3 CCU/mL and 50% (1/2) 10^4 CCU/mL; ≥51-year-olds with 50% (1/2) 10^4 CCU/mL and 50% (1/2) 10^6 CCU/mL. For *M. hominis*-positive female urine samples between age groups, samples with a bacterial load $\geq 10^5$ CCU/mL were only observed in two patient groups, the 16-20-year-olds (accounting for 20%; 2/10) and the 31-40-year-olds (accounting for 9.1%; 1/11). For the 41-50-year-old patient group, all female *M. hominis* urine-positives were comprised of samples with $<10^3$ CCU/mL. Male urine samples had only two age groups displaying *M. hominis* infection, the 16-20-year-olds and the 21-30-year-olds. The one urine positive for the 16-20-year-old group had a *M. hominis* load of $<10^3$ CCU/mL (100%; 1/1). Whereas the 12 positives isolated from the 21-30-year-old male patient group displayed bacterial loads of $<10^3$ CCU/mL in 83.33% (10/12) of cases, and 16.67% (2/12) 10^3 CCU/mL.

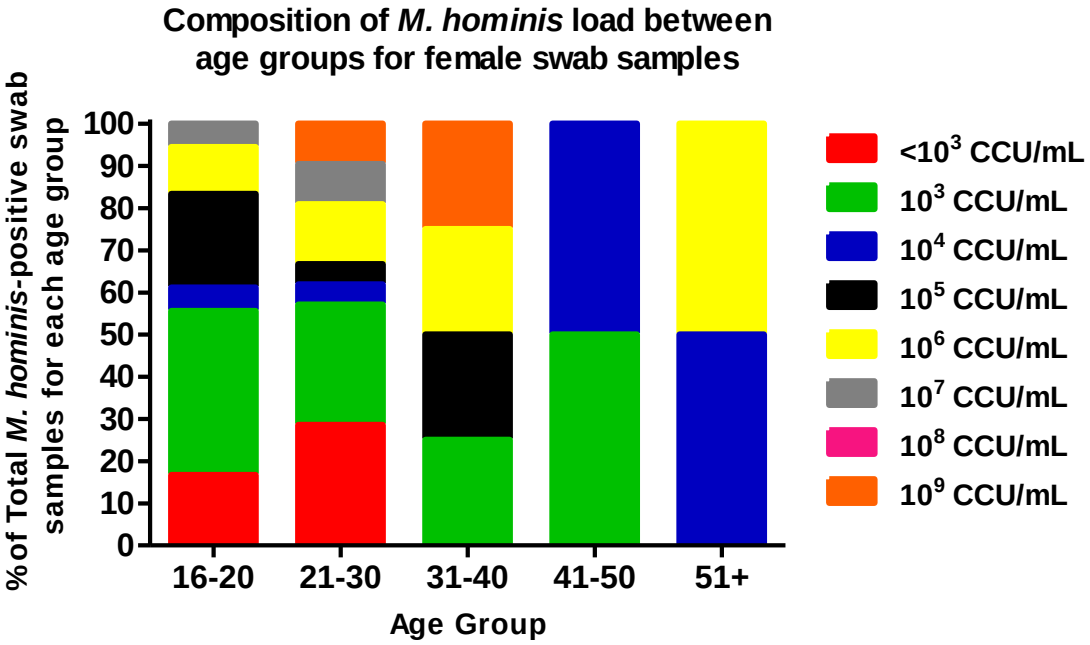


Figure 3.29 Bar graph displaying the composition of M. hominis-positive swab samples alongside bacterial load across age groups for female patients. For age groups 16-20 years old and 21-30 years old have bacterial loads ≥10^5 CCU/mL in 38.89% and 38.09% of M. hominis-positive swab samples, respectively. 75% of M. hominis-positive samples had a bacterial load of ≥10^5 CCU/mL.

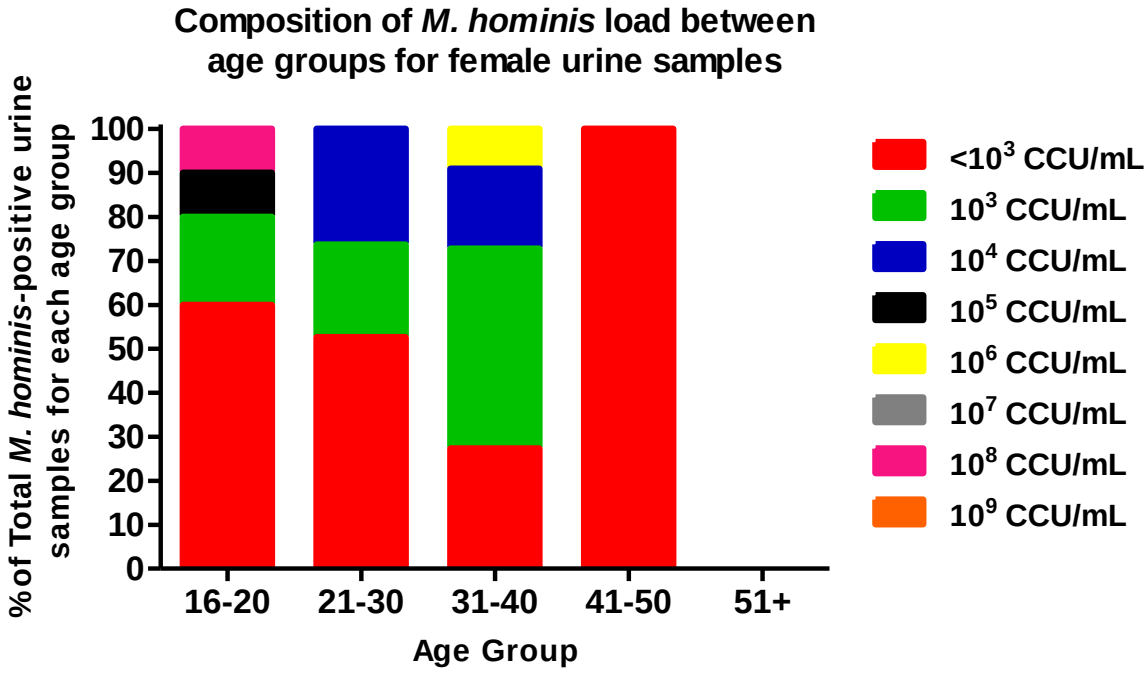


Figure 3.30 Bar graph displaying the composition of M. hominis-positive urine samples alongside bacterial load across age groups for male patients. Samples with a bacterial load ≥10^5 CCU/mL were only observed in two patient groups, the 16-20-year-olds (accounting for 20%; 2/10) and the 31-40-year-olds (accounting for 9.1%; 1/11).

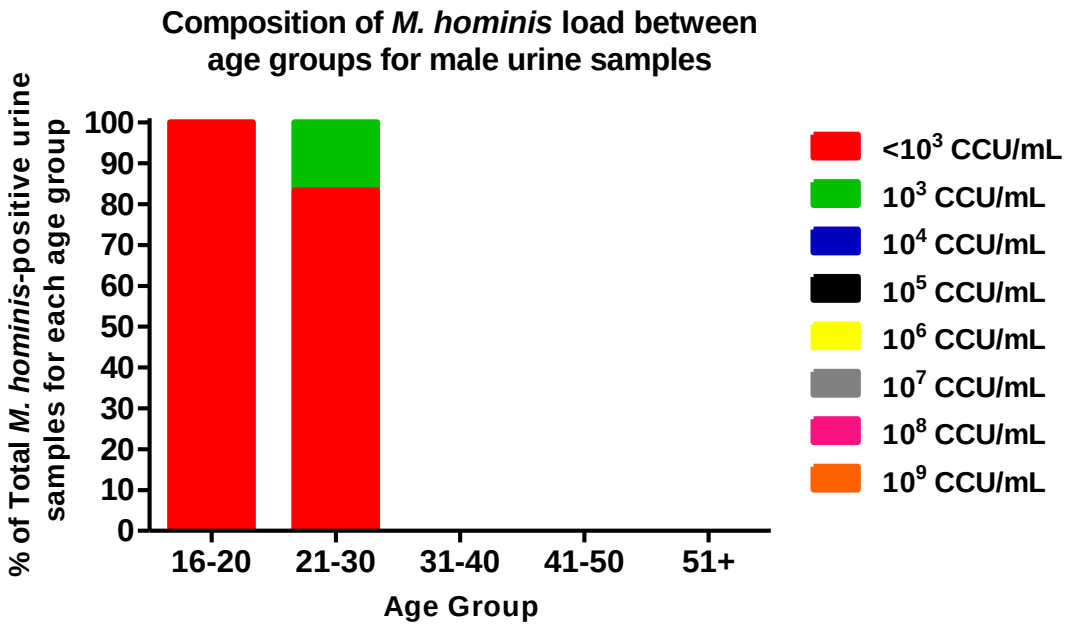


Figure 3.31 Bar graph displaying the composition of M. hominis-positive urine samples alongside bacterial load across age groups for male patients. 100% of M. hominis-positive male urine samples had a load of <10^3 CCU/mL for patients aged 16-20 years old. M. hominis-positive male urine samples from patients aged 21-30 had bacterial loads <10^3 CCU/mL in 83.33% of cases, with the remaining 16.67% of samples having a bacterial load of 10^3 CCU/mL.

3.15 Age and *Ureaplasma* spp. Composition

The composition of *Ureaplasma*-positive infections was analysed between age groups, for both male and female patients. Female patients aged 16-20 had the greatest portion of mixed *U. parvum* & *U. urealyticum* infections, accounting for 17.39% (12/69) of total *Ureaplasma* positives. This number gradually decreased for 21-30-year-olds (15.75%; 23/146) and 31-40-year-olds (8.47%; 5/59), with patients ≥41 having 0% mixed *Ureaplasma* spp. infections. Colonisation with *U. parvum*, steadily increased with regards to its percentage of total *Ureaplasma* spp. infection between the ages of 16 and 50. Accounting for 57.97% (40/69) of total *Ureaplasma* spp. infections in 16-20-year-olds, 66.44% (97/146) in the 21-30 female patient group, 74.57% (44/59) in 31-40-year-olds, and 100% (14/14) of 41-50-year-old *Ureaplasma*-positive females. However, *U. parvum* accounted for 77.78% of *Ureaplasma*-positive females in patients ≥51-years-old. For sole *U. urealyticum* infections, the % of the overall total *Ureaplasma*-positives they comprise steadily decreases between the ages of 16-50. Comprising 24.64% (17/69) of *Ureaplasma* spp. in female patients aged 16-20, 17.81% (23/146) in 21-30-year-olds, 16.95% (10/59) of *Ureaplasma*-positives in the 31-40-year-old female patient group. The 41-50-year-old female patients lacked a single *U. urealyticum* positive, however, 22.22% of female patients ≥51-years-old and *Ureaplasma*-positive were colonised by *U. urealyticum*.

For *Ureaplasma*-positive males, *U. parvum* accounts for the minority of positives between the ages of 16-30; 27.27% (3/11) for males aged 16-20 and 25.53% (12/47) of 21-30-year-olds. The reverse was true for male patients aged between 31 and 40, with *U. parvum* accounting for the most positives, 66.67% (4/6). This was again flipped for 41-50-year-old males with *U. urealyticum* infections comprising 66.67% (2/3) of *Ureaplasma*-positives. For male patients ≥51 years of age, the composition of *Ureaplasma*-positives was divided 66.67% (2/3) *U. parvum* to 33.33% (1/3) *U. urealyticum*.

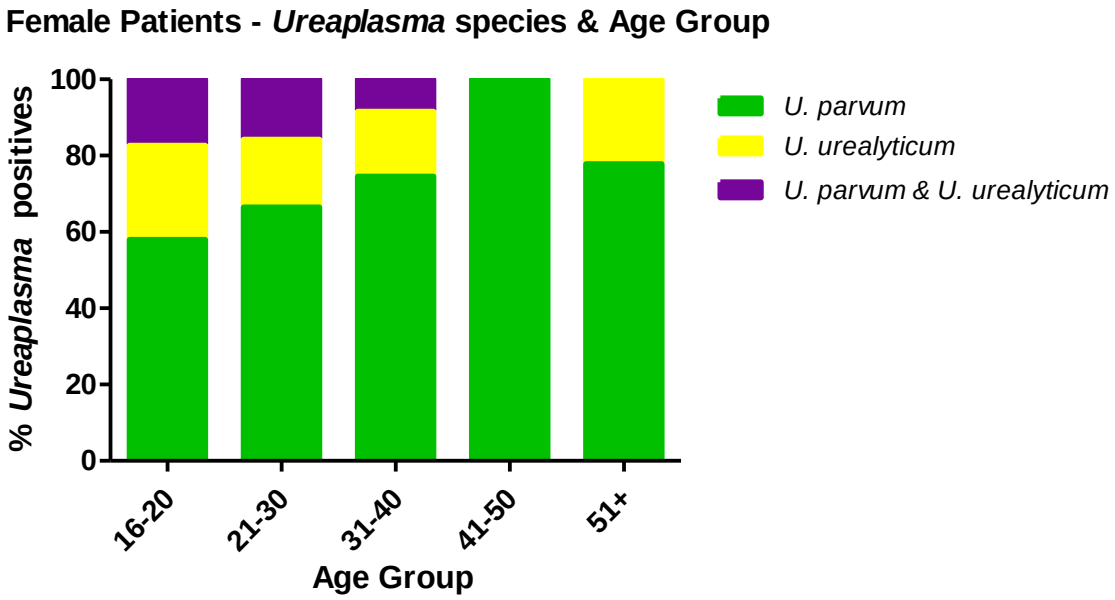


Figure 3.32 Bar graph displaying the composition of Ureaplasma-positive samples for female patients across age groups. Females aged 16-20: 57.97% U. parvum; 24.64% U. urealyticum; 17.39% U. Parvum and U. urealyticum. Females aged 21-30: 66.44% U. parvum; 17.81% U. urealyticum; 15.75% U. Parvum and U. urealyticum. Females aged 31-40: 74.57% U. parvum; 16.95% U. urealyticum; 8.47% U. parvum and U. urealyticum. Females aged 41-50: 100% U. parvum. Females aged ≥51: 77.78% U. parvum; 22.22% U. urealyticum.

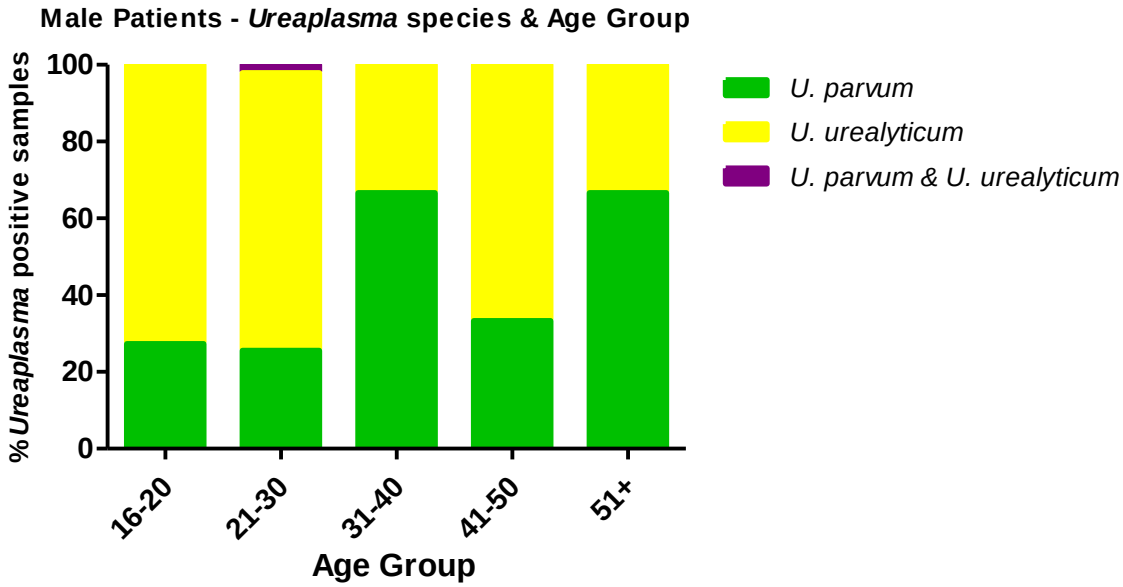


Figure 3.33 Bar graph displaying the composition of Ureaplasma-positive samples for male patients across age groups. Males aged 16-20: 27.27% U. parvum; 72.73% U. urealyticum. Males aged 21-30: 25.53% U. parvum; 72.34% U. urealyticum; 2.13% U. parvum and U. urealyticum. Males aged 31-40: 66.67% U. parvum; 33.33% U. urealyticum. Males aged 41-50: 33.33% U. parvum; 66.67% U. urealyticum. Males aged ≥51: 66.67% U. parvum; 33.33% U. urealyticum.

3.16 Species Prevalence Between Sex and Age Group

Prevalence rates of each *Ureaplasma* species between sex and age group were determined. For female *U. parvum* prevalence amongst the female population declined incrementally with age; 16-20 (52.25%; 52/99), 21-30 (48.97%; 97/245), 31-40 (44.14%; 49/111), 41-50 (37.84%; 14/37) and 51+ (30.43%; 7/23). The decreases in prevalence increased marginally (~1%) between each age group. Between the ages of 16-20 and 21-30, *U. parvum* prevalence decreased by 3.55%. Whereas between 21-30 and 31-40, the decrease in *U. parvum* prevalence was 4.84%. The *U. parvum* prevalence for females between 31-40 then decreased by 6.31%. Finally, the decrease in female *U. parvum* prevalence between the 41-50 and 50+ age group was 7.40%. Contrastingly, male *U. parvum* prevalence remained relatively consistent, fluctuating between 3 and 8%. Males aged between 16 and 20 had *U. parvum* prevalence rates of 7.5% (3/40). Followed by 21-30-year-olds with *U. parvum* prevalence of 6.78% (12/177) and 31-40-year-olds with 7.02% (4/57). Males between the ages of 41-50 and 51+ had *U. parvum* prevalence rates of 3.33% (1/30) and 8% (2/25), respectively.

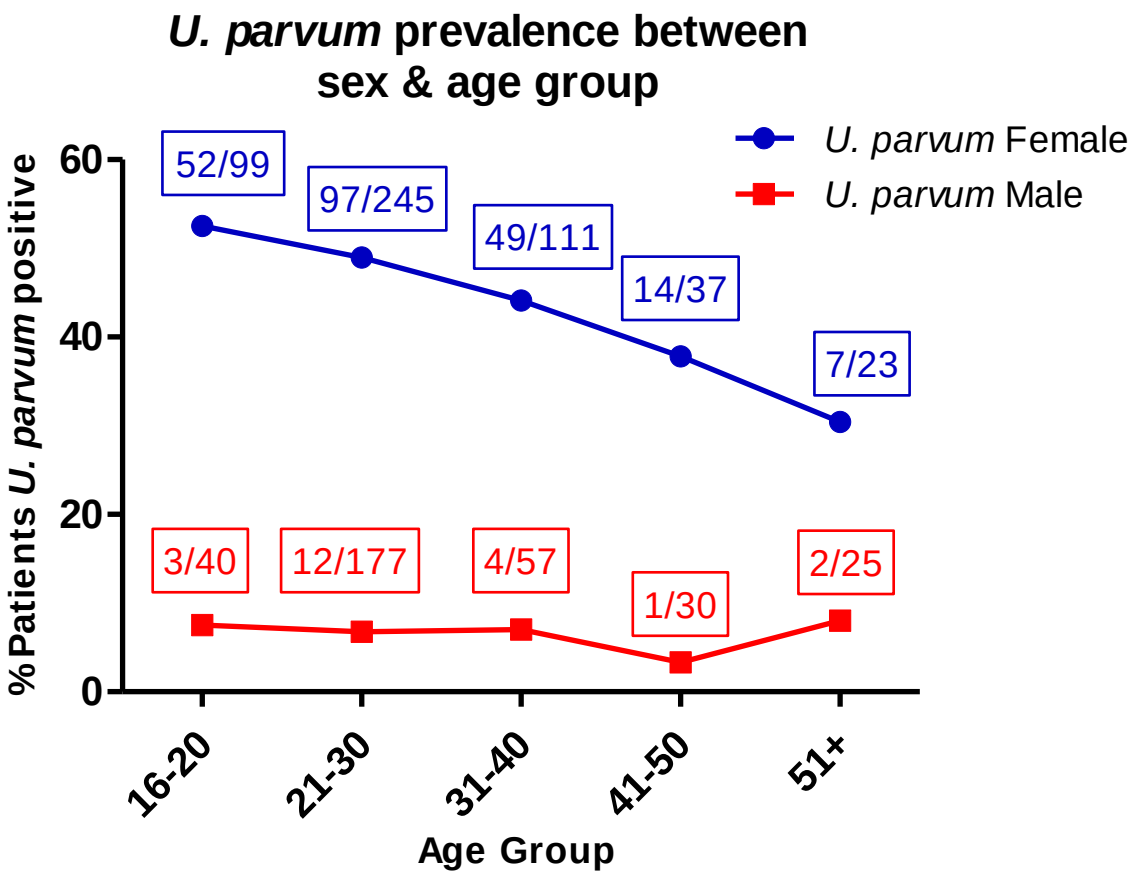


Figure 3.34 Line graph displaying U. parvum prevalence across age groups between males and females. U. parvum prevalence across age groups for females was as follows: 16-20, 52.25%; 21-30, 48.97%; 31-40, 44.14%; 41-50, 37.84%, ≥51, 30.43%. For males U. parvum prevalence was as follows: 16-20, 7.5%; 21-30, 6.78%; 31-40, 7.02%, 41-50, 3.33%; ≥51, 8%. Chi-squared analysis revealed neither trend to be statistically significant; females (p=0.1503), males (p=0.9556).

Unlike *U. parvum*, patterns of *U. urealyticum* prevalence between the sexes and across age groups aren't so distinct. Overall, both male and female *U. urealyticum* prevalence rates decrease as age increases. In males aged 16-20 and 21-30, *U. urealyticum* prevalence remained consistent at 20% (8/40) and 19.77% (35/177), respectively. For females at the same age group, *U. urealyticum* prevalence fell from 29.29% (29/99), for 16-20-year-olds, by 9.29% to 20% (49/245) for female patients aged 21-30. Beyond the age of 30, male *U. urealyticum* prevalence dropped sharply by 16.26% to 3.51% (4/57) for patients aged 31-40. Subsequently, *U. urealyticum* prevalence plateaued, at 6.67% (2/30) for patients between 41-50 and 4% (1/25) for patients aged ≥51. However, for female patients the decrease in *U. urealyticum* prevalence between 21-30-year-olds and patients aged between 31 and 40 was less severe. The prevalence between these age groups decreased by 6.49% from 20% (49/245) to 13.51% (15/111). No *U. urealyticum* infections were observed for the 41-50 age group (0%; 0/37), rising to a *U. urealyticum* prevalence rate of 8.69% (2/23) for female patients aged ≥51.

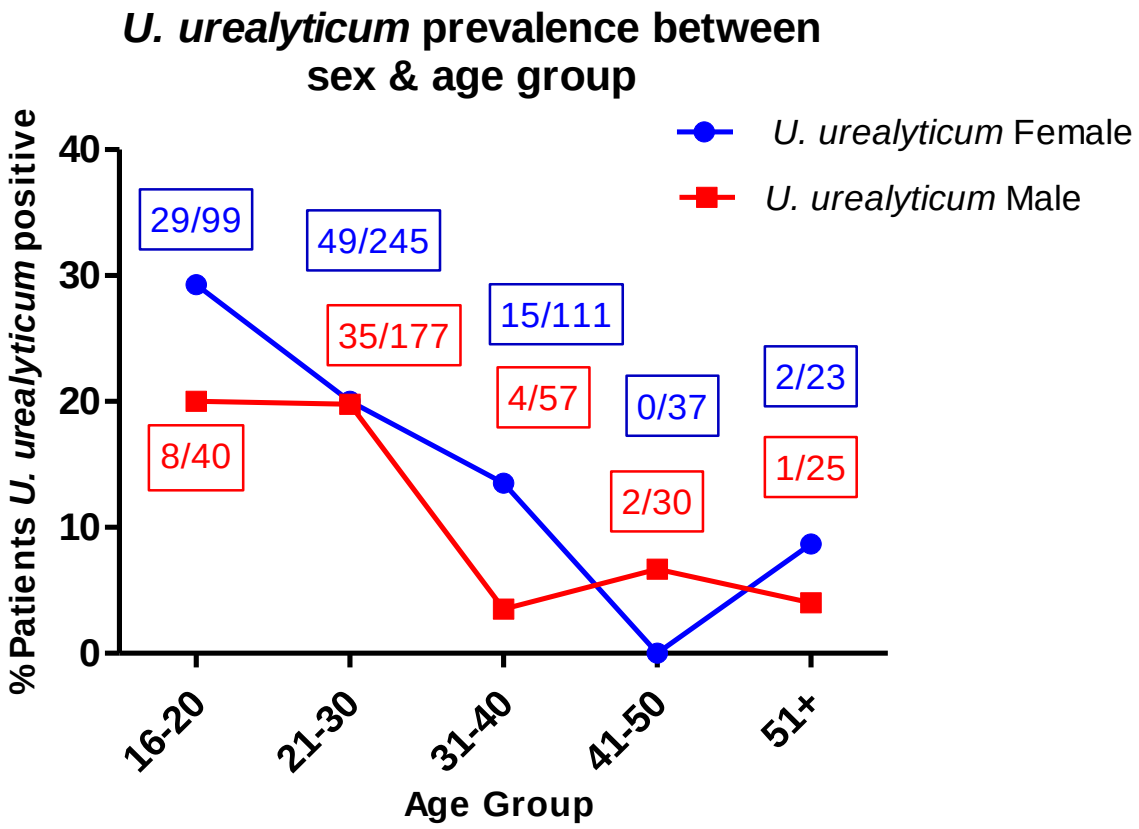


Figure 3.35 Line graph displaying U. urealyticum prevalence across age groups between males and females. U. urealyticum prevalence across age groups for females was as follows: 16-20, 29.29%; 21-30, 20%; 31-40, 13.51%; 41-50, 0%; ≥51, 8.69%. For males U. urealyticum prevalence was as follows: 16-20, 20%; 21-30, 19.77%; 31-40, 3.51%, 41-50, 6.67%; ≥51, 4%. Chi-squared analysis revealed the decline in prevalence across age groups for female patients was statistically significant (p=0.0006). Chi-squared analysis revealed the decline in prevalence across age groups for male patients was statistically significant (p=0.0304).

CHAPTER 4
DISCUSSION

4 Discussion

4.1 MYCO WELL D-ONE and Detection of Genital Mycoplasmas

Culturing of genital mycoplasmas in specially formulated selective media was the 'gold standard' for the detection of *Ureaplasma* spp. and *M. hominis*, until the establishment of novel, more sensitive, PCR-based detection methods. An example of such a method was published in 1994 by Teng *et al*., who presented their findings and methods for a more sensitive, more rapid, PCR methodology for the detection of *Ureaplasma* spp. in clinical specimens.[330] The subsequent development of real-time and multiplex PCR methods for the detection of *U. parvum*, *U. urealyticum* and *M. hominis* improved the clinical sensitivity of this method of detection, when compared with culture.[331,332] Alongside this, these multiplex methods offered a further advantage over culture-based detection methods, in that, they could discriminate between *Ureaplasma* spp. identifying *U. parvum*, *U. urealyticum* and mixed *Ureaplasma* infections.[333] Moreover, culture was sometimes limited by bacterial overgrowth due to the contamination of the clinical specimen with non-target organisms such as *Proteus* spp. or yeasts, a problem that is circumvented using molecular methods.[316] It is worth noting that though the overwhelming consensus is that PCR is a more sensitive and specific method, many studies still cite culture as the gold standard, determining PCR methodology sensitivities and specificities against it. One of the limitations of qPCR compared with culture is the inability of qPCR tests to accommodate large portions of clinical specimens. Also, these molecular methodologies require the use of specialist equipment, highly trained individuals and relatively expensive consumable items. Furthermore, the post-test analysis typically requires more complex analysis when compared with the simple binary yes/no answer provided by colour-changing media.

For the detection of *Ureaplasma* spp. the MYCO WELL D-ONE's overall sensitivity and specificity of 91.98% and 96.44%, compared with multiplex qPCR shows the MYCO WELL D-ONE to be an extremely sensitive culture-based method. This is consistent with a PCR versus culture study for *Ureaplasma* detection published in 2007, showing specificity and specificity of 92.4% and 93.8%,

respectively.[334] The culture-based methodology used in the study was the Mycotest commercial diagnostic assay. Though other studies have showed varying sensitivities and specificities for *Ureaplasma* detection when comparing PCR against culture, with such differences being wide-ranging and inconsistent. For instance, Stellrecht *et al.* showed that culture was 17% less sensitive than PCR.[331] But, it is important to highlight this study utilised a solid medium for the culture of *Ureaplasma* spp., which has been shown to be 8% less sensitive than broth-based culture.[335] That said, the sensitivity of the Mycoplasma IST2 (MYCO WELL D-ONE competitor) when compared with a commercially available PCR (Anyplex II) was only 44.9%, with a specificity of 87.9%.[327] This sensitivity value is in accordance with a recently published study, comparing the MYCO WELL D-ONE to the Anyplex II PCR system for *Ureaplasma* spp. and *M. hominis* detection. The researchers reported a sensitivity and specificity of 44.5% and 48.98% for *Ureaplasma* spp., when compared with the commercially available multiplex qPCR. These low sensitivity and specificity values were, in part, due to 4 positives in the MYCO WELL D-ONE not detected by the PCR-based method (Anyplex II). Yet, the researchers did not attempt to confirm the MYCO WELL D-ONE result by sub-culturing the positive wells or performing the multiplex qPCR on a DNA extraction obtained from the enriched culture of the positive well, as was performed in this study. The primer sequences for *Ureaplasma* spp. detection in Anyplex II PCR kit are proprietary and unpublished, however, previous studies comparing the multiplex qPCR kit to culture-based methodologies have shown the kit to have a sensitivity of 83.3% for the detection of *Ureaplasma* spp.[336] Furthermore, the limit of detection for the Anyplex II assay is 50 copies per reaction.[337] In addition to this the MYCO WELL D-ONE versus Anyplex II study does not provide any titre information for the *Ureaplasma* spp. positive wells. Therefore, it is entirely plausible that low concentrations of *Ureaplasma* spp. went undetected in the Anyplex II, as theoretically 1 (viable) CCU of *Ureaplasma* has the potential to result in a MYCO WELL D-ONE positive. These discordant results could have been resolved had the researchers followed up MYCO WELL D-ONE positives with a subsequent confirmatory method.

In my study we further analysed sensitivity and specificity between the sexes and samples types. No notable differences were discerned between urine, swab or female samples with sensitivities and specificities varying between 91.94-93.14% and 93.31-99.39%, respectively. However, sensitivity of *Ureaplasma* spp. detection in male samples dropped to 85.71%. This was due to the MYCO WELL D-ONE having a higher percentage of false negatives, when compared against true positives. For example, the false negative rate for male samples accounted for 14.28% of all true positive samples, compared with a false negative rate of 6.86% for female sample true positives. Possible explanations leading to this marginal reduction in sensitivity between male and female *Ureaplasma*-positive sample types are offered through the coupling of the low ureaplasmal titres of male-positives and the increased sensitivity of PCR methods, when compared with culture-based methods. For example, across age groups for males most *Ureaplasma*-positive samples possessed ureaplasmal concentrations $<10^3$ CCU/mL. Factoring in the lability of *Ureaplasma* spp. cells, their lack of a cell wall and inherent fragility, this predisposes them to become readily unculturable. Additionally, higher ureaplasmal titres present in female samples, provides a larger contingency of bacterium from which to culture, should a small portion of the cells become unculturable. The ability of qPCR to detect non-viable organisms, and the potential of low numbers of *Ureaplasma* cells to die during processing (to the point of undetectability via culture-based means) offers a likely explanation as to why the MYCO WELL D-ONE sensitivity, for the detection of *Ureaplasma*, is marginally lower for males. Furthermore, a loss in *Ureaplasma* spp. viability could potentially be caused if the patient depositing the sample were undergoing antibiotic treatment, however, there is no clear way to establish this at this point. As for PPV and NPV, the MYCO WELL D-ONE consistently had PPVs >95% and NPVs ranging from 88.98% for swab samples to 96.53% for male samples. This together with accuracy values of >92% across all sample types proves the MYCO WELL D-ONE to be an efficient means of identifying *Ureaplasma* spp. infections.

For the detection of *M. hominis*, the overall sensitivity and specificity was 78.23% and 98.84%, respectively. Like the above mentioned comparative study, in which a commercial culture-based

mycoplasmal diagnostic assay was compared with qPCR, sensitivity for *M. hominis* detection was reduced, when compared with *Ureaplasma* spp. detection sensitivity.[334] Petrikkos *et al*. found a 29.6% reduction in detection sensitivity between *Ureaplasma* spp. and *M. hominis*, sensitivity declined from 92.4% for *Ureaplasma* spp. to 62.8% for *M. hominis*. In this study, overall sensitivity declines between genital mycoplasmas for the MYCO WELL D-ONE was 13.75%. Petrikkos and colleagues also reported the specificity for *M. hominis* detection remained in relatively high concordance with molecular methods, at 98.8%. Similar results were found in my study with the MYCO WELL D-ONE *M. hominis* specificity being calculated to be 98.84%. Choe and colleagues found comparable specificity between the Mycoplasma IST2 (BioMerieux) and qPCR (Anyplex II) at 99.6%, however, the sensitivity was again extremely low at 44.7%.[327] Another study that supports the notion of culture being less sensitive for the detection of *M. hominis* – than it is for the detection of *Ureaplasma* spp. – is offered by Abele-Horn and colleagues.[338] Researchers analysed 726 clinical samples made up of 189 gynaecological samples, 362 urological samples, and 175 neonatal samples by both PCR and culture, for the detection of *Ureaplasma* spp. and *M. hominis*. The sensitivity results of culture when compared with PCR agreed with those of the MYCO WELL D-ONE with sensitivity values for *Ureaplasma* spp. and *M. hominis* detection at 91% and 84%, respectively. The disparity between sensitivity for the culture-based detection of *Ureaplasma* spp. and *M. hominis* has not been explained elsewhere. But it has been previously been determined that *M. hominis* cells are harder to recover than *Ureaplasma* spp. cells. Researchers discovered recovery rates of *Ureaplasma* spp. cells to be 95% using culture, compared with a recovery rate of only 60% for *M. hominis* cells.[339] So, it may simply be that through using culture-based assays, *M. hominis* is harder to recover than *Ureaplasma* spp. Moving on to specificity, across all sample types the specificity of *M. hominis* detection by the MYCO WELL D-ONE was consistently >97%, demonstrating the assay had an exceptionally low false positive rate. PPVs for *M. hominis* detection ranged between 89.9% for swab samples to 93.22% for urine samples. Alongside this NPVs across sample types ranges from 95.32-99.69%. These high values are aided by the large prevalence of these organisms within the

populations, but the accuracy of the assay — the probability the test will correctly classify a patient as positive or negative — is >94% across all sample types.

The MYCO WELL D-ONE displayed sensitivities and specificities that were extremely good for the detection of *Ureaplasma* spp., with the results being comparable to the widely-used and clinically validated Abbott real-time PCR for the detection of *C. trachomatis* (sensitivity, 92,4%; specificity 99.2%).[340] Though the sensitivity of the MYCO WELL D-ONE in the detection of *M. hominis* decreased slightly, this is seemingly a universal difference in the ability of culture-based methods to recover these organisms. Nevertheless, this decrease in sensitivity in no way negates the effectiveness of this assay to detect *M. hominis* infection, evidenced by the high values seen across specificity, PPV, NPV and accuracy. That said, PCR is the more sensitive method, and due to its ability to further speciate detected *Ureaplasma* infections — a useful tool given the association between *U. urealyticum*, and not *U. parvum*, with male NGU — it is the preferred method of detection for these organisms. Nonetheless, as stated previously these qPCR methods require specialist and expensive equipment, highly trained individuals due to the more complex and laborious sample processing needed prior to testing. Additionally, the current European guidelines that state routine screening for these organisms is undesirable in lieu of substantiating that the treatment of asymptomatic genital mycoplasmal infection is beneficial.[287] Meaning that sample numbers necessary for high-throughput molecular testing – as is currently performed for classic sexual health pathogens, such as *C. trachomatis* and *N. gonorrhoea* – are not generated during sexual health screening assessments. However, in cases where patients present pathologies like urethritis or cervicitis and are 'true' STI-negative, the MYCO WELL D-ONE offers a robust, simple, easy-to-use, economic, and more importantly accurate method for determining the presence of genital mycoplasmas in the urogenital tract. Moreover, its simplicity — requiring only an incubator — to perform the test mean it is well suited to the low-resource environments of sexual health clinics. Accordingly, it permits a clinician a more detailed view of the urogenital biome underlying certain pathologies, potentially further elucidating the aetiology of a specific condition, facilitating a more personalised treatment

approach. Currently, many sexual health pathologies are treated empirically which presents numerous problems. For urogenital infections, there are growing concerns around a number of issues related to empirical treatment, such as inappropriate antibiotic prescription leading to multi-drug resistant urogenital pathogens.[341] Examples of empirical treatment failing in the treatment of NGU, when patients were *U. urealyticum*-positive, are displayed by Walter and colleagues.[342] The researchers performed a randomised, double-blind study to determine the effectiveness of treating males suffering from NGU empirically, with Rifalazil, a rifamycin derivative. Rifalazil demonstrated remarkable effectiveness against *C. trachomatis* but failed to eradicate *U. urealyticum* infections. Consequently, *U. urealyticum*-positive NGU-sufferers symptoms remained unresolved with symptoms persisting post-intervention. This highlights the importance of species-specific antibiotic prescription for more effective treatment of pathologies that can be caused by one or more organism. The MYCO WELL D-ONE has the potential to improve sexual health patient care in this regard.

4.2 MYCO WELL D-ONE and *Ureaplasma* spp. Load Estimation

Although the MYCO WELL D-ONE proved to be an accurate means of detecting *Ureaplasma* spp. infections, the semi-quantitative enumeration well did not share the same level of accuracy. The MYCO WELL D-ONE quantitation wells only agreed with the culture titration methodology in 43.6% of cases. The main issue was the over-reporting of bacterial load by the MYCO WELL D-ONE, frequently indicating a ureaplasmal concentration of $\geq 10^5$ CCU/mL, when the more accurate method of enumeration by culture — the culture titration — determined the inoculum to be $\leq 10^4$ CCU/mL. Overall this occurred for 55.6% of MYCO WELL D-ONE and culture titration positives. Though the accuracy seemingly improved for swab samples, this could simply be due to the higher frequency of bacterial loads $\geq 10^5$ CCU/mL for these sample types. Therefore, the ease with which the $\geq 10^5$ MYCO WELL D-ONE well displayed a positive result and the higher frequency of $\geq 10^5$ CCU/mL *Ureaplasma*

spp. loads for swab samples means the increase in accuracy is likely coincidental. The method by which the two 10^4 CCU/mL wells and $\geq 10^5$ CCU/mL differentiate between ureaplasmal loads, without a further dilution step, is a proprietary function of the CPM SAS selective media used in the assay and as such it is difficult to speculate the reasons behind the well's low accuracy. It is reasonable to assume that the media employs selective inhibitors in the $\geq 10^5$ CCU/mL well, that aim to prevent the growth of *Ureaplasma* spp. loads $\leq 10^4$ CCU/mL. If this is the case, then the well must contain a concentration of inhibitor or inhibitors that binds to a concentration of cells $\leq 10^4$ CCU/mL, but beyond which is saturated. Therefore, the low sensitivity and over-reporting of bacterial loads in the semi-quantitative MYCO WELL D-ONE wells possibly indicates that the proprietary inhibitory substance does not consistently and effectively inhibit the growth of *Ureaplasma* spp. loads $\leq 10^4$ CCU/mL.

4.3 *Ureaplasma* spp. Prevalence

Utilising PHE's clinically validated multiplex qPCR for genital mycoplasma detection female urogenital *Ureaplasma* spp. prevalence was determined to be 57.2%. Typically, *Ureaplasma* spp. are isolated from the vagina at a prevalence rate of between 40-80%.[284] Therefore, the prevalence rate of *Ureaplasma* spp. isolated from women attending sexual health clinics throughout the Valley's convergence area is within the mid-range of rates cited elsewhere. Numerous factors seemingly influence *Ureaplasma* spp. prevalence rates such as younger age, lower socioeconomic status, sexual activity with multiple partners, African-American ethnicity, and oral contraceptive use.[134,265] It is safe to assume that from the nature of the clinics in which samples were collected – walk-in sexual health clinics – that patients are likely sexually active. Additionally, a prerequisite to patients consenting to this study was that participants be over the age of 16 and can therefore be considered of reproductive age. As a result, these prevalence rates support previously published data displaying that sexually active non-pregnant women have prevalence rates of 67%, compared with only 40% of sexually inactive females of reproductive age undergoing routine gynaecological assessments.[343,344]

For Males, *Ureaplasma* spp. colonisation was determined to be 21.6%, however, a direct comparison with other studies is difficult as it is generally accepted that the population prevalence of these organisms in males is unknown, with Horner *et al.* estimating it to be between 5-15%.[287] This is as studies on male *Ureaplasma* spp. have typically been performed on symptomatic or infertile populations.[232,283,286,323,345] However, it should be stated that the symptom status of males tested here – symptomatic or asymptomatic – is not known. This information, if made available from patient data collected as part of their screening could provide valuable insights into the prevalence levels of both male and female patients against the factors thought to influence colonisation rates, listed above. Females had significantly higher prevalence rates than males (p=<0.0001), supporting the consensus that female populations are at a higher risk of *Ureaplasma* spp. colonisation. An

explanation for the differences in prevalence between the sexes is offered through the significantly different levels of oestrogen they possess.

Oestrogen is a well-characterised though not fully understood hormone. Discovered more than 70 years ago by Edward Adelbert Doisy, whilst studying the oestrous cycle in animal models, oestrogen was first isolated from pig's urine and has been subsequently dubbed 'the female sex hormone'.[346] Though this group of compounds are renowned for their importance in the menstrual and oestrous reproductive cycles, alongside driving sexual development and maturity, their roles in infection mediation are currently being elucidated. It has previously been demonstrated in murine models, that pre-treatment with oestradiol (a form of oestrogen) increases *Ureaplasma* spp. colonisation in the genitourinary tract.[347] Interestingly, it was demonstrated that glucose-fermenting mycoplasma colonisation rates were only enhanced by progesterone treatment (unaffected by oestradiol), whereas non-glucose-fermenting mycoplasmas such as *Ureaplasma* spp. and *M. hominis* colonisation rates were, oppositely, unaffected by progesterone (enhanced by oestradiaol only). A noteworthy difference between progesterone-affected mycoplasmas and oestradiol-affected *Ureaplasma*s is that the former possess terminal structures that assist in adherence to cells.[348] Thus suggesting that the physiological response of urogenital epithelial cells to oestrogen favours the cytadherence of non-terminal-structure-possessing *Ureaplasma*s. Though the cytadherence mechanisms employed by *Ureaplasma* to establish infection are poorly investigated, virulence and colonisation mechanisms in other sexually transmitted infections have been shown to be oestrogen mediated. It is well established that gonococcal genital infections occur more frequently during, or in the lead up to menstruation, when oestrogen levels rise.[349] Whereas for chlamydial infections, researchers harvested luminal and glandular epithelial cells from the cervix, uterus and uterine horns of female swine and cultured them *ex vivo.* The researchers reported that cells in the proliferative, oestrogen-dominant phase, were more susceptible to *Chlamydia suis* S45 than those in the progesterone-dominant phase.[350] Subsequent work demonstrated that oestrogen-stimulated stromal cells release effector molecules that regulate the growth and maturation of the intrauterine

epithelia.[351,352] These stromal cell released effector molecules – which alongside the direct interactions between hormones, epithelial cells and stromal cells – regulate endometrial remodelling throughout the menstrual cycle.[353–356] Experiments on *C. trachomatis* in endometrial cell (Ishikawa, IK)/SHT-290 stromal cell co-cultures revealed three different mechanisms by which oestrogen enhances infectivity: membrane-bound oestrogen receptors directly facilitate entry of bacterium into epithelial cells; oestrogen receptor-signalling encourages intracellular development of *C. trachomatis*; oestrogen-stimulated endometrial stromal cell effector molecules indirectly accelerate chlamydial development in infected endometrial epithelial cells.[357] Repeating these experiments, substituting *C. trachomatis* for *Ureaplasma* spp. may reveal similar oestrogen-mediated mechanisms of epithelial infection. Both *Ureaplasma* spp. and *C. trachomatis* are reliant on host cell proliferation for survival; *C. trachomatis* are obligate intracellular pathogens[358] and *Ureaplasma* spp. has a limited genome – resulting in a reduced biosynthetic capacity – resigning them to parasitic/saprophytic host tissue-specificities.[6] Therefore it stands to reason that such organisms will have evolved to take advantage of the oestrogen-mediated pathways regulating the proliferation of the tissues contingent to their survival. To illustrate this idea, oestrogen drives the survival and proliferation of epithelial cells by: (1) upregulating the expression of cyclin D1 and various other cell cycle genes in endometrial epithelial cells; (2) inducing the secretion of insulin-like growth factor in stromal cells.[356,359] Conversely, progesterone thwarts the proliferation of endometrial epithelial cells induced by oestrogen stimulation, resulting in the maturation of these cells. Alongside this, rising progesterone levels in the endometrium inhibits the expression of oestrogen receptors, reducing the oestrogen-mediated transcription of specific genes and cell signalling in epithelial cells. Moreover, progesterone stimulated stromal cells secrete oestrogen-metabolising enzymes.[353–356] The expression of cellular adhesive compounds like fibronectin, integrins and metalloproteases, have been shown to be modulated by both hormones. Altering the expression of these compounds tempers and adjusts cell-signalling pathways, subsequently changing the contacts between adjacent epithelial cells and the extracellular matrix.[354,355,360,361] Thus, given

the contrasting effects of oestrogen and progesterone it would stand to reason that if the proliferative effects of oestrogen on epithelial cells are conducive to the cytadherence of ureaplasmal cells, the inhibitory effect of progesterone would antagonise *Ureaplasma* spp. colonisation of the urogenital epithelia. Support for this proposition is offered through research displaying that prolonged administration of vaginal progesterone was associated with lower rates of *U. urealyticum* infection in pregnant women.[362] Additionally, oestrogen production is associated with reduced rates of antimicrobial peptide and inflammatory cytokine secretion by the endometrial epithelia.[363] For instance, levels of lactoferrin — an antimicrobial iron-sequestering glycoprotein — have been shown to be inversely correlated to oestrogen levels.[364] Alongside this, the anti-inflammatory effects of oestrogen can be seen as it attenuates lipopolysaccharide-induced inflammatory responses in rat microglia.[365] Conversely, increased cytokine expression and immune cell recruitment are associated with elevated progesterone levels.[355,363] Furthermore, reproductive tract fluid levels of IgG and IgA antibodies are modulated by the different hormone-dominant stages of the oestrous cycle. In human cervical mucous, IgA levels rise alongside oestrogen levels in the lead up to ovulation.[366,367] In animal models – mouse and rat specifically – IgA concentrations peak in the vaginal lavage at oestrous, with the reverse being true for IgG.[368] *Ureaplasma* spp. has a known ability to degrade IgA through a novel host-tissue specific serine proteinase that cleaves IgA between proline and threonine residues in the hinge region of the immunoglobulins heavy chain.[218,369] Though the precise mechanisms of *Ureaplasma* spp. cytadherence are yet to be fully described, or understood, it has been demonstrated that pre-treatment of these organisms with N-acetylneuramic acid can block adherence to HeLa cells.[206] This suggests that adhesins – likely the MBA – recognise and bind to sialic acid containing receptors. Therefore, it is possible that mucins that contain sialic acid containing residues, such as MUC1 – a large, heavily glycosylated mucin secreted by the reproductive tract epithelium[370]– could bind to *Ureaplasma* spp. cells and prevent them adhering to cells. Interestingly, MUC1 expression has been shown to be progesterone dependent.[371,372] So, while there is much that remains to be explored as to how the complex hormonal regulation of the female

reproductive tract is exploited by *Ureaplasma* spp. in order to establish and sustain an infection in a host. It is seemingly a combination of enhancing the susceptibility of the urogenital tissues to infection, alongside providing a suitably traversable and favourable immune environment for *Ureaplasma* cells.

Additionally, patients were further sub-categorised by age group: 16-20, 21-30, 31-40, 41-50 and ≥51. Prevalence rates were highest amongst female patients aged 16-20-years-old at 70%, steadily decreasing by around 10% for each increasing age group, before plateauing beyond the age of 41 at between 38-39%. This supports the findings of several researchers associating younger age to an increased risk of *Ureaplasma* spp. infection, alongside colonisation rates decreasing with age.[24,304,373] Similarly, the prevalence of other sexually transmitted infections such as *C. trachomatis* has been shown to be age-associated in female populations, with increased infection rates in younger age groups.[374,375] Moreover, *Ureaplasma* spp. infection is frequently associated with concurrent infection with more 'traditional' sexually transmitted agents like *C. trachomatis*.[376,377] Correspondingly, a risk factor associated with both *C. trachomatis* infection and *Ureaplasma* spp. infection is number of sexual partners; an increase in number of sexual partners is associated with increased prevalence for both organisms.[378,379] Therefore, a possible explanation for the increase in female *Ureaplasma* spp. prevalence with younger age, is the association between younger age and multiple sexual partners.[380] It stands to reason that as younger people are more likely to have multiple sexual partners, alongside their increased propensity for risky sexual behaviours such as unprotected sex[381], they have an increased risk of acquiring sexually transmitted microbes, including *Ureaplasma* spp. Additionally, another important factor in colonisation rates between female age groups is hormonal status. As discussed previously, oestrogen levels likely play an important role in the susceptibility of females to *Ureaplasma* spp. colonisation, with increased oestrogen increasing infection likelihood. Female oestrogen levels begin to rise during and throughout puberty, initiating the development of female reproductive tissue and inducing menarche.[382,383] On average, serum oestrogen levels continue to rise in females of reproductive age peaking between the ages of 18-24

(~100pg/mL)[384,385], before steadily declining year on year until the onset of menopause at between 44.6-52 years old whereby serum oestrogen levels decline rapidly by around 80%.[386,387] Therefore, as overall oestrogen levels decline with age, so do *Ureaplasma* spp. colonisation rates. The physiological responses to oestrogen in female urogenital tract, that result in an increase in host-tissue susceptibility to *Ureaplasma* spp. infection are explored previously. It is worth noting that female ureaplasmal colonisation rates that coincide with, and extend beyond, menopausal age (≥41) plateau between 38-39%. Though this ostensibly 'post-menopausal' *Ureaplasma* spp. colonisation rate is much lower than that of the younger age groups assessed in this study – possibly indicating reduced oestrogen levels and a lower number of sexual partners – it is considerably higher than *Ureaplasma* spp. rates observed in post-menopausal females elsewhere, at 13%.[388] This may be owed to geographical or population variability, and of note, the post-menopausal women assessed in the Hillier and Lau study had not received hormone replacement therapy (HRT). Therefore, it would be interesting to determine whether post-menopausal patients receiving HRT had different ureaplasmal colonisation rates to those not receiving hormone therapy in my study, as HRT has been shown to influence the vaginal microbiome post-menopause.[389]

For male patients, a different trajectory for *Ureaplasma* spp. colonisation rates alongside age group was observed. Between the ages of 16-30, colonisation rates remained consistent at around 27%, before falling by 60% and plateauing for males aged ≥31 at between 10-12%. Similarly to female populations, younger age has been shown to be associated with male ureaplasmal colonisation rates.[390] Additionally, researchers discovered that alongside younger age, a recent change in sexual partner and fewer lifetime sexual partners was also significantly associated with male *Ureaplasma* spp. infection. Rates of carriage similar to those observed for males ≤30-years-old in this study were observed in males with two or more sexual partners in another study, with a colonisation rate of 26%.[391] Moreover, an assessment of infertile Chinese males revealed *Ureaplasma* spp. infection rates peaked between the ages of 25-29 (33.3%) and 30-34 (35.8%).[345]

Aside from increased sensitivity, when compared with culture-based detection methods, an additional advantage of using multiplex qPCR for the detection of *Ureaplasma* spp. is that it enables the differentiation between *Ureaplasma* spp. infection type: *U. parvum*, *U. urealyticum* or both (*U. parvum* and *U. urealyticum*). It is frequently reported that *U. parvum* is the dominant infection type in women, as was the case in my study, with a female *U. parvum* prevalence rate of 40.5%. Similarly, high prevalence rates for *U. parvum* colonisation have been stated elsewhere: 31%; Japan[377], 53.1%; Australia[392], 38.3%; Italy[292]. The data presented here and, in the literature, display that *U. parvum* is a prominent coloniser of the female urogenital tract and is the dominant species of *Ureaplasma* among the populous screened – GUM patients. There is a significant disparity between overall *U. parvum* and *U. urealyticum* prevalence, with rates dropping from 40.5% to 10.8% for each species, respectively. Again, the contrast in *U. parvum*-*U. urealyticum* prevalence rates is well established and the data presented here corroborates that of existing reports for *U. urealyticum* prevalence rates: 5.7%; Japan[377], 16.1%; Greece[393], 9%; Italy[292], 6.1%; Australia[392]. For mixed infections, prevalence rates were even lower among females at 5.89%, consistent with other studies describing concomitant *U. parvum* and *U. urealyticum* prevalence rates: 2.9%; Australia[392], 1.6%; Italy[292], 3.2%; Japan[377]. Typically, *U. urealyticum* is the species of *Ureaplasma* most associated with pathological conditions such as female infertility, cervicitis, PID and genital discomfort.[394] Whilst *U. urealyticum* prevalence is markedly reduced when compared with *U. parvum* — the less innocuous of the two — it's prevalence is not insignificant, being isolated from more than one in ten women attending GUM clinics, who as a result of this infection are at a higher risk of the previously described urogenital pathologies. Interestingly, with regards to species dominance, the reverse was true for males with *U. urealyticum* being the most prevalent with an overall colonisation rate of 14.3%, compared with only 6.9% *U. parvum*. This possibly contradicts the hypothesis held by Horner *et al*. whom surmised that *U. parvum* was likely the dominant ureaplasmal infection in males due to it being more frequently isolated from males without NGU.[287] Or, it could indicate high levels of male patients with NGU attending sexual-health clinics in the Valley's convergence area, due to *U. urealyticum's* association

with the condition.[290] Similar rates of *U. urealyticum* prevalence were observed among males determined to be symptomatic for microscopy-confirmed NGU, with 16% of NGU-positive patients, positive for *U. urealyticum*.[283] Though it should be stated that Cox *et al.* concluded that the elevated rate of *U. urealyticum* in NGU-confirmed patients was not statistically significant in their study. However, numerous other reports have determined a causative and statistically significant link between *U. urealyticum* infection and NGU.[228,395,396] Further patient information as to symptomatic status would permit more in-depth analysis, possibly substantiating or rebutting the link between symptomatic NGU and *U. urealyticum*. Additionally, the extreme differences observed in *U. parvum* colonisation rates between the sexes, dropping from 40.5% in females to 6.9% in males, could indicate that *U. parvum* is the sex dependent *Ureaplasma*, when compared with the consistent *U. urealyticum* rates of 14.3% in males and 10.8% in females. Furthermore, when mixed ureaplasmal infections are considered the overall percentage of male and female *U. urealyticum* prevalence is 14.3% to 16.7% respectively, narrowing the difference. Moreover, when *U. parvum* prevalence is determined by age group between the sexes an interesting trend is revealed. For males across all age ranges, prevalence rates remain consistent, fluctuating between 3-8% from the age of 16 to 51 and beyond. Whereas for female patients, prevalence rates begin at 52.25% for ages 16-20 and subsequently steadily decline to 30.43% for women aged ≥51. Conversely, the trends observed for *U. urealyticum* prevalence across age groups and between sexes followed similar trajectories. Prevalence rates peaked between 16-30 at between 20-30% for both sexes, before rapidly declining to between 0-16% beyond the age of the 31. The differences observed in the trends could indicate that *U. parvum* colonisation is mediated by oestrogen, as the colonisation patterns follow similar trends to oestrogen levels in both male and females. Male oestrogen levels remain relatively low and consistent throughout their entire lifespan,[397] reflecting the low and consistent prevalence rates of *U. parvum* across male age groups. Whereas the age-related decline in oestrogen observed in females is matched by the age-related decline in *U. parvum* prevalence. This is seemingly the first time that this species-specific observation has been made and the potential reasons as to why this

might be the case are not clear. The two *Ureaplasma* spp. are divided into 14 serovars; *U. parvum* comprises serovars 1, 3, 6 and 14 with *U. urealyticum* making up the remainder.[173] These existing serovars were established through metabolism inhibition and colony indirect epi-immunofluorescence testing, using rabbit antisera.[172] Serotyping techniques have been centred around the MBA, due the suggestion of serovar specific epitopes of this antigen.[134,317] Whilst conserved regions of the *mba* gene had been identified across all serovars, the variable region, also thought to be serotype-specific had only been partially characterised.[190,398] However, more recently, sequence analysis resulted in the discovery of *Ureaplasma* spp. propensity for inter-serovar horizontal gene transfer — generating hybrid serovars — calling the usefulness of serotyping into question.[399] Therefore, viewing the two species through the lens of serovar-specificities may not offer an insight into the differences in prevalence trends across age groups and between sexes. Further comparative genome analysis across all serovars revealed significant species differences, with those differences conserved throughout each species specific serovars.[227] Paralanov *et al.* identified 18 hypothetical proteins and 2 putative lipoproteins that were unique to all *U. parvum* serovars and absent from all *U. urealyticum* serovars. It was also highlighted that all *U. parvum* serovars possessed simpler MBA phase variation systems than those of *U. urealyticum* serovars. The conserved domain of the *mba* gene was surrounded by single base pair repeats, containing the 25-base pair putative recombination recognition site, in *U. parvum*. Additionally, *U. parvum* serovars possessed fewer putative recombinases than *U. urealyticum*. Such recombinases, like the tyrosine recombinase XerC of *U. parvum* serovar 3 have been shown to be potential mediators of DNA inversion events associated with MBA phase-variation.[201] The limited number of recombinases and relatively simplistic MBA phase-variation systems possessed by the smaller *Ureaplasma* genome of *U. parvum*, limits its capacity for MBA variety. MBAs have previously been shown to be recognised by TLRs and induce NF NF-κB, eliciting an immune response.[191] As such, it has been suggested that the ability of an organism to vary the MBAs expressed on their surface, possibly impacts the level of immune response produced, when recognised by TLRs.[400] More specifically, TLR2, 6 and 9 have been

the TLRs implicated in the initiation of an innate immune response to *U. parvum* and its MBA.[401] Interestingly, TLR2 activation results in a mollified inflammatory response, relative to TLR4 activation.[402] Moreover, oestrogen has been shown in numerous cases to mediate and suppress TLR-induced innate immune responses in human uterine and endometrial epithelial cells.[403,404] Additionally, following TLR2 activation — for which *U. parvum* and the MBA have displayed to be an agonist — oestrogen has been shown to attenuate chemokine production upon receptor stimulation, dampening the immune response.[405] Therefore, a possible explanation for the trends in *U. parvum* colonisation rates, proposed to follow the age-related decline in oestrogen for females, supported by the low and steady colonisation rates in males (low oestrogen throughout lifespan) across age groups, and that significantly differ from prevalence rate trends for *U. urealyticum* across both sexes, could be offered by *U. parvum's* limited *mba* gene, coupled with the anti-inflammatory environment generated by high oestrogen in younger females. The proposed mechanism for this is as follows: (i) the limited capacity for phase-variability in the *mba* gene of *U. parvum* results in an overall subdued response following TLR receptor stimulation (ii) elevated oestrogen levels present in the genitourinary tract of younger females attenuate the already subdued TLR-induced inflammatory response. Thus, relative to *U. urealyticum*, *U. parvum's* limited MBA phase variation machinery provides the appropriate amount of antigenic variation to avoid the adaptive immune system, whilst the organism's stimulation of innate immune receptors is attenuated by the high oestrogen environment. As such clearance of *U. parvum* is not achieved through innate immune cells in oestrogen-stimulated epithelia, generating conditions conducive to the establishment of persistent infection in the urogenital tract of younger/higher-oestrogen females, relative to older/lower-oestrogen females. This hypothesis supports the consensus that *U. parvum* is a commensal isolated from both symptomatic and asymptomatic individuals. Whereas *U. urealyticum* is often cited as the more virulent of the two, associated with symptomatic individuals in which a more pronounced inflammatory response has been triggered, manifesting clinically as NGU and NSC.[242,290,395,406–409] Allowing the speculative suggestion that the more complex *mba* genes isolated from

U. urealyticum, possessing more TRUs and recombinases have a more virulent motif, whereas the *U. parvum* MBAs have a more persistence/colonisation-based motif. For numerous other pathogenic bacteria, such as *C. trachomatis* and *Clostridium difficile*, the phase-variation strategies they employ have been directly associated to their virulence.[410–412] Therefore, it would be interesting to determine the immunogenicity of the various TRUs present in the *mba* genes of both *U. parvum* and *U. urealyticum*. Should TRUs be displayed to be differentially immunogenic, it could help to clarify the seeming differences in virulence between the two species of *Ureaplasma*, as *U. urealyticum* MBAs possess larger quantities of TRUs than those of *U. parvum*.[227] However, it should be stated that the notion of *U. urealyticum* being the more virulent of the two *Ureaplasma* spp. (particularly as a sexual health pathogen) though generally accepted, is not a universal one, with some researchers providing evidence to the contrary.[413,414] Moreover, the association between other, more chronic infections and conditions — neonatal- and pregnancy-related pathologies — and *U. parvum* are well-established.[134] Equally, the not-yet-characterised 18 hypothetical proteins unique to *U. parvum* and the 83 hypothetical proteins unique to various *U. urealyticum* serovars,[227] could potentially offer an explanation as to the observed differences in species prevalence trends between the sexes and across age groups. However, a function for these putative protein-encoding genes could not be assigned by Paralanov *et al.*, as they were genes for which orthologs coded for proteins of unknown function or for which an ortholog outside of the *Ureaplasma* genus did not exist. The only species-specific difference associated with a potential pathogenicity factor, noted by Paralanov and colleagues, was that each species employed different genes to produce H_2O_2 resistance-conferring compounds.

The current research surrounding the genetic differences between the *Ureaplasmas* and the molecular mechanisms that underlie adherence and virulence typically concludes that genomes of these organisms are poorly characterised.[209,227,415,416] Couple this with the continually growing evidence of the extensive level of horizontal gene transfer between *Ureaplasmas*, resulting in cross-reactivity, calling the appropriateness of serotyping for explaining the pathologic differences

between the species into question.[399] The findings of this study, together with the large number of clinical isolates it has generated, provide an extensive catalogue of both *U. parvum* and *U. urealyticum* cultures that could be screened/sequenced for the presence/absence of putative adherence or pathogenicity associated genes. Furthering this, the disease status of patients (if made available through patient notes taken as part of their routine sexual health screening) could be combined with the identification of such candidate genes for pathogenicity-associated products, to establish an association between specific genetic elements and clinical pathologies. As has been performed for other sexually transmitted agents, facilitating the characterisation of clinical manifestations and epidemiology of the *C. trachomatis* genetic variant nvCT.[417,418]

All comparative studies examined here utilised molecular methods for the detection and speciation of *Ureaplasma* spp. in urogenital samples. Caution must be exercised when reviewing the literature for *U. urealyticum* data, as many researchers were slow to adopt the post-2002 nomenclature that resulted in *U. parvum* becoming taxonomically distinct from other mycoplasmas.[173] It is extremely difficult to determine whether these previous, often culture-based approaches (unable to speciate), subsumed the detection *U. parvum* under *U. urealyticum*, or failed to detect it at all.

4.4 *M. hominis* Prevalence

As for *M. hominis* prevalence, the disparity between female and male prevalence was much the same, however the rates of prevalence were much lower for each patient group overall. For female patients, the prevalence of *M. hominis* colonisation was 16.3%. For sexually mature women rates of colonisation typically range between 20-50%.[284,285] Though below the typical range, *M. hominis* prevalence rates lower than 20% are not uncommon: 4.6%; Italian study[419], 4%; Turkish study[304], 4.3%; USA study[420], 1.01%; Korean study[421], 14.6%; Chinese Study[308], 2.5%; Italian study[422], 1.9%; Chinese study[423]. Again, an overall and general population prevalence is hard to obtain as most studies focus on *M. hominis* colonisation together with another factor, pathological or otherwise, that is linked to infection. Interestingly, the rate at which *M. hominis* was isolated from the female urogenital tract was 3.5 times less than *Ureaplasma* spp. infection. This was also reflected in Furr and Taylor-Robinson, who found *Ureaplasma* spp. to be isolated from the urogenital tracts of female patients four times more than *M. hominis*.[424]

Of males tested, only 4.3% of patients were positive for *M. hominis* corroborating reports of population prevalence to typically be between 2-4%, rendering it a relatively uncommon organism colonising the urethra of males.[283] However, ranges exceeding this have been documented in otherwise healthy, asymptomatic and fertile individuals. For example, prevalence rates of 9.27% have been observed in the semen samples of males in the Henan Province of China. Though the samples collected in our study were primarily urine samples, these have been shown to be equivalent to sperm for *M. hominis* detection, in a study reporting male *M. hominis* prevalence to be 10.6% *M. hominis*.[425] Also worth noting is that population prevalence has been described as high as 20% in some instances.[280] Aside from age (explored later), patient information such as sexuality, number of sexual partners, symptomatic/asymptomatic, fertility status etc. is unavailable at the time of writing. Therefore, the dataset available at present can neither confirm nor rebut the established but unconfirmed links between *M. hominis* infection and fertility status.[232] Controversy remains as to

whether *M. hominis* is a true causative agent, due to its strong association with 'true' STIs that have been previously established to cause infertility.[287] The association of *M. hominis* infection with these 'true' STIs could also be analysed if access to patient note data was granted in the future. Moreover, recent studies have demonstrated the sexual exchange of bacterial vaginosis (BV)-associated urogenital bacterial taxa between partners, alongside a significant association between partners urogenital biomes.[426,427] As such it begs the question, as a BV-associated microorganism with such low male prevalence, is male positivity for *M. hominis* a reflection of female sexual partner colonisation? Also, could it be used as a proxy/predictor of BV in the sexual partners of males deemed *M. hominis* positive? Further investigation into these research questions could provide a much-needed insight into *M. hominis* role in the male genital microbiome, as the recent and widespread recognition of the human microbiomes role in human health has led to the launch of projects such as the Human Microbiome Project.[428] Research has centred around the microbiomes of the intestinal tract, mouth, skin and female genital tract, with the male genital tract — thought to regulate reproductive tract homeostasis — being much neglected.[429] Additionally, if the hypothesis of male infection being largely transient and the consequence of intercourse with *M. hominis*-infected females is correct, it would be reasonable to assume that *M. hominis* prevalence in MSM populations would be lower than those of heterosexual (MSW) communities. This would be the opposite of what is observed for other urogenital mycoplasmas, such as *M. genitalium*, with prevalence rates for the MSM community estimated to be 3 times that of the general population.[430]

Overall, females had significantly higher rates of *M. hominis* colonisation than males (p=<0.0001; Fisher's exact test), as expected. Rates of colonisation were slightly lower than is typically seen for a sexually active populous (<20%) amongst females. Whereas the prevalence amongst males was at the upper end of what is routinely observed (2-4%), at 4.3%. Much the same as *Ureaplasma* spp., differences in prevalence rates between males and females could possibly be explained through the differing oestrogen levels females, compared with males. Whereas colonisation rates for *Ureaplasma* spp. have been shown to be enhanced by oestrogen in murine models,[129] *M. hominis*

colonisation of the genital tract is exclusively restricted to oestrogen-treated BALB/c mice.[431] Consequently, it has been suggested that HRT for post-menopausal women would increase colonisation rates of *M.* hominis, subsequently increasing the likelihood that these women would develop BV.[388] Several reasons for the role of oestrogen in the urogenital colonisation of *Ureaplasma* spp. were offered and explored previously. These could also explain and elucidate the reasons behind the strikingly similar disparities observed in the prevalence rates for *M. hominis* between men and women. As *M. hominis* and *Ureaplasma* both fall under the category of non-glucose-metabolising mycoplasmas, which have been shown to be oestrogen-dependent.[129] Additionally, another effect of oestrogen on the vaginal epithelia is the stimulation of glycogen production.[432] The main function of which is thought to be to promote the growth of *Lactobacillus* species,[288] which provide myriad benefits and were thought to help maintain a healthy vaginal pH through their production of lactic acid.[433,434] The low vaginal pH serves to prevent colonisation and infection by pathogenic bacteria.[435,436] Furthermore, the *Lactobacilli* also produce antimicrobial compounds like bacteriocins to deter infection with undesirable microorganisms.[437,438] However, though it was previously thought that the vaginal lactobacilli metabolised the majority of glycogen released by the vaginal epithelium and subsequently anaerobically fermented the resultant glucose.[433,434] It has recently been demonstrated that a number of vaginal lactobacilli isolates are unable to degrade glycogen and a host-derived α-amylase is now recognised as being responsible for the production of fermentable sugars present in vaginal secretions.[439–441] An *M. hominis*-related sexually-transmitted organism that also utilises glucose, through the production of endogenous amylases and glucose produced by host-derived α-amylase, is *Trichomonas vaginalis*.[442,443] This highly-prevalent STI has an estimated 200 million cases reported annually and as such is recognised and accepted as the most prevalent STI globally.[444,445] Additionally, 80% of *T. vaginalis* infections in the female populous are asymptomatic.[446–448] Identical to *M. hominis*, establishment of vaginal *T. vaginalis* infection in animal models is subject to pre-infection oestrogen treatment.[449] Moreover, trichomonad movement and attachment to mammalian cells *in vitro* has been shown to be oestrogen mediated.[450] Oestrogens

influence on *T. vaginalis* infection is reflected in the low carriage rates observed in males, much like *M. hominis*, estimated to be 10-fold less than the number of female cases.[445] But it has been noted that up to 73% of males with a *T. vaginalis*-infected female partner are positive for the infection.[451,452] However, researchers have noted that following exposure and detection of *T. vaginalis* in male patients, infections often resolve themselves, owing to the transient nature of *T. vaginalis* colonisation of the male urethra.[453] Therefore, the high-oestrogen, high-glucose environment of the female urogenital tract is thought to increase susceptibility to, and facilitate, *T. vaginalis* infection. Interestingly, a symbiotic relationship between *M. hominis* and *T. vaginalis* has long-been established. The founding of an association between these organisms occurred during a large clinical study, followed by the subsequent isolation of viable M. *hominis* from long-term *in-vitro* cultured *T. vaginalis* isolates.[454,455] Furthermore, it was later determined that >80% of clinically obtained *T. vaginalis* isolates tested were naturally infected with *M. hominis*, regardless of geographic location.[93,94,456] As such, these cases of symbiosis were the first of their kind to be described as it involved two distinct obligate human mucosal microbes, both of which are capable of causing disease in the anatomical area to which they reside, independent of one another. Though this symbiotic relationship remains to be fully resolved, certain characteristics of the relationship have been established. The capability of *M. hominis* to interact with *T. vaginalis* at both the cell surface level and intracellularly, alongside synchronised replication strategies, has been displayed.[94] These data, coupled with that of Rappelli and colleagues outlining how *M. hominis* infection can be transferred between infected and non-infected trichomonads and to epithelial cells of the cervix[93] led to the 'Trojan horse' hypothesis. The theory postulates that *M. hominis* intracellular location within the protozoan provides safe passage to the vaginal mucosa, shielding the mycoplasma from host immune cells and antibiotic compounds.[94,456] Additionally, the cytopathogenicity of *M. hominis*-harbouring *T. vaginalis* has been shown to be increased *in vitro*, compared to uninfected *T. vaginalis*.[457] Recently researchers analysing the metabolic results of this relationship found that *M. hominis*-infected *T. vaginalis* had higher replication rates, increased haemolytic activity and an

increased capacity for ATP production.[98] They also displayed how *M. hominis* modulated nitric oxide (NO) production in *T. vaginalis*-macrophage co-cultures through substrate competition for NOs precursor arginine. Therefore, further analysis of patient samples to determine *T. vaginalis* prevalence would illuminate the extent to which *M. hominis*-infection is *T. vaginalis*-mediated. Furthermore, it would provide valuable insight into whether oestrogen-mediated *M. hominis* colonisation is due to the following; oestrogen stimulates the release of glycogen by the vaginal epithelia providing a high-glucose environment conducive to *M. hominis*-harbouring *T. vaginalis* infection; (ii) oestrogen, due to factors independent of *T. vaginalis* infection, produces an environment conducive to mycoplasmal infection (as explored for *Ureaplasma* spp. earlier); (iii) the observed disparities, purportedly due to oestrogen, are as a consequence of the interplay between the two previous propositions. The third option is the most likely given that *M. hominis*-only infection has been shown to be oestrogen mediated in murine models, displaying it can act as a sole infective agent. Moreover, the high rates of prevalence and *M. hominis*-symbiosis observed for *T. vaginalis* will inevitably result in *T. vaginalis* infection being responsible for a large portion of *M. hominis* prevalence. Much like *Ureaplasma* spp. the colonisation prevalence for females decreased as age increased, steadily declining from between the ages of 16-20 before plateauing beyond the age of 41. Again, adding credence to the oestrogen-mediation theory of prevalence, as oestrogen levels steadily decline in females until the menopause reflecting the *M. hominis* prevalence rates across age groups.[382,383] For male patients, the distribution of prevalence rates across age groups did not follow the same trend. Males aged 16-20-year-old had a low colonisation rate of 2.5%, this increased to 7.34% for males aged 21-30, with 0 cases of male *M. hominis* positivity observed beyond the age of 31. This prevalence distribution supports the notion of male infection being only transient and is reflective of female partner infection due to the sharing of the urogenital microbiome between sexual partners.[426,429] Though this may seem counterintuitive in that the highest male detection rates are seen for males aged 21-30, whereas for females peak prevalence is

observed in the younger 16-20-year-old age group. This could be explained by a high proportion of females having male sexual partners that are 2-5 years their senior.[458,459]

4.5 Concomitant *Ureaplasma* spp. and *M. hominis*

The rates of dual infection for both *Ureaplasma* spp. and *M. hominis* were also determined, with sole *M. hominis* accounting for only 8.1% of *M. hominis*-colonised females. The remaining *M. hominis*- colonised females were co-infected with *Ureaplasma* spp., resulting in an overall co-colonisation rate of 15.02% (79/526). This is near double the coinfection rate (8.63%) observed in female gynaecology patients undergoing routine examinations in Sao Paolo City, Brazil.[373] Similarly, a 6-year study of symptomatic females at a sexual-health clinic in Belgrade, Serbia, has significantly lower rates of co-colonisation, with only 1% of females displaying positivity for both *Ureaplasma* spp. and *M. hominis*.[303] Additionally, the *Ureaplasma* spp. and *M. hominis* co-colonisation rate of patients presenting clinical vaginitis in Athens, Greece, was 2.92%.[297] A study of 100 pregnant females – typically cited as a population with an increased susceptibility to genital mycoplasmal colonisation – also only revealed a co-infection rate of 3%.[304] However, a study that is perhaps more comparable to this one found an overall co-colonisation rate of 10.1%, alongside higher incidences of genital mycoplasmal colonisation among individuals attending STI clinics.[460] Moreover, researchers recruiting sexually-active and symptomatic women at a Cameroon university hospital found a *Ureaplasma* spp. and *M. hominis* co-colonisation rate of 20%.[461] Thus the data presented here, indicates that women attending sexual-health clinics throughout the Valleys convergence area display elevated levels of *Ureaplasma* spp. and *M. hominis* co-infection. These rates are likely owed to increased levels of sexual activity, a known risk-factor associated with genital mycoplamsal colonisation, corroborating reports of STI clinic attendees being a high-risk group.[462] Also, a possible explanation for the disparity between the previously cited studies low co-colonisation rates (<5%) and those displayed here, is the method of detection. Most studies utilise rapid diagnostic culture-based assays which, as this study has shown for the MYCO WELL D-ONE, have markedly lower

sensitivity for the detection of *M. hominis* when compared with molecular methods, likely due to the organisms hard-to-recover nature.[339] A sentiment echoed in a 2009 paper utilising a multiplex PCR for the detection of genital mycoplasmas in sexually active females that reported 'higher-than-expected rates of *Mycoplasma, Ureaplasma..*', with concomitant *M. hominis-Ureaplasma* spp. infection rates similar to those presented here, at 11.4%.[392]

For males a similar trend to female concomitance was observed with the majority of *M. hominis* positive patients being co-colonised with *Ureaplasma* spp. though to a lesser extent, 71.4% versus 91.9%, respectively. Females were determined to have significantly higher rates of concomitant infection (p=0.0454; Fisher's exact test). Overall, the *Ureaplasma* spp. and *M. hominis* co-colonisation rate of male patients attending sexual health clinics in the Valleys convergence areas was 3.04%. This relatively low rate of co-infection is in accordance with several studies on male *Ureaplasma* spp. and *M. hominis* prevalence: 0.7%; Korea[421], 3.2%; China[463], 3%; Tunisia[131], 1%; China[345].

As expected, age-group analysis for concomitant *M. hominis-Ureaplasma* spp. infection showed an age-related decline in prevalence, likely due to the oestrogen-mediated increased susceptibility to genital mycoplasmal infection discussed previously. The composition of female *Ureaplasma* spp. and *M. hominis* dual infections was dominated by *M. hominis-U. parvum* infections, followed by *M. hominis-U. urealyticum* with the remainder being comprised of *M. hominis-U. parvum* and *U. urealyticum* colonisation, as has been described elsewhere.[292] Interestingly, no triple infections of *M. hominis-U. parvum* and *U. urealyticum* were observed for female patients aged ≥41, with the highest rates of triple infection being observed in patients between the ages of 16-20-years-old at 6.1%. Rates of triple infection declined with increasing age, perhaps indicating that triple infection cannot be supported in older females. However, comparing these data to other studies is difficult due to researchers failing to adopt the correct lexicon, often citing *Ureaplasma* spp. as *U. urealyticum* and failing to properly speciated *U. parvum* and *U. urealyticum* in the absence of molecular-based

detection methods.[287] Additionally, as patients were only categorised by age and sex no association between concomitant infection type and clinical outcome can be determined. It has been previously displayed that concomitant *M. hominis*-*Ureaplasma* spp. is associated with increased adverse pregnancy outcomes for women delivering preterm neonates, when compared with *Ureaplasma* spp. only infection.[464]

4.6 Bacterial Load and Sample Type

Alongside the identification of positivity for either *Ureaplasma* spp. and *M. hominis*, the culture titration methodology was utilised to determine the bacterial load of the urine- or swab-inoculated sterile saline. A striking disparity between sample type — urine or swab — was observed, with swab samples having consistently higher bacterial loads than urine samples for both *Ureaplasma* spp. and *M. hominis* positive samples. One of the primary reasons for determining the ureaplasmal or mycoplasmal load was due to the CLSI requirement for AST testing to be performed on titres $<10^5$ CCU/mL.[320] This is as it has been well-established that bacterial loads that do not conform to the CLSI guideline inoculum level produce false-positive resistance results.[328]

For *Ureaplasma* spp., of 422 culture-positive samples (168 swabs and 254 urine), 55.9% of swabs were determined to have a load $>10^5$ CCU/mL whereas the same load in urine samples was observed in only 5.9% of cases. There a few potential factors that could contribute to the significantly higher *Ureaplasma* spp. loads observed in swab samples, when compared with urine samples. Firstly, female patients comprised most swab samples (165/168 swabs), with these swab samples being endocervical, whereas most males deposited a urine sample for testing. A notable difference between endocervical samples and urine samples is that they sample different epithelial tissues; endocervical swabs sample the vaginal and cervical epithelia and urine analysis determines the colonisation status of the urethral epithelia. These tissues may be differentially affected by *Ureaplasma* spp. colonisation. It is possible that different infection mechanisms that are conducive

to increased bacterial loads occur during the infection of vaginal/cervical epithelial cells, when compared to those epithelial cells of the urinary tract. For example, other STIs have been shown to interact and infect epithelial tissues differentially, varying the infection pathway they deploy in response to the tissue they are infecting. Infection with *Neisseria gonorrhoeae* has been shown to be mediated by receptor-based endocytosis in the male urethra resulting in a proinflammatory response, whereas a different asymptomatic path to infection is observed in females. Cervical epithelial infection occurs through complement receptor 3 (CR3) mediated entry into cells, a receptor that does not exist in the urethral epithelia.[465,466] Moreover, and with regards to tissue specificities, in females, oestrogen receptor numbers vary throughout the tissues of the urogenital tract. For instance, oestradiol receptor concentrations in animal models show that the urethra and bladder contain between 10-20 times fewer oestradiol receptors, than the epithelia of the uterus at the higher portion of the urogenital tract.[467] As oestrogen seemingly mediates *Ureaplasma* spp. colonisation — explored previously — it is likely that the degree to which tissues are stimulated by oestrogen, as a consequence of their oestrogen receptor concentrations, directly impacts the bacterial load able to infect that tissue. As such, a possible explanation for the higher bacterial loads observed in swab samples, when compared with urine samples, is that the tissues being sampled (epithelia of the higher female urogenital tract) are more susceptible to *Ureaplasma* spp. increased colonisation than the urethral tracts of both male and female patients. Additionally, 127 patients supplied both a swab and urine sample made up of the following: 122 endocervical swab-urine pairings from females and 5 endourethral swab-urine pairings for males. For *Ureaplasma* spp. 71 patients were positive across both samples, with organism loads on swab samples being equal to or greater than their urine counterparts in 100% of cases, with statistical analysis determining the higher loads observed in swab samples to be statistically significant (p=0.0255). Most of the time — 43.7% of *Ureaplasma* spp. positive swab samples — had ureaplasmal loads between 100-1000 times greater than their urine counterparts. Currently, protocols for determining *Ureaplasma* spp. presence using commercially available rapid culture-based detections systems (Mycoplasma IST2,

Mycoplasma IES, Mycofast Revolution and MYCO WELL D-ONE) do not process urine or swab samples differently, in that both sample types receive the same initial dilution step. They are seeded into a volume of saline solution, before inoculating the test kit with the generated saline suspension.[321,468,469] Consequently, the results of this study have revealed significant differences in *Ureaplasma* spp. loads for swab samples, when compared with paired and unpaired urine samples, indicating that sample processing according to sample type is recommended. An additional dilution step for swab samples would result in more appropriate challenge inoculum ($<10^5$ CCU/mL) for concurrent detection and AST as per current rapid culture-based assays, and the MYCO WELL D-ONE more specifically. One of the primary issues raised by researchers is the concern that resistance data that is gathered using rapid culture-based screening systems leads to over-reported resistance, as high titres of challenge organisms overwhelm the antibiotic present in the kit's wells.[275,421,470] This results in resistance data being gathered, compiled and reported on in a non-CLSI complaint manner. It must be conceded that reporting a resistant isolate as susceptible has far greater implications than reporting a susceptible isolate as resistant. However, over-reporting antimicrobial resistance could lead to inappropriate treatment interventions being implemented, alongside unnecessary actions being taken such as the rejection of effective therapeutic strategies based on superficial 'resistance' to specific antibiotics. An example of this that has been rectified in UK clinical laboratories was the previous over-reporting of *Pseudomonas aeruginosa* resistance to ciprofloxacin, amending the AST broadened ciprofloxacin's applicability and potentially extended its lifespan as a therapeutic option for *P. aeruginosa* infection.[471]

Comparably, *M. hominis*-positive samples displayed a similar difference in bacterial load when swab samples were compared with urine samples. Much like *Ureaplasma* spp. positive samples, the vast majority (93%) of *M. hominis*-positive urine samples possessed titres $<10^5$ CCU/mL. Though most swab samples contained $<10^5$ CCU/mL (60%), a significantly higher portion of swabs (40%) contained titres $>10^5$ CCU/mL, outside of CLSI-compliant AST testing range.[320] Additionally, as there is no semi-quantitative well present in the MYCO WELL D-ONE for *M. hominis* any resistance is to be taken as

true. Therefore, a different dilution strategy should be implemented for swab samples in order to effectively test for *M. hominis* presence alongside AST. Further analysis of paired samples revealed that swab samples had bacterial titres equal to, or greater than, their urine counterparts in 76.2% of *M. hominis*-positive paired patient samples. However, most paired samples (42.9%) harboured loads equal to, or 10 times greater than, their urine counterparts with statistical analysis revealing the differences in paired sample loads to not be statistically significant. That said, due to the lower prevalence of *M. hominis*, compared with *Ureaplasma* spp., the number *M. hominis*-positive paired samples were limited at 21. Nevertheless, given the far greater number of undesirably high titres ($>10^5$ CCU/mL) present for *M. hominis* positive swab samples, coupled with the nature of commercial assays such as the MYCO WELL D-ONE — in that they aim to simultaneously detect *Ureaplasma* spp. and *M. hominis* infections and concurrently screen said organisms for resistance — the recommendation to process swab samples differently to urine samples remains valid.

Furthermore, for both male and female pathologies linked to *Ureaplasma* spp. infection bacterial load has routinely been cited as a contributory factor. For men, several reports have indicated an association between elevated *Ureaplasma* spp. loads and NGU. Utilising a quantitative culture methodology researchers displayed significantly elevated *Ureaplasma* concentrations in first-void urines of males suffering from NGU.[472] Likewise, a further study assessing the intensity of the clinical symptoms associated with NGU alongside the number of polymorphonuclear leukocytes in urethral smears found a correlation between both these factors and *Ureaplasma* spp. load.[473] Though it must be highlighted that contravening evidence has been presented. For example, elevated ureaplasmal concentrations in first-void urines were observed for men both with and without urethritis, with no significant differences in polymorphonuclear leukocyte number.[474] Following the advent of molecular methods of detection that were able to speciate and quantify *Ureaplasma* spp., researchers indicated that *U. urealyticum* load and not *U. parvum* load contributed to NGU.[242,246] Several other researchers reached the same conclusion regarding the importance of *U. urealyticum* load in an effort to resolve the controversy surrounding the link between *Ureaplasma* load and

NGU.[286,475] However, more recently, reports displaying that *U. parvum* load is related to clinical outcomes in males suffering NGU have been presented.[283,476] Though further efforts to resolve this have been made in displaying the different species association with NGU; *U. parvum* has been linked to chronic NGU with *U. urealyticum* associated with acute form of the condition.[477] Additionally, as the majority of these studies only compare the *Ureaplasma* spp. loads of the participants within them, it has been noted that there is a need for a consensus on what constitutes elevated/higher titres of *Ureaplasma* spp. in male urine.[287] Providing a cut-off level would standardise the reporting of quantitative detection methods and aid in identifying what constitutes a pathogenic level of infection. Whilst the literature base from which to deduce a cut-off is currently too thin for a consensus opinion to be reached by STI Guideline Editorial Boards,[287] a *U. urealyticum* load of $>10^3$ CCU/mL of urine is repeatedly associated with symptomatic NGU in male patients.[246,475] In our study a total of 41 urine samples were culture-positive for *U. urealyticum*, of which 30% had a bacterial load $>10^3$ CCU/mL placing these patients at a higher risk for NGU. This results in a population prevalence of 3.63% (12/330) for males, with urine samples positive for elevated *U. urealyticum* loads. Though this only accounts for a low portion of males carrying a high *U. urealyticum* load, from the number of males recruited (330), the number of patients presenting with NGU should be between 10-30% (33-99 patients).[478] Additionally, as a cross-sectional review of the literature revealed *U. urealyticum* to be causal in between 5-26% of NGU cases,[291] this prevalence of high-load *U. urealyticum* is to be expected. However, access to the symptomatic status of patient data gathered as part of their routine sexual health screen, alongside the microbiological data garnered through routine testing, would help to further establish or repudiate the association between *U. urealyticum* load and NGU. Interestingly, the % of elevated *U. parvum*-positive male urine samples ($>10^3$ CCU/mL) was very similar to that of *U. urealyticum*-positive male urine samples; 28% of *U. parvum*-positives and 30% of *U. urealyticum* positives possessed bacterial loads $>10^3$ CCU/mL. Though notably, bacterial loads $>10^4$ CCU/mL were only seen in *U. urealyticum*-positive male urine

samples. Again, the degree to which *U. parvum* load is a contributary factor in NGU could further be eluded to following access to symptomatic and microbial status of the patients recruited.

Likewise, *Ureaplasma* spp. load has been similarly associated with female pathologies. For instance, Abele-Horn and colleagues showed that high ureaplasmal loads colonising the lower genital tract of pregnant women were associated with adverse pregnancy outcomes, whereas lower loads were not.[479] This notion was further substantiated, particularly in the case of *U. parvum*, as researchers discovered that intrauterine inflammatory response determined by IL-8 level was significantly associated with *U. parvum* load.[480] This *Ureaplasma* spp. infection-induced increase in IL-8 in amniotic fluid would lead to PROM and pre-term delivery.[481,482] More specifically related to sexual health pathologies *U. parvum* load has been shown to be associated with NSC.[235] These findings were corroborated by reports of *U. parvum* load directly affecting cervical lesion development.[483] It is noted that the likely explanation for this is that more pronounced inflammatory response to higher Ureaplasmal loads at the site of infection leads to increased cell and tissue damage, as is observed for other pathogenic bacteria. *In vitro* studies of both *U. parvum* and *U. urealyticum* have shown their capacity to induce the release of pro-inflammatory chemokines and MMP-9 in a dose-dependent manner, from both neonatal and adult monocytes.[484,485] However, there was no association observed between women with abnormal and normal vaginal flora and *Ureaplasma* spp. load.[486] Again, comparing this present study with others in relation to *Ureaplasma* spp. load is inherently difficult given the lack of homogeneity in both sample processing and cut-off values for determining elevated vs 'normal' *Ureaplasma* spp. loads. Abele-Horn and colleagues processed swab samples in a similar way to my study, inoculating them into a volume of sterile saline and calculating ureaplasmal concentrations in CFU/mL.[479] They established significant associations for both *Ureaplasma* spp. at titres of $>10^4$ CFU/mL with PROM and preterm delivery, with concentrations $>10^5$ being associated with chorioamnionitis. Comparatively, of 168 female patients swab sampled and positive for *Ureaplasma* spp. by culture, 77.4% (130/168) possessed *Ureaplasma* titres $>10^4$ CCU/mL. Which, by Abele-Horn *et* al.'s analysis is predicted to increase the risk of these women

suffering PROM or preterm delivery, should they be or fall pregnant. Furthermore, as listed previously 55.9% (94/168) females swab-positive for *Ureaplasma* spp. had loads >10^5 CCU/mL, resulting in an increased risk of chorioamnionitis. Therefore, a considerable portion of the female populous attending GUM clinics in the Valleys convergence area are predicted to be predisposed to pregnancy complications, related to elevated *Ureaplasma* spp. load. Moreover, the consequences of the previously listed pathologies can severely affect neonates, with PROM, pre-term delivery and chorioamnionitis all associated with increased neonatal mortality and morbidities, particularly bronchopulmonary dysplasia (BPD) in the case of *Ureaplasma*.[487–490] Furthermore, symptomatic status of patients and coupling them with their respective *Ureaplasma* spp. loads could help to further confirm or rebut the association between load and clinical pathologies. Equating the *Ureaplasma* spp. loads isolated from swab-positive females, as part of this study, to previous studies linking *Ureaplasma* load to cervicitis and cervical lesions is problematic due to the way in which the swab samples are processed.[483] However, *Ureaplasma* spp. load is continually associated with often life-threatening pathologies, such as risk-factors contributing to neonatal morbidities and cervical cancer.[491] Therefore, further work to unequivocally substantiate these associations and better understand the role of *Ureaplasma* spp. load in these infections has the potential to have a significant impact on patient health.

Furthermore, age alongside bacterial loads were investigated. Interestingly for female swab samples, patients aged 30 or under positive for *Ureaplasma* spp. possessed bacterial loads ≥10^5 CCU/mL in 60.98% of cases. Whereas female swab samples for patients aged 31 and over, positive for *Ureaplasma* spp. only had bacterial loads ≥10^5 CCU/mL in 39.02% of cases. Therefore, females aged 30 and under had significantly higher *Ureaplasma* spp. titres for swab samples. In line with the Abele-Horn *et al.* study, our study has revealed that females aged ≤30 would be predicted to be at a significantly greater risk of *Ureaplasma*-associated pathologies, due to their increased ureaplasmal loads.[479] Once more the reason for this is likely due to elevated oestrogen levels in younger females. For males, there was no substantial differences in bacterial loads between the age groups analysed.

Possibly indicating that the risk of *Ureaplasma*-induced load-associated NGU (>10^3 CCU/mL) is equal throughout male lifespan.[246,475] This would be the opposite of what is observed in *C. trachomatis*-associated NGU, which is more frequently found in younger men.[492,493]

M. hominis load and disease pathogenesis is a seemingly under studied area, with only a few available studies investigating the relationship. Cox *et al.* discovered *M. hominis* loads were significantly increased in females with confirmed BV.[73] Interestingly, in my study, no particular female age-group was significantly associated with elevated *M. hominis* load. Similarly, age-group has not been shown to be associated with BV.[494] Further analysis of the current dataset including patient symptom status would potentially further elucidate *M. hominis*'s role in BV, particularly sample load – currently there is no established BV-associated threshold for *M. hominis* load. More comprehensive, large-scale studies, such as the one undertaken here could possibly provide a route to establishing such a threshold. The Nugent score is used to clinically diagnose BV at present.[76] However, though a simple-to-perform method, its reliability is frequently called into question due to its lack of sensitivity and subjectivity.[495] Therefore establishing a BV-related *M. hominis* threshold could offer clinicians better accuracy in diagnosing the condition. A *M. hominis* load threshold would be more appropriate than a presence/absence detection method for the diagnosis of BV. This is because effective BV therapies are associated with *M. hominis* load reduction, rather than clearance.[86,92]

4.7 Antibiotic Resistance

Of 372 *Ureaplasma* spp. isolated, the 106 identified as potentially resistant were subjected to AST (32 against levofloxacin), with two *U. parvum* isolates being determined to be resistant to levofloxacin (0.54%). This rate of levofloxacin resistance is considerably lower than rates published in other studies. For example, a study in Minnesota that subjected 250 clinically isolated *Ureaplasma* spp. to MIC testing determined a levofloxacin resistance rate of 6.4% and 5.2% for *U. parvum* and *U. urealyticum*, respectively.[496] Further molecular characterisation of the isolates in the Fernandez *et al.* study revealed the majority (13/14) of *Ureaplasma* spp. to possess the *parC* quinolone-associated resistance mutation, Ser83Leu. With the remaining *U. parvum* isolate harbouring a Glu87Lys mutation, conferring levofloxacin resistance. Whole genome sequencing of the two levofloxacin-resistant isolates identified in my study showed that they both possessed the Ser83Leu mutation in the *parC* gene. This Ser83Leu *parC* gene mutation is the most prevalent levofloxacin resistance mechanism identified in *Ureaplasma* spp. isolates, accounting for up to 87% of fluoroquinolone resistance.[275,496–498] A further US study identified a levofloxacin resistance rate more in line with my study at 1.4%, identifying a single (1/73) resistant isolate following the screening of 73 *Ureaplasma* spp. isolates obtained from college-aged females.[278] However, the mutation conferring levofloxacin resistance in the instance of the Valentine-King and Brown study was a Ser83Trp substitution. Recently, in France, an assessment of levofloxacin resistance in *Ureaplasma* spp. over a 6-year period revealed a similarly low prevalence rate of 1.2%.[499] Yet in China, levofloxacin resistance was identified in 75% of isolates through CLSI compliant testing.[279] Once more, Song *et al.* reported that the Ser83Leu *parC* mutation was responsible for the majority of *Ureaplasma* spp. levofloxacin resistance. Researchers attributed the extremely high levofloxacin resistance rates observed in China to the widespread and general use of fluoroquinolones. Interestingly, a study carried out on *Ureaplasma* spp. samples collected throughout England and Wales reported 0 levofloxacin resistant isolates.[500] The data generated by Beeton *et al.*, coupled with the rates of levofloxacin resistance

presented here, indicate extremely low levels of levofloxacin resistance for *Ureaplasma* spp. in Wales. Such resistance rates are very comforting when compared with those published elsewhere, indicating quinolones – levofloxacin and moxifloxacin in particular – remain effective and viable therapeutic options in the treatment of *Ureaplasma* spp. infections in Wales and the UK. This is echoed by a Japanese study, that assessed the clinical efficacy of 500mg levofloxacin daily for 7 days in the treatment of men symptomatic for NGU, reporting the complete clearance of *U. urealyticum*.[501] Furthermore, as the Ser83Leu substitution in the *parC* is mutation driven, and not acquired through horizontal transfer, the risk of it spreading between *Ureaplasma* spp. populations is limited.[500]

Additionally, two tetracycline resistant *U. parvum* isolates were also identified, resulting in a tetracycline resistance rate of 0.54% – particularly low. Molecular characterisation of the isolates revealed both harboured the Tn916 *tet(M)* mobile genetic element, the only known mechanism of tetracycline resistance discovered in *Ureaplasma* spp.[270] Tetracycline resistance rates for *Ureaplasma* spp. typically range between 0-14% globally: 0%; Croatia[324], 1%; China[308] 2%; Italy[502], 6%; Hungary[298], 14%; Turkey[503]. Though tetracycline resistance rates as high as 34% have been published following CLSI-compliant AST of *Ureaplasma* spp. isolates in the US.[497] However more recently, Valentine-King and Brown published a 1.4% tetracycline resistance rate for *Ureaplasma* spp. in Florida, USA.[278] Comparatively, screening for tetracycline resistance in *Ureaplasma* spp. populations in Wales and England has consistently (on two occasions) revealed tetracycline resistance rates of 2%.[328,500] Therefore, the findings of this study show that *tet(*M*)* prevalence in *Ureaplasma* spp. populations throughout Wales have not appreciably increased, and may have in fact decreased. Similarly, analysis of *Ureaplasma* spp. isolates in France over a 6-year period (2010-2015) did not note any increase in *tet(M)* prevalence, fluctuating at around 7.5%.[499] Nevertheless, previous data from France, between 1999 and 2002, displayed *Ureaplasma* spp. tetracycline resistance rates to be at 2.2%.[168] Therefore, whilst no significant change was seen in the most recent 6-year assessment there is a considerable rise in tetracycline resistance over the last 20 years, for

Ureaplasma spp. in France. Thus, highlighting the need for continued antibiotic resistance surveillance for *Ureaplasma* spp. to track such changes, should they occur in Wales and England. Especially as tetracycline is often prescribed as a first-line therapy in the treatment of adult urogenital *Ureaplasma* spp. infection. All *Ureaplasma* spp. screened were susceptible to erythromycin and moxifloxacin.

For *M. hominis*, a single tetracycline resistant strain was identified giving a resistance rate of 1%. This is a relatively low tetracycline resistance rate, however, rates cited elsewhere range from 0-100%, for *M. hominis*.[297,303,304] Though typically resistance rates are situated between 10-40% of *M. hominis* globally.[134,504] The single isolate determined to be tetracycline-resistant in this incidence harboured the Tn916 *tet(M)* gene. Like *Ureaplasma* spp., this is the most commonly described resistance mechanism for tetracycline resistance in *M. hominis*. Whilst the rate of tetracycline resistance for South Wales *M. hominis* is low, examples of rates between 0% and 5% are not uncommon.[309,310]

All *Ureaplasma* spp. screened were sensitive to erythromycin, moxifloxacin and josamycin. With all *M. hominis* isolates screened being sensitive to clindamycin, levofloxacin, moxifloxacin and josamycin. The geographic variability in both *Ureaplasma* spp. and *M. hominis* resistance rates (and resistance rates to other antimicrobials) is thought to be the consequence of varying degrees of antimicrobial exposure in different patient populations.[134,147] This highlights the need to continually survey resistance rates locally to better guide empirical treatment for these infections.[296] It is this need that commercial rapid diagnostics – such as the MYCO WELL D-ONE – have the potential to fill. As discussed previously in this thesis, commercial kits are often criticised for the over-reporting of antimicrobial resistance data.[470] The reasons for over-reporting are often due to the following: a failure to corroborate the result of a commercial kit with CLSI compliant testing;[323] a failure to separate mixed *Ureaplasma* spp. and *M. hominis* muddying macrolide resistance data;[137] a non-CLSI-compliant bacterial inoculum;[328] using breakpoints to determine resistance that differ from CLSI published concentrations.[421] Following up resistance identified by commercial kits using CLSI

compliant AST has revealed the significant over-reporting for a number of assays and antibiotics. For example, Beeton *et al.* showed the IST-2 over-reported ciprofloxacin resistance rates for *Ureaplasma* spp. isolates, when compared with the broth microdilution method.[500] Likewise, Meygret *et al.* displayed over-reporting of *Ureaplasma* spp. levofloxacin resistance for the MYCOFAST Revolution assay, against broth microdilution.[499] Analysis of the MYCO WELL D-ONE identified that the assay significantly overestimates levofloxacin and moxifloxacin resistance in *Ureaplasma* spp., clindamycin resistance for *M. hominis* was also significantly over-reported. Thus, highlighting the need to confirm such identified resistance with CLSI compliant methodologies if using the MYCO WELL D-ONE for *Ureaplasma* spp. and *M. hominis* AST.

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CHAPTER 5
CONCLUSION

5. Conclusion

5.1 Concluding Remarks

This study aimed to clinically validate a new rapid diagnostic – MYCO WELL D-ONE – for the detection of *Ureaplasma* spp. and *M. hominis*, against PHE's current qPCR detection method (gold standard), for patient samples collected during routine screenings at walk-in sexual health clinics throughout the Valley's convergence area. The assay proved to be a sensitive and specific means of detecting these bacteria – particularly *Ureaplasma* spp. – with sensitivity and specificity values comparable to clinically implemented qPCR methodologies for the detection of *C. trachomatis* (Abbott).[340] Though sensitivity was notably lower for the detection of *M. hominis* at 78.23%, when compared the assays sensitivity in detecting *Ureaplasma* spp. at 91.98%. The drop in sensitivity for *M. hominis* detection reiterated the results of similar studies comparing commercial rapid culture-based mycoplasma detection kits with molecular methods.[334] This was attributed to the harder-to-recover nature of *M. hominis* when compared with *Ureaplasma* spp., using culture-based methods.[339] The average mean time to a positive results was 27.57 and 34.09 hours for the detection of *Ureaplasma* spp. in female and male patients, respectively. Whilst the respective mean detection time for *M. hominis* female and male patient samples was 43.26 and 46.54 hours. Overall, for both *Ureaplasma* spp. and *M. hominis* the PPV and NPV was >90%. However, the accuracy of the semi-quantitative well for the estimation of *Ureaplasma* load was consistently inaccurate, frequently overestimating the titre of a sample inoculated suspension. The over-reporting of antibiotic resistance for *Ureaplasma* spp. and *M. hominis*, due to the use of commercial diagnostic kits has frequently been raised.[470,499,500] CLSI compliant screening of isolates identified as resistant by the MYCO WELL D-ONE determined the assay significantly overestimated levofloxacin and moxifloxacin resistance for *Ureaplasma* spp., alongside over-reporting clindamycin resistance for *M. hominis*. Currently the MYCO WELL D-ONE is identified as the only commercially available assay that employs CLSI recommended breakpoints,[470] however, to ensure compliant testing a bacterial load of $\leq 10^5$

CCU/mL is required. In relation to this, it was noted that different sample types – swab or urine – possessed significantly different bacterial loads, across both *Ureaplasma* spp. and *M. hominis*. The majority (94.1%) of *Ureaplasma*-positive urine samples possessed CLSI compliant titres of $\leq 10^5$ CCU/mL, whereas the majority *Ureaplasma*-positive of swab samples (55.9%) harboured loads $>10^5$ CCU/mL, CLSI non-compliant. Whilst the difference in bacterial titres between sample types for *M. hominis* was less pronounced, a large portion of *M. hominis*-positive swab samples (40%) possessed CLSI non-compliant titres. Additional confirmation for the disparity in titres for *Ureaplasma*-positive samples between swabs and urine was determined following the analysis of paired samples. It has been previously demonstrated that *Ureaplasma* spp. titres greater than that recommended by the CLSI produces false-positive resistance results.[328] Therefore, the results of this study indicate that swab samples and urine samples require different dilution methodologies to ensure effective detection and simultaneous CLSI-compliant screening on the MYCOWELL D-ONE assay. Currently the same initial dilution step (10mL of sterile saline) is applied to both swab and urine samples, it is recommended that swab samples receive a greater initial dilution to accommodate the increased *Mycoplasma*/*Ureaplasma* burden they possess.

Employing PHE's multiplex qPCR for the detection of genital mycoplasmas prevalence data for both *Ureaplasma* spp. and *M. hominis* were generated. An overall prevalence rate of 43.46% for *Ureaplasma* spp. was determined with females having a significantly higher prevalence rate than males at 57.2% and 21.6%, respectively. It is well established that the incidence of *Ureaplasma* spp. infection is notably higher in the female populous, with the rates presented here corroborating that of numerous other reports citing female *Ureaplasma* prevalence at between 40 and 80%.[73,134,287,292,308,331,419] Similarly, *M. hominis* was more prevalent in females than males, with respective prevalence rates of 16.3% and 4.3%, again, largely in agreement with previously published prevalence data.[73,284,285,308] The greater prevalence rates found in females for both genital mycoplasmas is due, at least in part, to oestrogens effect in enhancing *Ureaplasma* spp. infection.[129,130,347] To further elucidate the mechanisms by which oestrogen enhances the

colonisation of genital mycoplasmas in the female urogenital tract extensive research is required. Such research has been carried out for other established sexual health pathogens such as *C. trachomatis*.[357,505] Similar studies analysing the effects of oestrogen on the colonisation of *Ureaplasma* spp. and *M. hominis* on endometrial cell lines *in vitro*, alongside the effect oestrogen has on the transcriptome of both organisms. A more comprehensive understanding of the effect of sex hormones on genital mycoplasma infections may aid in the development of more effective therapies in treating these infections. Furthermore, most *M. hominis*-positive patients were co-infected with *Ureaplasma* spp., however, *M. hominis* positive females had significantly higher co-infection rates than males. Overall, *U. parvum* was the dominant *Ureaplasma* spp. infecting females with a 40.5% prevalence rate, followed by 10.8% *U. urealyticum* and 5.89% mixed *U. parvum* and *U. urealyticum*. This is in line with the species-specific *Ureaplasma* female prevalence rates listed in the literature.[292,377,392,393] Therefore overall *Ureaplasma* spp. prevalence, and species specific prevalence, for females attending sexual health clinics in the Valley's convergence area are consistent with those published elsewhere. For males, *U. urealyticum* was the dominant *Ureaplasma* spp. infection for males with a prevalence rate of 14.3%, followed by *U. parvum* at 6.9% and 0.3% mixed *U. parvum* and *U. urealyticum* infection. This is at the higher end of *U. urealyticum* infection rates for males previously described, typically ranging from 5-15%.[283,286,289] Most earlier studies did not differentiate between *U. urealyticum* and *U. parvum*, which still continues if culture-based detection methods are used alone.[290,291] Therefore, the prevalence of *U. parvum* in male patients is not known, yet, several researchers have suggested that as *U. parvum* is detected more often in controls than cases in numerous studies it is more prevalent than *U. urealyticum*.[287] The results of this study disagree with such a proposal with *U. parvum* prevalence in males being significantly lower than that of *U. urealyticum*, for male patients. Furthermore, comparison between male and female patient groups revealed *U. urealyticum* prevalence to be consistent between sexes at 14.6% and 16.7% (p=0.0865; Fisher's exact test). Whereas there was a significant difference in *U. parvum* colonisation rates (inclusive mixed *Ureaplasma* infections) at 46.39% for females and 7.2% for males, indicating that *U.*

parvum infection only and not *U. urealyticum*, is sex-dependent (p=< 0.0001). Following an extensive review of the literature, to the best of my knowledge, this is the first time this observation has been made. Additionally, it was noted the age had a significant effect on the colonisation of *Ureaplasma* spp. for both male and female patients, with age having a significant impact on *M. hominis* prevalence in female patients. Younger persons were at a greater risk of *Ureaplasma* spp. infection, with younger females possessing greater *M. hominis* prevalence rates. These age-associated risks had been previously identified and the results presented here corroborated these reports[24,304,373], showing age plays a significant role in *Ureaplasma* spp. infection for males and females through the South Wales Valley's. Along with increased *M. hominis* prevalence being associated with younger age, for the same population. Moreover, resistance rates determined through CLSI-compliant testing for both *Ureaplasma* spp. and *M. hominis* displayed that no significant increases in antimicrobial resistance have occurred in the last 10 years in Wales.[500] Indicating that the currently prescribed antibiotic therapies for these infections remain largely effective and appropriate. However, the large geographic variability in antibiotic resistance for these organisms highlights the need for routine surveillance of these populations, locally, to continually monitor resistance rates. This is imperative, given the already limited therapeutic options that are effective against *Mollicutes*[7,10] – given their unique and limited physiology – particularly for the treatment of neonates. Although the results of this study also emphasised the need to confirm resistance identified through commercial rapid diagnostic culture-based assays, given their tendency to over-report antibiotic resistance in these organisms.[328,470,499]

Much controversy remains as to whether *Ureaplasma* spp. and *M. hominis* are 'true' urogenital pathogens or sexual transmitted infections, currently the European Guidelines STI Editorial Board does not recommend routine screening.[287] However, their associations with, and role within, the most common symptomatic clinical pathologies presented by patients at GUM clinics – NGU and BV – are well-established.[73,92,244,280,284,286,290,413,475,477,506] Additionally, they pose a significant risk to pregnant females, unequivocally contributing to pre-term birth, chorioamnionitis, PPROM and

neonatal morbidities such as BPD.[186,203,209,284,464,480,487,507] Given their relatively high prevalence rates – particularly in younger people – to underplay their clinical importance due to a lack of understanding as to how these organisms cause disease, would be extremely detrimental. The MYCO WELL D-ONE offers a sensitive, specific, easy-to-use, robust, economical and timely means of identifying these infections in the low-resource environment of sexual health clinics. Thus, in the absence of a classically recognised STI, the MYCO WELL D-ONE affords clinicians a higher-resolution picture of the underlying microbiological agents contributing to the aetiology of symptomatic patient pathologies. Thereby facilitating guided and informed prescription of empirical treatments, potentially improving patient outcomes.

5.2 Future Work

At the time of writing an ethics application has been submitted and accepted, under IRAS 253889 *"Do genitourinary Mycoplasma species cause disease''*. Work is currently underway, compiling and correlating the copious amounts of clinical patient information, recorded as part of the routine sexual health screening, with the results of patients screened for *Ureaplasma* spp. and *M. hominis*. This will permit the analysis of the following against *Ureaplasma* spp. and *M. hominis* infection: symptom status (asymptomatic/symptomatic), symptoms present (warts, discharge etc.), sexuality, type of intercourse (vaginal, oral etc.), treatment status, number of sexual partners, parity (total pregnancies), previous or current STI, patient medical history and contraception status. Moreover, all patient samples have been screened for *M. genitalium* using the SpeeDx ResistancePlus MG, to compare rates with the data presented in this study. Such analysis will give an insight into, and enhance the current understanding of, the role *Ureaplasma* spp. and *M. hominis* play in sexual health pathologies alongside determining factors that increase or decrease individual's susceptibility to these infections. For example, in relation to *Ureaplasma* spp. role in male NGU and patient symptomatic status the literature is marred by inconsistent reporting, with earlier studies failing to effectively speciate infections and/or failed to determine the bacterial load.[287,290,291] Such failings have made establishing concrete associations difficult, however, more recent studies utilising molecular methods have begun to establish and solidify the links between *Ureaplasma* spp. – particularly *U. urealyticum* – and NGU, with bacterial load determined to be a contributory factor.[246,475] The data generated and presented here, coupled with the listed patient data, will help to further solidify or rebut the association between *Ureaplasma* spp. infection, load, and urethritis. Moreover, it will provide an insight into any other associations between *Ureaplasma* spp. infection and any additional syndromic clinical manifestations such as testicular pain, lower abdominal pain, dysuria etc., as has been performed for other STIs such as *C. trachomatis*.[508] There are several

further research questions that analysis of the newly-formed dataset will permit. Oestrogen has been shown to modulate and enhance genital mycoplasma infection[129,130,347], therefore do oestrogen-containing contraceptives effect genital mycoplasma infection rates? If so, are there any significant differences observed between different types of contraceptive (i.e. implant vs. pill)? Additionally, it has previously been established that *T. vaginalis* infected with *M. hominis* has increased virulence *in vitro*.[457] There are currently >1000 patient collected DNA extractions containing sufficient volumes of DNA for further molecular analysis stored at -80^{o}C at the Heath hospital laboratory. Several clinically-validated qPCR methods for the detection of *T. vaginalis* exist.[509,510] Applying these or other appropriate qPCR methodologies for the detection of *T. vaginalis* to the collection of cryopreserved samples would further described the relationship between *M. hominis* and *T. vaginalis* in determining the prevalence of concomitant infections. It would additionally permit analysis into whether the increased virulence observed in *M. hominis*-*T. vaginalis* co-cultures *in vitro* translates into increased or more severe symptomatic reporting by patients co-infected with these organisms. Similarly, in patients suffering from BV *M. hominis* isolation is associated with *G. vaginalis*, with researchers suggesting a synergistic relationship between the two.[73,506] Again, applying qPCR methods, such as those outlined in Cox *et al.* for the detection of *G. vaginalis* to the collection of held cryopreserved samples would aid in further understanding the relationship between *M. hominis* and *G. vaginalis* co-infection and clinical outcomes. Furthermore, a clinically relevant load for *M. hominis* has yet to be established, *M. hominis* load analysis against clinical symptoms may reveal such a threshold.

Broadening the scope of future work, due to the high number of patients recruited throughout this study a huge number of clinical *Ureaplasma* spp. and *M. hominis* isolates have been sub-cultured and cryopreserved. Organisms contained within this large library of clinical isolates can be recovered and further characterised in the future, following the findings reported from the analysis of patient symptom status. Whilst virulence-associated genes have been identified for both *Ureaplasma* spp. and *M. hominis* such as *mba* and *vaa* respectively, their role in clinical outcomes has yet to be

established leading several researchers suggesting the presence of yet undetected pathogenicity factors – genes, mutations, transposons, integrated phages etc. Due to the now relatively low cost of sequencing nowadays, sequencing large numbers of clinical isolates is now feasible. The correlation of patient symptom status with *Ureaplasma* spp. or *M. hominis* infection will highlight potentially virulent isolates. Comparative genome analysis, such as that performed by Momynaliev *et al.* and Paralanov *et al.*, will facilitate the identification of genetic elements responsible for the potential virulence factors that are conserved amongst isolates associated with symptomatic infection.[226,227] Furthermore, it has been suggested that the assortment of MBA variable domains and the capacity of *Ureaplasma* spp. to vary their sizes and switch between domains determines the organisms ability to activate TLRs, and cause inflammation.[400] It has been shown that one such unique variable domain can be transferred between *Ureaplasma* spp. by horizontal gene transfer, indicating that the *mba* gene can lose and acquire variable domains.[399] Sequencing, alignment and comparative analysis of a large library of clinical isolates – those generated in this study – will potentially facilitate the identification of unique variable *mba* domains associated with virulence, alongside the genes involved in the horizontal transfer of such elements between organisms.

The genital mycoplasmas – *Ureaplasma* spp. and *M. hominis* – are unique, prevalent and a currently understudied group of organisms. Further large-scale molecular characterisation of these organisms will inevitably precipitate an increased understanding of the unique genetic mechanisms they utilise to colonise and infect a significant portion of the population. An increased understanding of such mechanisms may result in the development of unique and novel therapeutic options effective in tackling these inherently antibiotic resistant organisms.

References

1. Taylor-Robinson, D. Infections due to species of Mycoplasma and Ureaplasma: An update. *Clin. Infect. Dis.* **23,** 671–684 (1996).
2. Taylor-Robinson, D. & Waites, K. B. Mycoplasma and Ureaplasma. in *Manual of Clinical Microbiology, 11th Edition* 1088–1105 (American Society of Microbiology, 2015). doi:10.1128/9781555817381.ch62
3. Bhimji, S. S. & Unakal, C. G. *Bacteria, Gram Positive*. *StatPearls* (StatPearls Publishing, 2017).
4. Razin, S., Yogev, D. & Naot, Y. Molecular biology and pathogenicity of mycoplasmas. *Microbiol. Mol. Biol. Rev.* (1998). doi:1092-2172/98
5. Wolf, M., Müller, T., Dandekar, T. & Pollack, J. D. Phylogeny of Firmicutes with special reference to Mycoplasma (Mollicutes) as inferred from phosphoglycerate kinase amino acid sequence data. *Int. J. Syst. Evol. Microbiol.* **54,** 871–875 (2004).
6. Rottem, S. Interaction of Mycoplasmas With Host Cells. *Physiol. Rev.* **83,** 417–432 (2003).
7. McCormack, W. M. Susceptibility of mycoplasmas to antimicrobial agents: clinical implications. *Clin. Infect. Dis.* **17 Suppl 1,** S200-1 (1993).
8. Miles, R. J. Catabolism in mollicutes. *J. Gen. Microbiol.* **138,** 1773–1783 (1992).
9. Jeske, O. *et al.* Planctomycetes do possess a peptidoglycan cell wall. *Nat. Commun.* **6,** 7116 (2015).
10. Bermingham, A. & Derrick, J. P. The folic acid biosynthesis pathway in bacteria: Evaluation of potential for antibacterial drug discovery. *BioEssays* **24,** 637–648 (2002).
11. Carnrot, C., Wehelie, R., Eriksson, S., Bölske, G. & Wang, L. Molecular characterization of thymidine kinase from Ureaplasma urealyticum: Nucleoside analogues as potent inhibitors of mycoplasma growth. *Mol. Microbiol.* **50,** 771–780 (2003).
12. Grosjean, H. *et al.* Predicting the Minimal Translation Apparatus: Lessons from the Reductive Evolution of Mollicutes. *PLoS Genet.* **10,** (2014).
13. Inamine, J. M., Ho, K. C., Loechel, S. & Hu, P. C. Evidence that UGA is read as a tryptophan codon rather than as a stop codon by Mycoplasma pneumoniae, Mycoplasma genitalium, and Mycoplasma gallisepticum. *J. Bacteriol.* **172,** 504–506 (1990).
14. Smiley, B. K. & Minion, F. C. Enhanced readthrough of opal (UGA) stop codons and production of Mycoplasma pneumoniae P1 epitopes in Escherichia coli. *Gene* **134,** 33–40 (1993).
15. Waites, K. B. & Talkington, D. F. Mycoplasma pneumoniae and its role as a human pathogen. *Clin. Microbiol. Rev.* **17,** 697–728, table of contents (2004).
16. Walker Vergara, R. *et al.* Prevalence, risk factor analysis, and hematological findings of hemoplasma infection in domestic cats from Valdivia, Southern Chile. *Comp. Immunol. Microbiol. Infect. Dis.* **46,** 20–26 (2016).
17. Anabestani, A. *et al.* Identification of putative effector genes and their transcripts in three strains related to 'Candidatus Phytoplasma aurantifolia'. *Microbiol. Res.* **199,** 57–66 (2017).
18. Rosales, R. S., Puleio, R., Loria, G. R., Catania, S. & Nicholas, R. A. J. Mycoplasmas: Brain invaders? *Research in Veterinary Science* **113,** 56–61 (2017).

19. Fisunov, G. Y., Evsyutina, D. V, Manuvera, V. A. & Govorun, V. M. Binding site of restriction-modification system controller protein in Mollicutes. *BMC Microbiology* **17,** 1–7 (2017).

20. Fisunov, G. Y. *et al.* Binding site of MraZ transcription factor in Mollicutes. *Biochimie* **125,** 59–65 (2016).

21. Lluch-Senar, M. *et al.* Defining a minimal cell: essentiality of small ORFs and ncRNAs in a genome-reduced bacterium. *Mol. Syst. Biol.* **11,** 780–780 (2015).

22. Giono-cerezo, S., Estrada-gutiérrez, G., Rivera-tapia, J. A., Yáñez-santos, J. A. & Díaz-garcía, F. J. Current Status of the Mollicute (Mycoplasma) Lung Disease : Pathogenesis , Diagnostics , Treatment and Prevention. *Lung Dis. – Sel. State Art Rev.* **Chapter 15,** (2012).

23. Dienes, L. & Edsall, G. Observations on the L-Organism of Klieneberger. *Exp. Biol. Med.* **36,** 740–744 (1937).

24. Govender, S., Theron, G. B., Odendaal, H. J. & Chalkley, L. J. Prevalence of genital mycoplasmas, ureaplasmas and chlamydia in pregnancy. *J. Obstet. Gynaecol. (Lahore).* **29,** 698–701 (2009).

25. Meyer, R. D. & Clough, W. Extragenital Mycoplasma hominis Infections in Adults: Emphasis on Immunosuppression. *Clin. Infect. Dis.* **17,** S243–S249 (1993).

26. Thorsen, P. *et al.* Few microorganisms associated with bacterial vaginosis may constitute the pathologic core: A population-based microbiologic study among 3596 pregnant women. *Am. J. Obstet. Gynecol.* **178,** 580–587 (1998).

27. Wong, S. S. Y. & Yuen, K. Y. Acute pyelonephritis caused by mycoplasma hominis. *Pathology* **27,** 61–63 (1995).

28. Ness, R. B. *et al.* A cluster analysis of bacterial vaginosis-associated microflora and pelvic inflammatory disease. *Am. J. Epidemiol.* **162,** 585–590 (2005).

29. Díaz, L. *et al.* Frequency and antimicrobial sensitivity of ureaplasma urealyticum and mycoplasma hominis in patients with vaginal discharge. *MEDICC Rev.* **15,** 45–47 (2013).

30. Pingmin, W., Yuepu, P. & Jiwen, Z. Prevalence survey on condom use and infection of urogenital mycoplasmas in female sex workers in China. *Contraception* **72,** 217–220 (2005).

31. Mufson, M. A. Mycoplasma hominis: a review of its role as a respiratory tract pathogen of humans. *Sex. Transm. Dis.* **10,** 335–340 (1983).

32. Madoff, S. & Hooper, D. C. Nongenitourinary infections caused by mycoplasma hominis in adults. *Rev. Infect. Dis.* **10,** 602–611 (1988).

33. McMahon, D. K., Dummer, J. S., Pasculle, A. W. & Cassell, G. Extragenital Mycoplasma hominis infections in adults. *Am. J. Med.* **89,** 275–281 (1990).

34. Mattila, P. S. *et al.* Life-threatening Mycoplasma hominis mediastinitis. *Clin. Infect. Dis.* **29,** 1529–37 (1999).

35. Correspondence, F. P. & Parsonson, F. Mycoplasma hominis infection following neurosurgical intervention in a patient with spinal cord compression. doi:10.1099/jmmcr.0.005023

36. Razin, S. The mycoplasmas. *Microbiol. Rev.* **42,** 414–470 (1978).

37. Pereyre, S. *et al.* Life on arginine for Mycoplasma hominis: Clues from its minimal genome and comparison with other human urogenital mycoplasmas. *PLoS Genet.* **5,** (2009).

38. Maniloff, J. & Morowitz, H. J. Cell biology of the mycoplasmas. *Bacteriol. Rev.* **36,** 263–290 (1972).

39. Meloni, G. A., Bertoloni, G., Busolo, F. & Conventi, L. Colony Morphology, Ultrastructure and Morphogenesis in Mycoplasma hominis, Acholeplasma laidlawii and Ureaplasma urealyticum. *Microbiology* **116,** 435–443 (1980).

40. Razin, S. *Mycoplasmas*. *Medical Microbiology* (University of Texas Medical Branch at Galveston, 1996).

41. Krause, D. C. & Balish, M. F. Structure, function, and assembly of the terminal organelle of Mycoplasma pneumoniae. *FEMS Microbiology Letters* **198,** 1–7 (2001).

42. Henrich, B., Feldmann, R. C. & Hadding, U. Cytoadhesins of Mycoplasma hominis. *Infect. Immun.* **61,** 2945–2951 (1993).

43. Olson, L. D. *et al.* Monoclonal antibodies to surface antigens of a pathogenic Mycoplasma hominis strain. *Infect. Immun.* **59,** 1683–1689 (1991).

44. Kitzerow, A., Hadding, U. & Henrich, B. Cyto-adherence studies of the adhesin P50 of Mycoplasma hominis. *J. Med. Microbiol.* **48,** 485–493 (1999).

45. Hopfe, M., Hoffmann, R. & Henrich, B. P80, the HinT interacting membrane protein, is a secreted antigen of Mycoplasma hominis. *BMC Microbiol.* **4,** (2004).

46. Henrich, B., Kretzmer, F., Deenen, R. & Köhrer, K. Validation of a novel Mho microarray for a comprehensive characterisation of the Mycoplasma hominis action in HeLa cell infection. *PLoS One* **12,** e0181383 (2017).

47. Tola, S. *et al.* Sequence, cloning, expression and characterisation of the 81-kDa surface membrane protein (P80) of Mycoplasma agalactiae. *FEMS Microbiol. Lett.* **202,** 45–50 (2001).

48. Saadat, S., Sajadi, M. M., Alikhani, M. Y., Rikhtegaran Tehrani, Z. & Yousefi Mashouf, R. Production of a chimeric protein and its potential application in sero-diagnosis of Mycoplasma hominis infection. *J. Microbiol. Methods* **144,** 186–191 (2018).

49. Parker, C. T. & Sperandio, V. Cell-to-cell signalling during pathogenesis. *Cellular Microbiology* **11,** 363–369 (2009).

50. Moxon, R., Bayliss, C. & Hood, D. Bacterial Contingency Loci: The Role of Simple Sequence DNA Repeats in Bacterial Adaptation. *Annu. Rev. Genet.* **40,** 307–333 (2006).

51. Wise, K. S. Adaptive surface variation in mycoplasmas. *Trends in Microbiology* **1,** 59–63 (1993).

52. Woude, M. W. Van Der & Bäumler, A. J. Phase and Antigenic Variation in Bacteria Phase and Antigenic Variation in Bacteria. *Clin. Microbiol. Rev.* **17,** 581–611 (2004).

53. Olson, L. D., Renshaw, C. A., Shane, S. W. & Barile, M. F. Successive synovial Mycoplasma hominis isolates exhibit apparent antigenic variation. *Infect. Immun.* **59,** 3327–3329 (1991).

54. Olson, L. D. *et al.* Monoclonal antibodies to surface antigens of a pathogenic Mycoplasma hominis strain. *Infect. Immun.* **59,** 1683–1689 (1991).

55. Levinson, G. & Gutman, G. A. Slipped-strand mispairing: a major mechanism for DNA sequence evolution. *Mol. Biol. Evol.* **4,** 203–221 (1987).

56. Zhang, Q. & Wise, K. S. Localized reversible frameshift mutation in an adhesin gene confers a phase-variable adherence phenotype in mycoplasma. *Mol. Microbiol.* **25,** 859–869 (1997).

57. Wise, K. I. M. S. Molecular basis of size and antigenic variation of a Mycoplasma hominis adhesin encoded by divergent vaa genes . These include : Molecular Basis of Size and Antigenic Variation of a Mycoplasma hominis Adhesin Encoded by Divergent vaa Genes. *Infect. Immun.* **64,** 2737–2744 (1996).

58. Feldmann, R. C., Henrich, B., Kolb-Bachofen, V. & Hadding, U. Decreased metabolism and viability of Mycoplasma hominis induced by monoclonal antibody-mediated agglutination. *Infect. Immun.* **60,** 166–174 (1992).

59. Brown, R. J., Chalker, V. J. & Spiller, O. B. *Mycoplasma hominis* Variable Adherence-Associated Antigen: A Major Adhesin and Highly Variable Surface Membrane Protein. *Adv. Microbiol.* **4,** 736–746 (2014).

60. Boesen, T. *et al.* The Mycoplasma hominis vaa gene displays a mosaic gene structure. *Mol. Microbiol.* **29,** 97–110 (1998).

61. Feavers, I. M., Heath, A. B., Bygraves, J. A. & Maiden, M. C. J. Role of horizontal genetic exchange in the antigenic variation of the class 1 outer membrane protein of Neisseria meningitidis. *Mol. Microbiol.* **6,** 489–495 (1992).

62. Kong, Y. *et al.* Homologous recombination drives both sequence diversity and gene content variation in Neisseria meningitidis. *Genome Biol. Evol.* **5,** 1611–1627 (2013).

63. Boesen, T., Fedosova, N. U., Kjeldgaard, M., Birkelund, S. & Christiansen, G. Molecular design of Mycoplasma hominis Vaa adhesin. doi:10.1101/ps.31901

64. Tashiro, M. *et al.* High-resolution solution NMR structure of the Z domain of staphlococcal protein A. *J. Mol. Biol.* **272,** 573–590 (1997).

65. Henrich, B., Hopfe, M., Kitzerow, A. & Hadding, U. The adherence-associated lipoprotein P100, encoded by an opp operon structure, functions as the oligopeptide-binding domain OppA of a putative oligopeptide transport system in Mycoplasma hominis. *J. Bacteriol.* **181,** 4873–4878 (1999).

66. Hopfe, M. & Henrich, B. OppA, the Substrate-Binding Subunit of the Oligopeptide Permease, Is the Major Ecto-ATPase of Mycoplasma hominis. *J. Bacteriol.* **186,** 1021–1028 (2004).

67. Redegeld, F., Filippini, A. & Sitkovsky, M. Comparative studies of the cytotoxic T lymphocyte-mediated cytotoxicity and of extracellular ATP-induced cell lysis. Different requirements in extracellular Mg2+ and pH. *J. Immunol.* **147,** 3638–45 (1991).

68. Plesner, L. Ecto-ATPases: identities and functions. *Int. Rev. Cytol.* **158,** 141–214 (1995).

69. Clifford, E. E., Martin, K. A., Dalal, P., Thomas, R. & Dubyak, G. R. Stage-specific expression of P2Y receptors, ecto-apyrase, and ecto-5'-nucleotidase in myeloid leukocytes. *Am. J. Physiol.* **273,** C973–C987 (1997).

70. Bisaggio, D. F. R., Peres-Sampaio, C. E., Meyer-Fernandes, J. R. & Souto-Padrón, T. Ecto-ATPase activity on the surface of Trypanosoma cruzi and its possible role in the parasite-host cell interaction. *Parasitol. Res.* **91,** 273–282 (2003).

71. Hopfe, M., Dahlmanns, T. & Henrich, B. In Mycoplasma hominis the OppA-mediated cytoadhesion depends on its ATPase activity. *BMC Microbiol.* **11,** 185 (2011).

72. Hopfe, M. & Henrich, B. OppA, the ecto-ATPase of Mycoplasma hominis induces ATP release and cell death in HeLa cells. *BMC Microbiol.* **8,** (2008).

73. Cox, C., Watt, A. P., McKenna, J. P. & Coyle, P. V. Mycoplasma hominis and Gardnerella

vaginalis display a significant synergistic relationship in bacterial vaginosis. *Eur. J. Clin. Microbiol. Infect. Dis.* **35,** 481–487 (2016).

74. Sobel, J. D. Vaginitis. *N. Engl. J. Med.* **337,** 1896–1903 (1997).

75. Onderdonk, A. B., Delaney, M. L. & Fichorova, R. N. The human microbiome during bacterial vaginosis. *Clin. Microbiol. Rev.* **29,** 223–238 (2016).

76. Nugent, R. P., Krohn, M. A. & Hillier, S. L. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *J. Clin. Microbiol.* **29,** 297–301 (1991).

77. Taha, T. E. *et al.* Bacterial vaginosis and disturbances of vaginal flora: Association with increased acquisition of HIV. *AIDS* **12,** 1699–1706 (1998).

78. Martin, H. L. *et al.* Vaginal Lactobacilli, Microbial Flora, and Risk of Human Immunodeficiency Virus Type 1 and Sexually Transmitted Disease Acquisition. *J. Infect. Dis.* **180,** 1863–1868 (1999).

79. Sha, B. E. *et al.* Female Genital-Tract HIV Load Correlates Inversely with Lactobacillus Species but Positively with Bacterial Vaginosis and Mycoplasma hominis. *J. Infect. Dis.* **191,** 25–32 (2005).

80. al-Harthi, L. *et al.* Bacterial vaginosis-associated microflora isolated from the female genital tract activates HIV-1 expression. *J. Acquir. Immune Defic. Syndr.* **21,** 194–202 (1999).

81. Crouse, D. T., English, B. K., Livingston, L. & Meals, E. A. Genital mycoplasmas stimulate tumor necrosis factor-α and inducible nitric oxide synthase production from a murine macrophage cell line. *Pediatr. Res.* **44,** 785–790 (1998).

82. Taylor-Robinson, D. & McCormack, W. M. The genital mycoplasmas (second of two parts). *N.Engl.J.Med.* **302,** 1063–1067 (1980).

83. Krohn, M. A., Hillier, S. L. & Eschenbach, D. A. Comparison of methods for diagnosing bacterial vaginosis among pregnant women. *J. Clin. Microbiol.* **27,** 1266–1271 (1989).

84. Martius, J. *et al.* Relationships of vaginal Lactobacillus species, cervical Chlamydia trachomatis, and bacterial vaginosis to preterm birth. *Obstet. Gynecol.* **71,** 89–95 (1988).

85. Holst, E., Wathne, B., Hovelius, B. & M??rdh, P. A. Bacterial vaginosis: Microbiological and clinical findings. *Eur. J. Clin. Microbiol.* **6,** 536–541 (1987).

86. Koutsky, L. A. *et al.* Persistence of Mycoplasma hominis after therapy: importance of tetracycline resistance and of coexisting vaginal flora. *Sex. Transm. Dis.* **10,** 374–381 (1983).

87. Spiegel, C. A. Bacterial vaginosis. *Clinical Microbiology Reviews* **4,** 485–502 (1991).

88. Taylor-Robinson, D. & Rosenstein, I. J. Is Mycoplasma hominis a vaginal pathogen? *Sex. Transm. Infect.* **77,** 302 (2001).

89. Sha, B. E. *et al.* Utility of amsel criteria, nugent score, and quantitative PCR for Gardnerella vaginalis, Mycoplasma hominis, and Lactobacillus spp. for diagnosis of bacterial vaginosis in human immunodeficiency virus-infected women. *J. Clin. Microbiol.* **43,** 4607–4612 (2005).

90. Zariffard, M. R. *et al.* Induction of Tumor Necrosis Factor–α Secretion and Toll-Like Receptor 2 and 4 mRNA Expression by Genital Mucosal Fluids from Women with Bacterial Vaginosis. *J. Infect. Dis.* **191,** 1913–1921 (2005).

91. Hasebe, A., Mu, H.-H. & Cole, B. C. A Potential Pathogenic Factor from *Mycoplasma hominis* is

a TLR2-Dependent, Macrophage-Activating, P50-Related Adhesin. *Am. J. Reprod. Immunol.* **72,** 285–295 (2014).

92. Austin, M. N., Beigi, R. H., Meyn, L. A. & Hillier, S. L. Microbiologic Response to Treatment of Bacterial Vaginosis with Topical Clindamycin or Metronidazole. *J. Clin. Microbiol.* **43,** 4492–4497 (2005).

93. Rappelli, P. *et al.* Mycoplasma hominis and Trichomonas vaginalis symbiosis: multiplicity of infection and transmissibility of M. hominis to human cells. *Arch. Microbiol.* **175,** 70–4 (2001).

94. Dessì, D. *et al.* Long-term survival and intracellular replication of Mycoplasma hominis in Trichomonas vaginalis cells: potential role of the protozoon in transmitting bacterial infection. *Infect. Immun.* **73,** 1180–6 (2005).

95. Yarlett, N., Martinez, M. P., Ali Moharrami, M. & Tachezy, J. The contribution of the arginine dihydrolase pathway to energy metabolism by Trichomonas vaginalis. *Mol. Biochem. Parasitol.* **78,** 117–125 (1996).

96. Morada, M. *et al.* Arginine metabolism in Trichomonas vaginalis infected with Mycoplasma hominis. *Microbiology* **156,** 3734–3743 (2010).

97. Dillon, B. J., Holtsberg, F. W., Ensor, C. M., Bomalaski, J. S. & Clark, M. A. Biochemical characterization of the arginine degrading enzymes arginase and arginine deiminase and their effect on nitric oxide production. *Med. Sci. Monit.* **8,** BR248-53 (2002).

98. Margarita, V. *et al.* Symbiotic association with Mycoplasma hominis can influence growth rate, ATP production, cytolysis and inflammatory response of Trichomonas vaginalis. *Front. Microbiol.* **7,** (2016).

99. Fiori, P. L., Diaz, N., Cocco, A. R., Rappelli, P. & Dessì, D. Association of Trichomonas vaginalis with its symbiont Mycoplasma hominis synergistically upregulates the in vitro proinflammatory response of human monocytes. *Sex. Transm. Infect.* **89,** 449–54 (2013).

100. Fürnkranz, U., Henrich, B. & Walochnik, J. Mycoplasma hominis impacts gene expression in Trichomonas vaginalis. *Parasitol. Res.* **117,** 841–847 (2018).

101. Soper, D. E. Pelvic Inflammatory Disease. *Obstet. Gynecol.* **116,** 419–428 (2010).

102. Brunham, R. C., Gottlieb, S. L. & Paavonen, J. Pelvic Inflammatory Disease. *N. Engl. J. Med.* **372,** 2039–2048 (2015).

103. Wiesenfeld, H. C. *et al.* Lower genital tract infection and endometritis: insight into subclinical pelvic inflammatory disease. *Obstet. Gynecol.* **100,** 456–63 (2002).

104. Weström, L., Joesoef, R., Reynolds, G., Hagdu, A. & Thompson, S. E. Pelvic inflammatory disease and fertility: A cohort study of 1,844 women with laparoscopically verified disease and 657 control women with normal laparoscopic results. *Sex. Transm. Dis.* **19,** 185–192 (1992).

105. Mårdh, P. A. & Weström, L. Tubal and cervical cultures in acute salpingitis with special reference to Mycoplasma hominis and T-strain mycoplasmas. *Br. J. Vener. Dis.* **46,** 179–86 (1970).

106. Moller, B. R. The role of mycoplasmas in the upper genital tract of women. *Sex. Transm. Dis.* **10,** 281–284 (1983).

107. Moller, B. R., Freundt, E. A., Black, F. T. & Frederiksen2, P. Experimental Infection of the Genital Tract of Female Grivet Monkeys by Mycoplasma hominis. *Infect. Immun.* **20,** 248–257

(1978).

108. Chatwani, A., Harmanli, O. H., Nyirjesy, P. & Albert Reece, E. Significance of Genital Mycoplasmas in Pelvic Inflammatory Disease: Innocent Bystander! *Infect. Dis. Obstet. Gynecol.* **4,** 263–268 (1996).

109. Taylor-Robinson, D. The role of mycoplasmas in pregnancy outcome. *Best Practice and Research: Clinical Obstetrics and Gynaecology* **21,** 425–438 (2007).

110. Jurstrand, M., Jensen, J. S., Magnuson, A., Kamwendo, F. & Fredlund, H. A serological study of the role of Mycoplasma genitalium in pelvic inflammatory disease and ectopic pregnancy. *Sex. Transm. Infect.* **83,** 319–323 (2007).

111. Hillier, S. L. *et al.* Association between Bacterial Vaginosis and Preterm Delivery of a Low-Birth-Weight Infant. *N. Engl. J. Med.* **333,** 1737–1742 (1995).

112. Taylor-Robinson, D. & Lamont, R. F. Mycoplasmas in pregnancy. *BJOG: An International Journal of Obstetrics and Gynaecology* **118,** 164–174 (2011).

113. Pararas, M. V., Skevaki, C. L. & Kafetzis, D. A. Preterm birth due to maternal infection: Causative pathogens and modes of prevention. *European Journal of Clinical Microbiology and Infectious Diseases* **25,** 562–569 (2006).

114. Hosny, A. E.-D. M. S., El-khayat, W., Kashef, M. T. & Fakhry, M. N. Association between preterm labor and genitourinary tract infections caused by Trichomonas vaginalis , Mycoplasma hominis , Gram-negative bacilli, and coryneforms. *J. Chinese Med. Assoc.* **80,** 575–581 (2017).

115. Almagor, M., Kahane, I., Gilon, C. & Yatziv, S. Protective effects of the glutathione redox cycle and vitamin E on cultured fibroblasts infected by Mycoplasma pneumoniae. *Infect. Immun.* **52,** 240–4 (1986).

116. Truchetet, M. E. *et al.* Potential role of Mycoplasma hominis in interleukin (IL)-17-producing CD4+ T-cell generation via induction of IL-23 secretion by human dendritic cells. *J Infect Dis* **204,** 1796–1805 (2011).

117. Fossiez, F. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J. Exp. Med.* **183,** 2593–2603 (1996).

118. Inoue, H. *et al.* Regulation by PGE2 of the production of interleukin-6, macrophage colony stimulating factor, and vascular endothelial growth factor in human synovial fibroblasts. *Br. J. Pharmacol.* **136,** 287–95 (2002).

119. Carbonne, B. *et al.* Synthesis of glycosaminoglycans by human cervical fibroblasts in culture: effects of prostaglandian E2 and cyclic AMP. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **70,** 101–105 (1996).

120. Novy, M. J. *et al.* Ureaplasma parvum or Mycoplasma hominis as sole pathogens cause chorioamnionitis, preterm delivery, and fetal pneumonia in rhesus macaques. *Reprod. Sci.* **16,** 56–70 (2009).

121. Kotani, M. *et al.* Multiple signal transduction pathways through two prostaglandin E receptor EP3 subtype isoforms expressed in human uterus. *J Clin Endocrinol Metab* **85,** 4315–4322 (2000).

122. Lappas, M. *et al.* MAPK and AP-1 proteins are increased in term pre-labour fetal membranes overlying the cervix: Regulation of enzymes involved in the degradation of fetal membranes. *Placenta* **32,** 1016–1025 (2011).

123. Mishima, K. *et al.* Overexpression of the ERK/MAP kinases in oral squamous cell carcinoma. *Mod. Pathol.* **11,** 886–91 (1998).

124. Loda, M. *et al.* Expression of mitogen-activated protein kinase phosphatase-1 in the early phases of human epithelial carcinogenesis. *Am. J. Pathol.* **149,** 1553–64 (1996).

125. Branca, M. *et al.* Activation of the ERK/MAP Kinase Pathway in Cervical Intraepithelial Neoplasia Is Related to Grade of the Lesion but not to High-Risk Human Papillomavirus, Virus Clearance, or Prognosis in Cervical Cancer. *Am. J. Clin. Pathol.* **122,** 902–911 (2004).

126. Lukic, A. *et al.* Determination of cervicovaginal microorganisms in women with abnormal cervical cytology: The role of Ureaplasma urealyticum. *Anticancer Res.* **26,** 4843–4849 (2006).

127. Il Ji, Y. Co-infections with human papillomavirus and mycoplasma/ureaplasma spp. in women with abnormal cervical cytology. *Res Rep Gynaecol Obs.* **1,** (2017).

128. Walboomers, J. M. M. *et al.* Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol.* **189,** 12–19 (1999).

129. Furr, P. M. & Taylor-Robinson, D. Oestradiol-induced infection of the genital tract of female mice by Mycoplasma hominis. *J. Gen. Microbiol.* **135,** 2743–2749 (1989).

130. Taylor-Robinson, D. & Furr, P. M. Elimination of mycoplasmas from the murine genital tract by hormone treatment. *Epidemiol. Infect.* **105,** 163–168 (1990).

131. Gdoura, R. *et al.* Ureaplasma urealyticum, Ureaplasma parvum, Mycoplasma hominis and Mycoplasma genitalium infections and semen quality of infertile men. *BMC Infect. Dis.* **7,** 129 (2007).

132. Huang, C. *et al.* Ureaplasma urealyticum and Mycoplasma hominis infections and semen quality in 19,098 infertile men in China. *World J. Urol.* **34,** 1039–1044 (2016).

133. Taylor-Robinson, D. Mycoplasmas: Respiratory and genital tract infections. in *Medical Microbiology: Eighteenth Edition* 400–410 (Elsevier, 2012). doi:10.1016/B978-0-7020-4089-4.00056-1

134. Waites, K. B., Katz, B. & Schelonka, R. L. Mycoplasmas and ureaplasmas as neonatal pathogens. *Clin. Microbiol. Rev.* **18,** 757–789 (2005).

135. Sánchez, A. R., Rogers, R. S. & Sheridan, P. J. Tetracycline and other tetracycline-derivative staining of the teeth and oral cavity. *International Journal of Dermatology* **43,** 709–715 (2004).

136. Patel, K. & Goldman, J. L. Safety Concerns Surrounding Quinolone Use in Children. *Journal of Clinical Pharmacology* **56,** 1060–1075 (2016).

137. Pereyre, S. *et al.* Mutations in 23S rRNA account for intrinsic resistance to macrolides in Mycoplasma hominis and Mycoplasma fermentans and for acquired resistance to macrolides in M. hominis. *Antimicrob. Agents Chemother.* **46,** 3142–3150 (2002).

138. Oates, J. A., Wood, A. J. J., Hooper, D. C. & Wolfson, J. S. Fluoroquinolone Antimicrobial Agents. *N. Engl. J. Med.* **324,** 384–394 (1991).

139. Blondeau, J. M. Expanded activity and utility of the new fluoroquinolones: A review. *Clinical Therapeutics* **21,** 3–40 (1999).

140. Wang, J. C. & Lynch, A. S. Transcription and DNA supercoiling. *Curr Opin Genet Dev* **3,** 764–8. (1993).

141. Blondeau, J. M. Fluoroquinolones: Mechanism of action, classification, and development of resistance. *Survey of Ophthalmology* **49,** S73–S78 (2004).

142. Pan, X. S. & Fisher, L. M. Targeting of DNA gyrase in Streptococcus pneumoniae by sparfloxacin: Selective targeting of gyrase or topoisomerase IV by quinolones. *Antimicrob. Agents Chemother.* **41,** 471–474 (1997).

143. Zechiedrich, E. L. & Cozzarelli, N. R. Roles of topoisomerase IV and DNA gyrase in DNA unlinking during replication in Escherichia coli. *Genes Dev.* **9,** 2859–2869 (1995).

144. Kato, J., Suzuki, H. & Ikeda, H. Purification and characterization of DNA topoisomerase IV in Escherichia coli. *J. Biol. Chem.* **267,** 25676–25684 (1992).

145. Ezelarab, H. A. A., Abbas, S. H., Hassan, H. A. & Abuo-Rahma, G. E.-D. A. Recent updates of fluoroquinolones as antibacterial agents. *Arch. Pharm. (Weinheim).* 1800141 (2018). doi:10.1002/ardp.201800141

146. Hooper, D. C. & Jacoby, G. A. Mechanisms of drug resistance: Quinolone resistance. *Ann. N. Y. Acad. Sci.* **1354,** 12–31 (2015).

147. Bebear, C. M. *et al.* DNA gyrase and topoisomerase IV mutations in clinical isolates of Ureaplasma spp. and Mycoplasma hominis resistant to fluoroquinolones. *Antimicrob. Agents Chemother.* **47,** 3323–3325 (2003).

148. Gruson, D. *et al.* In vitro development of resistance to six and four fluoroquinolones in Mycoplasma pneumoniae and Mycoplasma hominis, respectively. *Antimicrob. Agents Chemother.* **49,** 1190–1193 (2005).

149. Bebear, C. M. *et al.* Alterations in topoisomerase IV and DNA gyrase in quinolone-resistant mutants of Mycoplasma hominis obtained in vitro. *Antimicrob. Agents Chemother.* **42,** 2304–2311 (1998).

150. Spahn, C. M. T. & Prescott, C. D. Throwing a spanner in the works: Antibiotics and the translation apparatus. *Journal of Molecular Medicine* **74,** 423–439 (1996).

151. Nissen, P., Hansen, J., Ban, N., Moore, P. B. & Steitz, T. A. The structural basis of ribosome activity in peptide bond synthesis. *Science (80-.).* **289,** 920–930 (2000).

152. Moore, P. B. & Steitz, T. A. The involvement of RNA in ribosome function. *Nature* **418,** 229–235 (2002).

153. Porse, B. T. & Garrett, R. A. Sites of interaction of streptogramin A and B antibiotics in the peptidyl transferase loop of 23 S rRNA and the synergism of their inhibitory mechanisms. *J. Mol. Biol.* **286,** 375–387 (1999).

154. Rodriguez-Fonseca, C., Amils, R. & Garrett, R. A. Fine Structure of the Peptidyl Transferase Centre on 23 S-like rRNAs Deduced from Chemical Probing of Antibiotic-Ribosome Complexes. *J. Mol. Biol.* **247,** 224–235 (1995).

155. Chopra, I., Hawkey, P. M. & Hinton, M. Tetracyclines, molecular and clinical aspects. *Journal of Antimicrobial Chemotherapy* **29,** 245–277 (1992).

156. Pioletti, M. *et al.* Crystal structures of complexes of the small ribosomal subunit with tetracycline, edeine and IF3. *EMBO J.* **20,** 1829–1839 (2001).

157. Dibner, J. J. & Richards, J. D. Antibiotic Growth Promoters in Agriculture : History and Mode of Action Actual Usage of Antimicrobials in Denmark Voluntary and Legislated Bans. *Poult. Sci.* **84,** 634–643 (2005).

158. Chopra, I. & Roberts, M. Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. *Microbiol. Mol. Biol. Rev.* **65,** 232–260 (2001).

159. Roberts, M. C. Update on acquired tetracycline resistance genes. *FEMS Microbiology Letters* **245,** 195–203 (2005).

160. Burdett, V. *THE JOURNAL OF BIOLOGICAL CHEMISTRY Purification and Characterization of Tet(M), a Protein That Renders Ribosomes Resistant to Tetracycline**. **266,** (1991).

161. Taylor, D. E. & Chau, A. *MINIREVIEW Tetracycline Resistance Mediated by Ribosomal Protection*. *ANTIMICROBIAL AGENTS AND CHEMOTHERAPY* **40,** (1996).

162. Sanchez-Pescador, R., Brown, J. T., Roberts, M. & Urdea, M. S. Homology of the tetm with translational elongation factors: Implications for potential modes of tetm conferred tetracycline resistance. *Nucleic Acids Res.* **16,** 1218 (1988).

163. Dantley, K. A., Dannelly, H. K. & Burdett, V. Binding interaction between Tet(M) and the ribosome: requirements for binding. *J. Bacteriol.* **180,** 4089–92 (1998).

164. Donhofer, A. *et al.* Structural basis for TetM-mediated tetracycline resistance. *Proc. Natl. Acad. Sci.* **109,** 16900–16905 (2012).

165. Calcutt, M. J. & Foecking, M. F. An Excision-Competent and Exogenous Mosaic Transposon Harbors the tetM Gene in Multiple Mycoplasma hominis Lineages. *Antimicrob. Agents Chemother.* **59,** 6665–6 (2015).

166. Johnson, C. M. & Grossman, A. D. Integrative and Conjugative Elements (ICEs): What They Do and How They Work. *Annu. Rev. Genet.* **49,** 577–601 (2015).

167. Dordet-Frisoni, E. *et al.* Chromosomal transfers in mycoplasmas: When minimal genomes go mobile. *MBio* **5,** (2014).

168. Dégrange, S., Renaudin, H., Charron, A., Bébéar, C. & Bébéar, C. M. Tetracycline resistance in Ureaplasma spp. and Mycoplasma hominis: Prevalence in Bordeaux, France, from 1999 to 2002 and description of two tet(M)-positive isolates of M. hominis susceptible to tetracyclines. *Antimicrob. Agents Chemother.* **52,** 742–744 (2008).

169. Su, Y. A., He, P. & Clewell, D. B. Characterization of the tet (M) determinant of Tn916: evidence for regulation by transcription attenuation. *Antimicrob. Agents Chemother.* **36,** 769–778 (1992).

170. SHEPARD, M. C. The Recovery of Pleuropneumonia-Like Organisms from Negro Men with and without Nongonococcal Urethritis. *Am. J. Syph.* **38,** 113–24 (1954).

171. Ford, D. K. CULTURE OF HUMAN GENITAL "T-STRAIN" PLEUROPNEUMONIA-LIKE ORGANISMS. *J. Bacteriol.* **84,** 1028–34 (1962).

172. Robertson, J. A. & Stemke, G. W. Expanded serotyping scheme for Ureaplasma urealyticum strains isolated from humans. *J. Clin. Microbiol.* **15,** 873–878 (1982).

173. Robertson, J. A. *et al.* Proposal of Ureaplasma parvum sp. nov. and emended description of Ureaplasma urealyticum (Shepard et al. 1974) Robertson et al. 2001. *Int. J. Syst. Evol. Microbiol.* **52,** 587–597 (2002).

174. Benstein, B. D., Crouse, D. T., Shanklin, D. R. & Ourth, D. D. Ureaplasma in lung: 1. Localization by in situ hybridization in a mouse model. *Exp. Mol. Pathol.* **75,** 165–170 (2003).

175. Pollack, J. D., Merola, A. J., Platz, M. & Booth, R. L. Respiration-associated components of Mollicutes. *J. Bacteriol.* **146,** 907–913 (1981).

176. Miles, R. J. Catabolism in mollicutes. *J. Gen. Microbiol.* **138,** 1773–1783 (1992).

177. Smith, D. G. E., Russell, W. C., Ingledew, W. J. & Thirkell, D. Hydrolysis of urea by Ureaplasma urealyticum generates a transmembrane potential with resultant ATP synthesis. *J. Bacteriol.* **175,** 3253–3258 (1993).

178. Nagata, K., Takagi, E., Satoh, H., Okamura, H. & Tamura, T. Growth inhibition of Ureaplasma urealyticum by the proton pump inhibitor lansoprazole: Direcrt attribution to inhibition by lansoprazole of urease activity and urea-induced ATP synthesis in U. urealyticum. *Antimicrob. Agents Chemother.* **39,** 2187–2192 (1995).

179. Hedelin, H., Brorson, J. -E, Grenabo, L. & Pettersson, S. Ureaplasma urealyticum and Upper Urinary Tract Stones. *Br. J. Urol.* **56,** 244–249 (1984).

180. Reyes, L., Reinhard, M., O'Donell, L. J., Stevens, J. & Brown, M. B. Rat strains differ in susceptibility to Ureaplasma parvum-induced urinary tract infection and struvite stone formation. *Infect. Immun.* **74,** 6656–6664 (2006).

181. Ligon, J. V & Kenny, G. E. Virulence of ureaplasmal urease for mice. *Infect. Immun.* **59,** 1170–1171 (1991).

182. Volgmann, T., Ohlinger, R. & Panzig, B. Ureaplasma urealyticum-harmless commensal or underestimated enemy of human reproduction? A review. *Arch. Gynecol. Obstet.* **273,** 133–139 (2005).

183. Namba, F. *et al.* Placental features of chorioamnionitis colonized with ureaplasma species in preterm delivery. *Pediatr. Res.* **67,** 166–172 (2010).

184. Sweeney, E. L. *et al.* Placental Infection with Ureaplasma species is Associated with Histologic Chorioamnionitis and Adverse Outcomes in Moderately Preterm and Late-Preterm Infants. *J. Infect. Dis.* **213,** 1340–1347 (2016).

185. Naessens, A., Foulon, W., Cammu, H., Goossens, A. & Lauwers, S. Epidemiology and Pathogenesis of Ureaplasma Urealyticum in Spontaneous Abortion and Early Preterm Labor. *Acta Obstet. Gynecol. Scand.* **66,** 513–516 (1987).

186. DiGiulio, D. B. *et al.* Prevalence and Diversity of Microbes in the Amniotic Fluid, the Fetal Inflammatory Response, and Pregnancy Outcome in Women with Preterm Pre-Labor Rupture of Membranes. *Am. J. Reprod. Immunol.* **64,** 38–57 (2010).

187. Gerber, S., Vial, Y., Hohlfeld, P. & Witkin, S. S. Detection of Ureaplasma urealyticum in Second-Trimester Amniotic Fluid by Polymerase Chain Reaction Correlates with Subsequent Preterm Labor and Delivery. *J. Infect. Dis.* **187,** 518–521 (2003).

188. De Francesco, M. A., Negrini, R., Pinsi, G., Peroni, L. & Manca, N. Detection of Ureaplasma biovars and polymerase chain reaction-based subtyping of Ureaplasma parvum in women with or without symptoms of genital infections. *Eur. J. Clin. Microbiol. Infect. Dis.* **28,** 641–646 (2009).

189. Eun, H. S. *et al.* Serological investigation of Ureaplasma urealyticum in Korean preterm infants. *Korean J. Pediatr.* **56,** 477–481 (2013).

190. Zheng, X., Watson, H. L., Waites, K. B. & Cassell, G. H. Serotype diversity and antigen variation among invasive isolates of Ureaplasma urealyticum from neonates. *Infection and Immunity* **60,** 3472–3474 (1992).

191. Shimizu, T., Kida, Y. & Kuwano, K. Ureaplasma parvum lipoproteins, including MB antigen, activate NF-??B through TLR1, TLR2 and TLR6. *Microbiology* **154,** 1318–1325 (2008).

192. Watson, H. L., Blalock, D. K. & Cassell, G. H. Variable antigens of Ureaplasma urealyticum containing both serovar-specific and serovar-cross-reactive epitopes. *Infect. Immun.* **58,** 3679–3688 (1990).

193. ZHENG, X. *et al.* Size Variation of a Major Serotype-Specific Antigen of Ureaplasma urealyticum. *Ann. N. Y. Acad. Sci.* **730,** 299–301 (1994).

194. Teng, L. J. *et al.* Ureaplasma urealyticum biovar specificity and diversity are encoded in multiple-banded antigen gene. *J. Clin. Microbiol.* **32,** 1464–1469 (1994).

195. Zheng, X. *et al.* Small repeating units within the Ureaplasma urealyticum MB antigen gene encode serovar specificity and are associated with antigen size variation. *Infect. Immun.* **63,** 891–898 (1995).

196. Monecke, S., Helbig, J. H. & Jacobs, E. Phase variation of the multiple banded protein in Ureaplasma urealyticum and Ureaplasma parvum. *Int. J. Med. Microbiol.* **293,** 203–211 (2003).

197. Lysnyansky, I., Ron, Y. & Yogev, D. Juxtaposition of an active promoter to vsp genes via site-specific DNA inversions generates antigenic variation in Mycoplasma bovis. *J. Bacteriol.* **183,** 5698–5708 (2001).

198. Bhugra, B., Voelker, L. R. L., Zou, N., Yu, H. & Dybvig, K. Mechanism of antigenic variation in Mycoplasma pulmonis: Interwoven, site-specific DNA inversions. *Mol. Microbiol.* **18,** 703–714 (1995).

199. Zimmerman, C. U. R., Stiedl, T., Rosengarten, R. & Spergser, J. Alternate phase variation in expression of two major surface membrane proteins (MBA and UU376) of Ureaplasma parvum serovar 3. *FEMS Microbiol. Lett.* **292,** 187–193 (2009).

200. Zimmerman, C. U. R., Rosengarten, R. & Spergser, J. Ureaplasma antigenic variation beyond MBA phase variation: DNA inversions generating chimeric structures and switching in expression of the MBA N-terminal paralogue UU172. *Mol. Microbiol.* **79,** 663–676 (2011).

201. Zimmerman, C. U. R., Rosengarten, R. & Spergser, J. Interaction of the putative tyrosine recombinases RipX (UU145), XerC (UU222), and CodV (UU529) of Ureaplasma parvum serovar 3 with specific DNA. *FEMS Microbiol. Lett.* **340,** 55–64 (2013).

202. Knox, C. L. *et al.* Ureaplasma parvum and Ureaplasma urealyticum are detected in semen after washing before assisted reproductive technology procedures. *Fertil. Steril.* **80,** 921–929 (2003).

203. Viscardi, R. M. & Hasday, J. D. Role of Ureaplasma species in neonatal chronic lung disease: Epidemiologic and experimental evidence. *Pediatric Research* **65,** 84R–90R (2009).

204. Saada, A.-B., Terespolski, Y., Adoni, A. & Kahane', I. *Adherence of Ureaplasma urealyticum to Human Erythrocytes*. *INFECTION AND IMMUNITY* **59,** (1991).

205. Torres-Morquecho, A. *et al. African journal of microbiology research. African Journal of Microbiology Research* **4,** (Academic Journals, 2010).

206. Smith, D. G. E., Russell, W. C. & Thirkell, D. Adherence of Ureaplasma urealyticum to human epithelial cells. *Microbiology* **140,** 2893–2898 (1994).

207. Knox, C. L. *et al.* The Severity of Chorioamnionitis in Pregnant Sheep Is Associated with In Vivo

Variation of the Surface-Exposed Multiple-Banded Antigen/Gene of Ureaplasma parvum1. *Biol. Reprod.* **83,** 415–426 (2010).

208. Dando, S. J. *et al.* The role of the multiple banded antigen of Ureaplasma parvum in intra-amniotic infection: Major virulence factor or decoy? *PLoS One* **7,** 29856 (2012).

209. Sweeney, E. L. *et al.* Ureaplasma Species Multiple Banded Antigen (MBA) Variation Is Associated with the Severity of Inflammation In vivo and In vitro in Human Placentae. *Front. Cell. Infect. Microbiol.* **7,** 123 (2017).

210. Istivan, T. S. & Coloe, P. J. Phospholipase A in Gram-negative bacteria and its role in pathogenesis. *Microbiology* **152,** 1263–1274 (2006).

211. Songer, J. G. Bacterial phospholipases and their role in virulence. *Trends in Microbiology* **5,** 156–161 (1997).

212. De Silva, N. S. & Quinn, P. A. Localization of endogenous activity of phospholipases A and C in Ureaplasma urealyticum. *J. Clin. Microbiol.* **29,** 1498–1503 (1991).

213. Bejar, R., Curbelo, V., Davis, C. & Gluck, L. Premature labor. II. Bacterial sources of phospholipase. *Obstet. Gynecol.* **57,** 479–482 (1981).

214. Goldenberg, R. L. *et al.* The Alabama Preterm Birth Study: Umbilical cord blood Ureaplasma urealyticum and Mycoplasma hominis cultures in very preterm newborn infants. *Am. J. Obstet. Gynecol.* **198,** 43.e1-43.e5 (2008).

215. Glass, J. I. *et al.* The complete sequence of the mucosal pathogen Ureaplasma urealyticum. *Nature* **407,** 757–762 (2000).

216. Schrama, A. J. J. *et al.* Phospholipase A 2 is present in meconium and inhibits the activity of pulmonary surfactant : an in vitro study. *Acta Paediatr.* **90,** 412–416 (2001).

217. Cultrera, R., Seraceni, S., Germani, R. & Contini, C. Molecular evidence of Ureaplasma urealyticum and ureaplasma parvum colonization in preterm infants during respiratory distress syndrome. *BMC Infect. Dis.* **6,** 166 (2006).

218. Spooner, R. K., Russell, W. C. & Thirkell, D. Characterization of the immunoglobulin A protease of Ureaplasma urealyticum. *Infection and Immunity* **60,** 2544–2546 (1992).

219. MacPherson, A. J., McCoy, K. D., Johansen, F. E. & Brandtzaeg, P. The immune geography of IgA induction and function. *Mucosal Immunology* **1,** 11–22 (2008).

220. Kapatais-Zoumbos, K., Chandler, D. K. F. & Barile, M. F. Survey of immunoglobulin A protease activity among selected species of ureaplasma and mycoplasma: Specificity for host immunoglobulin A. *Infect. Immun.* **47,** 704–709 (1985).

221. Menestrina, G., Dalla Serra, M., Pederzolli, C., Bregante, M. & Gambale, F. Bacterial hemolysins and leukotoxins affect target cells by forming large exogenous pores into their plasma membrane. Escherichia coli hemolysin a as a case example. *Biosci. Rep.* **15,** 543–551 (1995).

222. Hacker, J. *et al.* Deletions of chromosomal regions coding for fimbriae and hemolysins occur in vitro and in vivo in various extra intestinal Escherichia coli isolates. *Microb. Pathog.* **8,** 213–225 (1990).

223. High, N. J., Hales,', B. A., Jann, K. & Boulnoisl, G. J. *A Block of Urovirulence Genes Encoding Multiple Fimbriae and Hemolysin in Escherichia coli 04:K12:H*. *INFECTION AND IMMUNITY* **56,** (1988).

224. Schmidt, H. & Hensel, M. Pathogenicity Islands in Bacterial Pathogenesis. *Clinical Microbiology Reviews* **17,** 14–56 (2004).

225. Messerer, M., Fischer, W. & Schubert, S. Investigation of horizontal gene transfer of pathogenicity islands in Escherichia coli using next-generation sequencing. *PLoS One* **12,** e0179880 (2017).

226. Momynaliev, K. *et al.* Comparative genome analysis of Ureaplasma parvum clinical isolates. *Res. Microbiol.* **158,** 371–378 (2007).

227. Paralanov, V. *et al.* Comparative genome analysis of 19 Ureaplasma urealyticum and Ureaplasma parvum strains. *BMC Microbiol.* **12,** 88 (2012).

228. Couldwell, D. L. *et al.* Ureaplasma urealyticum is significantly associated with non-gonococcal urethritis in heterosexual Sydney men. *Int. J. STD AIDS* **21,** 337–341 (2010).

229. Taylor, S. N. Epididymitis. *Clin. Infect. Dis.* **61,** S770–S773 (2015).

230. Ito, S. *et al.* Prevalence of genital mycoplasmas and ureaplasmas in men younger than 40 years-of-age with acute epididymitis. *Int. J. Urol.* **19,** 234–238 (2012).

231. Taylor-Robinson, D. Evaluation of the role of Ureaplasma urealyticum in infertility. *Pediatr. Infect. Dis.* **5,** S262-5 (1986).

232. Huang, C. *et al.* Mycoplasma and ureaplasma infection and male infertility: A systematic review and meta-analysis. *Andrology* **3,** 809–816 (2015).

233. Hillier, S. L. *et al.* A Case–Control Study of Chorioamnionic Infection and Histologic Chorioamnionitis in Prematurity. *N. Engl. J. Med.* **319,** 972–978 (1988).

234. Maher, C. F., Haran, M. V., Farrell, D. J. & Cave, D. G. Ureaplasma Urealyticum Chorioamnionitis. *Aust. New Zeal. J. Obstet. Gynaecol.* **34,** 477–479 (1994).

235. Liu, L., Cao, G., Zhao, Z., Zhao, F. & Huang, Y. High bacterial loads of Ureaplasma may be associated with non-specific cervicitis. *Scand. J. Infect. Dis.* **46,** 637–641 (2014).

236. Kundsin, R. B., Leviton, A., Allred, E. N. & Poulin, S. A. Ureaplasma urealyticum infection of the placenta in pregnancies that ended prematurely. *Obstet. Gynecol.* **87,** 122–127 (1996).

237. Brus, F., van Waarde, W. M., Schoots, C. & Bambang Oetomo, S. Fatal ureaplasmal pneumonia and sepsis in a newborn infant. *Eur. J. Pediatr.* **150,** 782–783 (1991).

238. Waites, K. B., Crouse, D. T., Philips, J. B. 3rd, Canupp, K. C. & Cassell, G. H. Ureaplasmal pneumonia and sepsis associated with persistent pulmonary hypertension of the newborn. *Pediatrics* **83,** 79–85 (1989).

239. Lillis, R. A. & Martin, D. H. Persistent and Recurrent Nongonococcal Urethritis. in *Infectious Diseases* 603–604.e1 (2017). doi:10.1016/B978-0-7020-6285-8.00215-X

240. Frieden, T. R. *et al.* department of health and human services Centers for Disease Control and Prevention Morbidity and Mortality Weekly Report Sexually Transmitted Diseases Treatment Guidelines, 2010 Centers for Disease Control and Prevention. *MMWR* **5959,** (2009).

241. Martin, D. H. Nongonococcal urethritis: New views through the prism of modern molecular microbiology. *Current Infectious Disease Reports* **10,** 128–132 (2008).

242. Deguchi, T. *et al.* Association of Ureaplasma urealyticum (Biovar 2) with Nongonococcal Urethritis. *Sex. Transm. Dis.* **31,** 192–195 (2004).

243. Yoshida, T. *et al.* Polymerase chain reaction-based subtyping of Ureaplasma parvum and Ureaplasma urealyticum in first-pass urine samples from men with or without urethritis. *Sex. Transm. Dis.* **32,** 454–457 (2005).

244. Taylor-Robinson, D., Csonka, G. W. & Prentice, M. J. Human intra-urethral inoculation of ureaplasmas. *QJM* **46,** 309–326 (1977).

245. Brown, M. B., Cassell, G. H., Taylor-Robinson, D. & Shepard, M. C. Measurement of antibody to Ureaplasma urealyticum by an enzyme-linked immunosorbent assay and detection of antibody responses in patients with nongonococcal urethritis. *J. Clin. Microbiol.* **17,** 288–95 (1983).

246. Yoshida, T. *et al.* Quantitative detection of ureaplasma parvum (biovar 1) and ureaplasma urealyticum (biovar 2) in urine specimens from men with and without urethritis by real-time polymerase chain reaction. *Sex. Transm. Dis.* **34,** 416–419 (2007).

247. Montagut, J. M., Leprêtre, S., Degoy, J. & Rousseau, M. Ureaplasma in semen and IVF. *Hum. Reprod.* **6,** 727–729 (1991).

248. Dadoune, J. P. The nuclear status of human sperm cells. *Micron* **26,** 323–45 (1995).

249. Reichart, M., Kahane, I. & Bartoov, B. In vivo and in vitro impairment of human and ram sperm nuclear chromatin integrity by sexually transmitted Ureaplasma urealyticum infection. *Biol. Reprod.* **63,** 1041–8 (2000).

250. Lusk, M. J. & Konecny, P. Cervicitis: A review. *Current Opinion in Infectious Diseases* **21,** 49–55 (2008).

251. Workowski, K. A., Berman, S. & CDC, C. for D. C. and P. *Sexually transmitted diseases treatment guidelines, 2010. MMWR. Recommendations and reports : Morbidity and mortality weekly report. Recommendations and reports / Centers for Disease Control* **59,** (2010).

252. Corey, L., Wald, A., Celum, C. L. & Quinn, T. C. The Effects of Herpes Simplex Virus-2 on HIV-1 Acquisition and Transmission: A Review of Two Overlapping Epidemics. *Journal of Acquired Immune Deficiency Syndromes* **35,** 435–445 (2004).

253. Kreiss, J. *et al.* Association between cervical inflammation and cervical shedding of human immunodeficiency virus dna. *J. Infect. Dis.* **170,** 1597–1601 (1994).

254. McClelland, R. S. *et al.* Treatment of cervicitis is associated with decreased cervical shedding of HIV-1. *AIDS* **15,** 105–110 (2001).

255. Castle, P. E. Beyond human papillomavirus: The cervix, exogenous secondary factors, and the development of cervical precancer and cancer. *Journal of Lower Genital Tract Disease* **8,** 224–230 (2004).

256. Rodrigues, M. M. *et al.* Frequency of Chlamydia trachomatis, Neisseria gonorrhoeae, Mycoplasma genitalium, Mycoplasma hominis and Ureaplasma species in cervical samples. *J. Obstet. Gynaecol. (Lahore).* **31,** 237–241 (2011).

257. Lusk, M. J. *et al.* Mycoplasma genitalium is associated with cervicitis and HIV infection in an urban Australian STI clinic population. *Sex. Transm. Infect.* **87,** 107–109 (2011).

258. Schlicht, M. J. *et al.* High prevalence of genital mycoplasmas among sexually active young adults with urethritis or cervicitis symptoms in La Crosse, Wisconsin. *J. Clin. Microbiol.* **42,** 4636–4640 (2004).

259. Chaim, W. *et al.* Ureaplasma urealyticum in the development of postpartum endometritis.

Eur. J. Obstet. Gynecol. Reprod. Biol. **109,** 145–148 (2003).

260. Yoon, B. H. *et al.* Microbial invasion of the amniotic cavity with Ureaplasma urealyticum is associated with a robust host response in fetal, amniotic, and maternal compartments. *Am. J. Obstet. Gynecol.* **179,** 1254–1260 (1998).

261. Peltier, M. R. Immunology of term and preterm labor. *Reproductive Biology and Endocrinology* **1,** 122 (2003).

262. Aaltonen, R., Heikkinen, J., Vahlberg, T., Jensen, J. S. & Alanen, A. Local inflammatory response in choriodecidua induced by Ureaplasma urealyticum. *BJOG An Int. J. Obstet. Gynaecol.* **114,** 1432–1435 (2007).

263. Mazor, M., Chaim, W., Horowitz, S., Leiberman, J. R. & Glezerman, M. Successful treatment of preterm labour by eradication of Ureaplasma urealyticum with erythromycin. *Arch. Gynecol. Obstet.* **253,** 215–218 (1993).

264. Gaurivaud, P., Laigret, F. & Bove, J. M. Insusceptibility of members of the class Mollicutes to rifampin: Studies of the Spiroplasma citri RNA polymerase β-subunit gene. *Antimicrob. Agents Chemother.* **40,** 858–862 (1996).

265. Cassell, G. H., Waites, K. B., Watson, H. L., Crouse, D. T. & Harasawa, R. Ureaplasma urealyticum intrauterine infection: Role in prematurity and disease in newborns. *Clinical Microbiology Reviews* **6,** 69–87 (1993).

266. Geißdörfer, W. *et al.* Ureaplasma urealyticum meningitis in an adult patient. *J. Clin. Microbiol.* **46,** 1141–1143 (2008).

267. Matlow, A. *et al.* Susceptibilities of neonatal respiratory isolates of Ureaplasma urealyticum to antimicrobial agents. *Antimicrob. Agents Chemother.* **42,** 1290–1292 (1998).

268. Pereyre, S. *et al.* Characterisation of in vitro-selected mutants of Ureaplasma parvum resistant to macrolides and related antibiotics. *Int. J. Antimicrob. Agents* **29,** 207–211 (2007).

269. De Barbeyrac, B., Dupon, M., Rodriguez, P., Renaudin, H. & Bébéar, C. A Tn1545-like transposon carries the tet (M) gene in tetracycline resistant strains of Bacteroides ureolyticus as well as Ureaplasma urealyticum but not Neisseria gonorrhoeae. *J. Antimicrob. Chemother.* **37,** 223–232 (1996).

270. Mardassi, B. B. A. *et al.* Evidence for the predominance of a single tet(M) gene sequence type in tetracycline-resistant Ureaplasma parvum and Mycoplasma hominis isolates from Tunisian patients. *J. Med. Microbiol.* **61,** 1254–1261 (2012).

271. Roberts, M. C. & Kenny, G. E. Dissemination of the tetM tetracycline resistance determinant to Ureaplasma urealyticum. *Antimicrob. Agents Chemother.* **29,** 350–352 (1986).

272. Evans, R. T. & Taylor-robinson, D. The incidence of tetracycline-resistant strains of ureaplasma urealyticum. *J. Antimicrob. Chemother.* **4,** 57–63 (1978).

273. Taylor-Robinson, D. & Furr, P. M. Clinical antibiotic resistance of Ureaplasma urealyticum. *Pediatr. Infect. Dis.* **5,** S335-7 (1986).

274. Roberts, M. C. & Kenny, G. E. TetM tetracycline-resistant determinants in Ureaplasma urealyticum. *Pediatr. Infect. Dis.* **5,** S338-40 (1986).

275. Beeton, M. L., Chalker, V. J., Kotecha, S. & Spiller, O. B. Comparison of full gyrA, gyrB, parC and parE gene sequences between all Ureaplasma parvum and Ureaplasma urealyticum serovars to separate true fluoroquinolone antibiotic resistance mutations from non-

resistance polymorphism. *J. Antimicrob. Chemother.* **64,** 529–538 (2009).

276. Piddock, L. J. Mechanisms of fluoroquinolone resistance: an update 1994-1998. *Drugs* **58 Suppl 2,** 11–18 (1999).

277. Schmitz, F. J., Higgins, P., Mayer, S., Fluit, A. & Dalhoff, A. Activity of quinolones against gram-positive cocci: Mechanisms of drug action and bacterial resistance. *European Journal of Clinical Microbiology and Infectious Diseases* **21,** 647–659 (2002).

278. Valentine-King, M. A. & Brown, M. B. Antibacterial Resistance in Ureaplasma Species and Mycoplasma hominis Isolates from Urine Cultures in College-Aged Females. *Antimicrob. Agents Chemother.* **61,** (2017).

279. Song, J. *et al.* Frequent topoisomerase IV mutations associated with fluoroquinolone resistance in Ureaplasma species. *J. Med. Microbiol.* **64,** 1315–1320 (2015).

280. Bowie, W. R. *et al.* Bacteriology of the urethra in normal men and men with nongonococcal urethritis. *J. Clin. Microbiol.* **6,** 482–488 (1977).

281. Andrade-Rocha, F. T. Ureaplasma urealyticum and Mycoplasma hominis in Men Attending for Routine Semen Analysis. *Urol. Int.* **71,** 377–381 (2003).

282. Zdrodowska-Stefanow, B., Kłosowska, W. M., Ostaszewska-Puchalska, I., Bułhak-Kozioł, V. & Kotowicz, B. Mycoplasma hominis and Ureaplasma urealyticum infections in male urethritis and its complications. *Adv. Med. Sci.* **51,** 254–7 (2006).

283. Cox, C., McKenna, J. P., Watt, A. P. & Coyle, P. V. Ureaplasma parvum and Mycoplasma genitalium are found to be significantly associated with microscopy-confirmed urethritis in a routine genitourinary medicine setting. *Int. J. STD AIDS* **27,** 861–867 (2016).

284. Taylor-Robinson, D. Mollicutes in vaginal microbiology: Mycoplasma hominis, Ureaplasma urealyticum, Ureaplasma parvum and Mycoplasma genitalium. *Res. Microbiol.* **168,** 875–881 (2017).

285. Clegg, A., Passey, M., Yoannes, M. & Michael, A. High rates of genital mycoplasma infection in the highlands of Papua New Guinea determined both by culture and by a commercial detection kit. *J. Clin. Microbiol.* **35,** 197–200 (1997).

286. Frølund, M. *et al.* Urethritis-associated pathogens in urine from men with nongonococcal urethritis: A case-control study. *Acta Derm. Venereol.* **96,** 689–695 (2016).

287. Horner, P. *et al.* Should we be testing for urogenital Mycoplasma hominis, Ureaplasma parvum and Ureaplasma urealyticum in men and women? - a position statement from the European STI Guidelines Editorial Board. *Journal of the European Academy of Dermatology and Venereology* **32,** 1845–1851 (2018).

288. Mirmonsef, P. *et al.* Free glycogen in vaginal fluids is associated with Lactobacillus colonization and low vaginal pH. *PLoS One* **9,** (2014).

289. Jensen, A. J. *et al.* Chlamydia trachomatis, Mycoplasma genitalium and Ureaplasma urealyticum among students in northern Norway. *J. Eur. Acad. Dermatology Venereol.* **27,** e91–e96 (2013).

290. Zhang, N. *et al.* Are Ureaplasma spp. a cause of nongonococcal urethritis? A systematic review and meta-analysis. *PLoS One* **9,** e113771 (2014).

291. Horner, P. J., Blee, K., Falk, L., van der Meijden, W. & Moi, H. 2016 European guideline on the management of non-gonococcal urethritis. *Int. J. STD AIDS* **27,** 928–937 (2016).

292. Leli, C. *et al.* Prevalence of cervical colonization by Ureaplasma parvum, Ureaplasma urealyticum , Mycoplasma hominis and Mycoplasma genitalium in childbearing age women by a commercially available multiplex real-time PCR: An Italian observational multicentre study. *J. Microbiol. Immunol. Infect.* **51,** 220–225 (2018).

293. Esen, B. *et al.* Ureaplasma urealyticum: Presence among sexually transmitted diseases. *Jpn. J. Infect. Dis.* **70,** 75–79 (2017).

294. Romero, P. *et al.* Ureaplasmas and mycoplasmas in vaginal samples from prepubertal girls and the reasons for gynecological consultation. *J. Pediatr. Adolesc. Gynecol.* **27,** 10–13 (2014).

295. Marovt, M. *et al.* Ureaplasma parvum and Ureaplasma urealyticum detected with the same frequency among women with and without symptoms of urogenital tract infection. *Eur. J. Clin. Microbiol. Infect. Dis.* **34,** 1237–1245 (2015).

296. Krausse, R. & Schubert, S. In-Vitro Activities of Tetracyclines, Macrolides, Fluoroquinolones and Clindamycin against *Mycoplasma hominis* and *Ureaplasma* ssp. isolated in Germany over 20 years. *Clin. Microbiol. Infect.* **16,** 1649–55 (2009).

297. Kechagia, N., Bersimis, S. & Chatzipanagiotou, S. Incidence and antimicrobial susceptibilities of genital mycoplasmas in outpatient women with clinical vaginitis in Athens, Greece. *J. Antimicrob. Chemother.* **62,** 122–125 (2008).

298. Pónyai, K. *et al.* Incidence and antibiotic susceptibility of genital mycoplasmas in sexually active individuals in Hungary. *Eur. J. Clin. Microbiol. Infect. Dis.* **32,** 1423–1426 (2013).

299. Pignanelli, S., Pulcrano, G., Schiavone, P., Iula, V. D. & Catania, M. R. In vitro antimicrobial susceptibility of Mycoplasma hominis genital isolates. *Indian J. Dermatol. Venereol. Leprol.* **81,** 286–8 (2015).

300. De Francesco, M. A., Caracciolo, S., Bonfanti, C. & Manca, N. Incidence and antibiotic susceptibility of Mycoplasma hominis and Ureaplasma urealyticum isolated in Brescia, Italy, over 7 years. *J. Infect. Chemother.* **19,** 621–627 (2013).

301. Leli, C. *et al.* Prevalence and antimicrobial susceptibility of Ureaplasma urealyticum and Mycoplasma hominis in a population of Italian and immigrant outpatients. *Le Infez. Med.* **20,** 82–7 (2012).

302. Mihai, M. *et al.* Antibiotic susceptibility profiles of mycoplasma hominis and ureaplasma urealyticum isolated during a population-based study concerning women infertility in northeast Romania. *Brazilian J. Microbiol.* **42,** 256–260 (2011).

303. Skiljevic, D., Mirkov, D. & Vukicevic, J. Prevalence and antibiotic susceptibility of Mycoplasma hominis and Ureaplasma urealyticum in genital samples collected over 6 years at a Serbian university hospital. *Indian J. Dermatology, Venereol. Leprol.* **82,** 37 (2016).

304. Bayraktar, M. R., Ozerol, I. H., Gucluer, N. & Celik, O. Prevalence and antibiotic susceptibility of Mycoplasma hominis and Ureaplasma urealyticum in pregnant women. *Int. J. Infect. Dis.* **14,** e90–e95 (2010).

305. Karou, S. D. *et al.* Antimicrobial resistance of abnormal vaginal discharges microorganisms in Ouagadougou, Burkina Faso. *Asian Pac. J. Trop. Biomed.* **2,** 294–297 (2012).

306. Kouegnigan Rerambiah, L., Ndong, J. C., Medzegue, S., Elisee-Ndam, M. & Djoba Siawaya, J. F. Genital Mycoplasma infections and their resistance phenotypes in an African setting. *Eur. J. Clin. Microbiol. Infect. Dis.* **34,** 1087–1090 (2015).

307. Domingues, D. *et al.* Genital mycoplasmas in women attending a family planning clinic in

Guiné-Bissau and their susceptibility to antimicrobial agents. *Acta Tropica* **86,** 19–24 (2003).

308. Song, T. *et al.* Epidemiological investigation and antimicrobial susceptibility analysis of ureaplasma species and *Mycoplasma* hominis in outpatients with genital manifestations. *J. Clin. Pathol.* **67,** 817–820 (2014).

309. Ye, G. *et al.* The Resistance Analysis of Ureaplasma urealyticum and Mycoplasma hominis in Female Reproductive Tract Specimens. *Cell Biochem. Biophys.* **68,** 207–210 (2014).

310. Ling, Y. *et al.* Prevalence and antimicrobial susceptibility of Ureaplasma urealyticum and Mycoplasma hominis in Chinese women with genital infectious diseases. *Indian J. Dermatology, Venereol. Leprol.* **78,** 406 (2012).

311. Shepard, M. C. & Lunceford, C. D. Differential agar medium (A7) for identification of Ureaplasma urealyticum (human T mycoplasmas) in primary cultures of clinical material. *J. Clin. Microbiol.* **3,** 613–625 (1976).

312. Aguilera-Arreola, M. G., González-Cardel, A. M., Tenorio, A. M., Curiel-Quesada, E. & Castro-Escarpulli, G. Highly specific and efficient primers for in-house multiplex PCR detection of Chlamydia trachomatis, Neisseria gonorrhoeae, Mycoplasma hominis and Ureaplasma urealyticum. *BMC Res. Notes* **7,** 433 (2014).

313. Baczynska, A., Svenstrup, H. F., Fedder, J., Birkelund, S. & Christiansen, G. Development of real-time PCR for detection of Mycoplasma hominis. *BMC Microbiol.* **4,** (2004).

314. Férandon, C. *et al.* Development of a real-time PCR targeting the yidC gene for the detection of Mycoplasma hominis and comparison with quantitative culture. *Clin. Microbiol. Infect.* **17,** 155–159 (2011).

315. Menard, J., Fenollar, F., Henry, M., Bretelle, F. & Raoult, D. Molecular Quantification of Gardnerella vaginalis and Atopobium vaginae Loads to Predict Bacterial Vaginosis. *Clin. Infect. Dis.* **47,** 33–43 (2008).

316. Mallard, K., Schopfer, K. & Bodmer, T. Development of real-time PCR for the differential detection and quantification of Ureaplasma urealyticum and Ureaplasma parvum. *J. Microbiol. Methods* **60,** 13–19 (2005).

317. Xiao, L. *et al.* Detection and characterization of human Ureaplasma species and serovars by real-time PCR. *J. Clin. Microbiol.* **48,** 2715–2723 (2010).

318. Waites, K. B., Xiao, L., Paralanov, V., Viscardi, R. M. & Glass, J. I. Molecular methods for the detection of mycoplasma and ureaplasma infections in humans: A paper from the 2011 William Beaumont Hospital symposium on molecular pathology. *Journal of Molecular Diagnostics* **14,** 437–450 (2012).

319. Jao, R. L. Susceptibility of Mycoplasma pneumoniae to 21 antibiotics in vitro. *Am. J. Med. Sci.* **253,** 639–650 (1967).

320. M43-A: Methods for Antimicrobial Susceptibility Testing for Human Mycoplasmas; Approved Guideline. (2011).

321. Redelinghuys, M. J., Ehlers, M. M., Dreyer, A. W., Lombaard, H. A. & Kock, M. M. Comparison of the new Mycofast Revolution assay with a molecular assay for the detection of genital mycoplasmas from clinical specimens. *BMC Infect. Dis.* **13,** (2013).

322. Cheah, F. C., Anderson, T. P., Darlow, B. A. & Murdoch, D. R. Comparison of the mycoplasma duo test with PCR for detection of Ureaplasma species in endotracheal aspirates from premature infants. *J. Clin. Microbiol.* **43,** 509–510 (2005).

323. Huang, C. *et al.* Ureaplasma urealyticum and Mycoplasma hominis infections and semen quality in 19,098 infertile men in China. *World J. Urol.* **34,** 1039–1044 (2016).

324. Hunjak, B. *et al.* Ureaplasma urealyticum and Ureaplasma parvum in women of reproductive age. *Arch. Gynecol. Obstet.* **289,** 407–412 (2014).

325. Pignanelli, S. *et al.* In vitro antimicrobial profile of Ureaplasma urealyticum from genital tract of childbearing-aged women in Northern and Southern Italy. *APMIS* **122,** 552–555 (2014).

326. Schneider, S. C. *et al.* Antibiotic susceptibility and sequence type distribution of ureaplasma species isolated from genital samples in Switzerland. *Antimicrob. Agents Chemother.* **59,** 6026–6031 (2015).

327. Choe, H. S. *et al.* Performance of Anyplex™ II multiplex real-time PCR for the diagnosis of seven sexually transmitted infections: Comparison with currently available methods. *Int. J. Infect. Dis.* **17,** e1134–e1140 (2013).

328. Beeton, M. L., Chalker, V. J., Maxwell, N. C., Kotecha, S. & Spiller, O. B. Concurrent titration and determination of antibiotic resistance in Ureaplasma species with identification of novel point mutations in genes associated with resistance. *Antimicrob. Agents Chemother.* **53,** 2020–2027 (2009).

329. Payne, M. S. *et al.* A quantitative analysis of Ureaplasma urealyticum and Ureaplasma parvum compared with host immune response in preterm neonates at risk of developing bronchopulmonary dysplasia. *J. Clin. Microbiol.* **50,** 909–914 (2012).

330. Teng, K. *et al.* Comparison of PCR with culture for detection of Ureaplasma urealyticum in clinical samples from patients with urogenital infections. *J. Clin. Microbiol.* **32,** 2232–2234 (1994).

331. Stellrecht, K. A., Woron, A. M., Mishrik, N. G. & Venezia, R. A. Comparison of Multiplex PCR Assay with Culture for Detection of Genital Mycoplasmas. *J. Clin. Microbiol.* **42,** 1528–1533 (2004).

332. Keskin, F. *et al.* Comparison of culture and real-time polymerase chain reaction methods for detection of Mycoplasma hominis in amniotic fluids samples. *Niger. J. Clin. Pract.* **21,** 1127–1131 (2018).

333. Jensen, J. S., Ahrens, P., Björnelius, E., Frølund, M. & Lidbrink, P. Comparison between Culture and a Multiplex Quantitative Real-Time Polymerase Chain Reaction Assay Detecting Ureaplasma urealyticum and U. parvum. *PLoS One* **9,** e102743 (2014).

334. Petrikkos, G. L., Hadjisoteriou, M. & Daikos, G. L. PCR versus culture in the detection of vaginal Ureaplasma urealyticum and Mycoplasma hominis. *Int. J. Gynecol. Obstet.* **97,** 202–203 (2007).

335. Fiacco, V., Miller, M. J., Carney, E. & Martin, W. J. Comparison of media for isolation of Ureaplasma urealyticum and genital Mycoplasma species. *J. Clin. Microbiol.* **20,** 862–865 (1984).

336. Berçot, B. *et al.* Assessment of coinfection of sexually transmitted pathogen microbes by use of the Anyplex II STI-7 molecular kit. *J. Clin. Microbiol.* **53,** 991–993 (2015).

337. Avolio, M., Modolo, M. L., Stano, P., De Rosa, R. & Camporese, A. Molecular evaluation of 7 sexually transmissible microorganisms in symptomatic and asymptomatic Italian childbearing age women: is Ureaplasma parvum a real innocent bystander? *Microbiol. Medica* **31,** (2016).

338. Abele-Horn, M. *et al.* Polymerase chain reaction versus culture for detection of ureaplasma

urealyticum and mycoplasma hominis in the urogenital tract of adults and the respiratory tract of newborns. *Eur. J. Clin. Microbiol. Infect. Dis.* **15,** 595–598 (1996).

339. Cavicchini, S., Biffi, M. R., Brezzi, A. & Alessi, E. Mycoplasma hominis and Ureaplasma urealyticum. Comparison of media for isolation and prevalence among patients with nongonococcal nonchlamydial genital infections. *G. Ital. di Dermatologia e Venereol.* **124,** 321–324 (1989).

340. Gaydos, C. A. *et al.* Performance of the Abbott RealTime CT/NG for detection of Chlamydia trachomatis and Neisseria gonorrhoeae. *J. Clin. Microbiol.* **48,** 3236–3243 (2010).

341. Bader, M. S., Loeb, M. & Brooks, A. A. An update on the management of urinary tract infections in the era of antimicrobial resistance. *Postgraduate Medicine* **129,** 242–258 (2017).

342. Stamm, W. E. *et al.* A randomized, double-blind study comparing single-dose rifalazil with single-dose azithromycin for the empirical treatment of nongonococcal urethritis in men. *Sex. Transm. Dis.* **34,** 545–552 (2007).

343. Iwasaka, T., Wada, T., Kidera, Y. & Sugimori, H. Hormonal status and mycoplasma colonization in the female genital tract. *Obstet. Gynecol.* **68,** 263–6 (1986).

344. Viscardi, R. M. Ureaplasma species: Role in diseases of prematurity. *Clinics in Perinatology* **37,** 393–409 (2010).

345. Zhu, X., Li, M., Cao, H., Yang, X. & Zhang, C. Epidemiology of Ureaplasma urealyticum and Mycoplasma hominis in the semen of male outpatients with reproductive disorders. *Exp. Ther. Med.* **12,** 1165–1170 (2016).

346. Wright, F. L. An Autobiography. *Annual Review of Biochemistry* **45,** 1–12 (1943).

347. Furr, P. M. & Taylor-Robinson, D. Factors influencing the ability of different mycoplasmas to colonize the genital tract of hormone-treated female mice. *Int. J. Exp. Path* **74,** 97–101 (1993).

348. Tully, J. G., Taylor-Robinson, D., Rose, D. L., Cole,' A N D, R. M. & Bove4, J. M. *Mycoplasma genitalium, a New Species from the Human Urogenital Tract*. *INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY* (1983).

349. Sonnex, C. Influence of ovarian hormones on urogenital infection. *Sexually Transmitted Infections* **74,** 11–19 (1998).

350. Guseva, N. V, Knight, S. T., Whittimore, J. D. & Wyrick, P. B. Primary cultures of female swine genital epithelial cells in vitro: A new approach for the study of hormonal modulation of chlamydia infection. *Infect. Immun.* **71,** 4700–4710 (2003).

351. Tsutsumi, A. *et al.* Estrogen induces stromal cell-derived factor 1 (SDF-1/CXCL12) production in human endometrial stromal cells: A possible role of endometrial epithelial cell growth. *Fertil. Steril.* **95,** 444–447 (2011).

352. Ren, C. E. *et al.* Microarray analysis on gene regulation by estrogen, progesterone and tamoxifen in human endometrial stromal cells. *Int. J. Mol. Sci.* **16,** 5864–5885 (2015).

353. Arnold, J. T., Kaufman, D. G., Seppälä, M. & Lessey, B. A. Endometrial stromal cells regulate epithelial cell growth in vitro: A new co-culture model. *Hum. Reprod.* **16,** 836–845 (2001).

354. Arnold, J. T., Lessey, B. A., Seppälä, M. & Kaufman, D. G. Effect of normal endometrial stroma on growth and differentiation in Ishikawa endometrial adenocarcinoma cells. *Cancer Res.* **62,** 79–88 (2002).

355. King, A. E. & Critchley, H. O. D. Oestrogen and progesterone regulation of inflammatory processes in the human endometrium. *J. Steroid Biochem. Mol. Biol.* **120,** 116–126 (2010).

356. Kim, J. J., Kurita, T. & Bulun, S. E. Progesterone action in endometrial cancer, endometriosis, uterine fibroids, and breast cancer. *Endocrine Reviews* **34,** 130–162 (2013).

357. Kintner, J., Schoborg, R. V, Wyrick, P. B. & Hall, J. V. Progesterone antagonizes the positive influence of estrogen on Chlamydia trachomatis serovar E in an Ishikawa/SHT-290 co-culture model. *Pathog. Dis.* **73,** 15 (2015).

358. Wyrick, P. B. Intracellular survival by Chlamydia. *Cellular Microbiology* **2,** 275–282 (2000).

359. Wang, Y. *et al.* Progesterone inhibition of Wnt/β-catenin signaling in normal endometrium and endometrial cancer. *Clin. Cancer Res.* **15,** 5784–5793 (2009).

360. Castelbaum, A. J. *et al.* Characterization of integrin expression in a well differentiated endometrial adenocarcinoma cell line (Ishikawa). *J. Clin. Endocrinol. Metab.* **82,** 136–142 (1997).

361. Cao, W., Mah, K., Carroll, R. S., Slayden, O. D. & Brenner, R. M. Progesterone withdrawal up-regulates fibronectin and integrins during menstruation and repair in the rhesus macaque endometrium. *Hum. Reprod.* **22,** 3223–3231 (2007).

362. Koucký, M. *et al.* Prolonged progesterone administration is associated with less frequent cervicovaginal colonization by Ureaplasma urealyticum during pregnancy - Results of a pilot study. *J. Reprod. Immunol.* **116,** 35–41 (2016).

363. Beagley, K. W. & Gockel, C. M. Regulation of innate and adaptive immunity by the female sex hormones oestradiol and progesterone. *FEMS Immunology and Medical Microbiology* **38,** 13–22 (2003).

364. Walmer, D. K., Wrona, M. A., Hughes, C. L. & Nelson, K. G. Lactoferrin expression in the mouse reproductive traci during the natural estrous cycle: Correlation with circulating estradiol and progesterone. *Endocrinology* **131,** 1458–1466 (1992).

365. Vegeto, E. *et al.* Estrogen Prevents the Lipopolysaccharide-Induced Inflammatory Response in Microglia. *J. Neurosci.* **21,** 1809–1818 (2001).

366. Kutteh, W. H., Prince, S. J., Hammond, K. R., Kutteh, C. C. & Mestecky, J. Variations in immunoglobulins and IgA subclasses of human uterine cervical secretions around the time of ovulation. *Clin. Exp. Immunol.* **104,** 538–542 (1996).

367. Kutteh, W. H., Moldoveanu, Z. & Mestecky, J. Mucosal immunity in the female reproductive tract: correlation of immunoglobulins, cytokines, and reproductive hormones in human cervical mucus around the time of ovulation. *AIDS Res. Hum. Retroviruses* **14 Suppl 1,** S51-5 (1998).

368. Gallichan, W. S. & Rosenthal, K. L. Effects of the estrous cycle on local humoral immune responses and protection of intranasally immunized female mice against herpes simplex virus type 2 infection in the genital tract. *Virology* **224,** 487–497 (1996).

369. Robertson, J. A., Stemler, M. E. & Stemke, G. W. Immunoglobulin A protease activity of Ureaplasma urealyticum. *J. Clin. Microbiol.* **19,** 255–258 (1984).

370. Gendler, S. J. MUC1, The renaissance molecule. *Journal of Mammary Gland Biology and Neoplasia* **6,** 339–353 (2001).

371. Hey, N. A., Graham, R. A., Seif, M. W. & Aplin, J. D. The polymorphic epithelial mucin MUC1 in

human endometrium is regulated with maximal expression in the implantation phase. *J. Clin. Endocrinol. Metab.* **78,** 337–342 (1994).

372. Hild-Petito, S., Fazleabas, A. T., Julian, J. & Carson, D. D. Mucin (Muc-1) expression is differentially regulated in uterine luminal and glandular epithelia of the baboon (Papio anubis). *Biol. Reprod.* **54,** 939–947 (1996).

373. Milanezi, F. *et al.* Prevalence of Mycoplasma hominis and ureaplasma spp. In routine gynecological care in Sao Paulo City, Brazil. *Arch. Clin. Infect. Dis.* **11,** (2016).

374. Adams, E. J., Charlett, A., Edmunds, W. J. & Hughes, G. Chlamydia trachomatis in the United Kingdom: A systematic review and analysis of prevalence studies. *Sexually Transmitted Infections* **80,** 354–362 (2004).

375. Fenton, K. A. *et al.* Sexual behaviour in Britain: Reported sexually transmitted infections and prevalent genital Chlamydia trachomatis infection. *Lancet* **358,** 1851–1854 (2001).

376. Denks, K. *et al.* Coinfection of Chlamydia trachomatis, Ureaplasma urealyticum and human papillomavirus among patients attending STD clinics in Estonia. *Scand. J. Infect. Dis.* **39,** 714–718 (2007).

377. Yamazaki, T. *et al.* Frequency of Chlamydia trachomatis in Ureaplasma-positive healthy women attending their first prenatal visit in a community hospital in Sapporo, Japan. *BMC Infect. Dis.* **12,** 82 (2012).

378. Hocking, J. S. *et al.* A chlamydia prevalence survey of young women living in Melbourne, Victoria. *Sex. Health* **3,** 235–240 (2006).

379. McCormack, W. M., Almeida, P. C., Bailey, P. E., Grady, E. M. & Lee, Y. H. Sexual Activity and Vaginal Colonization with Genital Mycoplasmas. *JAMA J. Am. Med. Assoc.* **221,** 1375–1377 (1972).

380. Adimora, A. A. *et al.* Concurrent sexual partnerships among women in the United States. *Epidemiology* **13,** 320–7 (2002).

381. Mazzaferro, K. E. *et al.* Depression, Stress, and Social Support as Predictors of High-Risk Sexual Behaviors and STIs in Young Women. *J. Adolesc. Heal.* **39,** 601–603 (2006).

382. Alonso, L. C. & Rosenfield, R. L. Oestrogens and puberty. *Best Practice and Research: Clinical Endocrinology and Metabolism* **16,** 13–30 (2002).

383. Sehested, A. *et al.* Serum inhibin A and inhibin B in healthy prepubertal, pubertal, and adolescent girls and adult women: Relation to age, stage of puberty, menstrual cycle, follicle-stimulating hormone, luteinizing hormone, and estradiol levels. *J. Clin. Endocrinol. Metab.* **85,** 1634–1640 (2000).

384. Dimitrakakis, C. Androgens and Breast Cancer in Men and Women. *Endocrinology and Metabolism Clinics of North America* **40,** 533–547 (2011).

385. Glaser, R. & Dimitrakakis, C. Testosterone therapy in women: Myths and misconceptions. *Maturitas* **74,** 230–234 (2013).

386. Dratva, J. *et al.* Is age at menopause increasing across Europe? Results on age at menopause and determinants from two population-based studies. *Menopause* **16,** 385–394 (2009).

387. Viña, J., Sastre, J., Pallardó, F. V., Gambini, J. & Borrás, C. Role of mitochondrial oxidative stress to explain the different longevity between genders. Protective effect of estrogens. *Free Radic. Res.* **40,** 1359–1365 (2006).

388. Hillier, S. L. & Lau, R. J. Vaginal microflora in postmenopausal women who have not received estrogen replacement therapy. *Clin. Infect. Dis.* **25 Suppl 2,** S123-6 (1997).

389. Brotman, R. M. *et al.* Association between the vaginal microbiota, menopause status, and signs of vulvovaginal atrophy. *Menopause* **25,** 1321–1330 (2018).

390. Horner, P., Thomas, B., Gilroy, C. B., Egger, M. & Taylor-Robinson, D. Role of Mycoplasma genitalium and Ureaplasma urealyticum in acute and chronic nongonococcal urethritis. *Clin Infect Dis* **32,** 995–1003 (2001).

391. McCormack, W. M., Lee, Y. H. & Zinner, S. H. Sexual experience and urethral colonization with genital mycoplasmas. A study in normal men. *Ann. Intern. Med.* **78,** 696–698 (1973).

392. McIver, C. J. *et al.* Multiplex PCR testing detection of higher-than-expected rates of cervical Mycoplasma, Ureaplasma, and Trichomonas and viral agent infections in sexually active australian women. *J. Clin. Microbiol.* **47,** 1358–1363 (2009).

393. T.Z., K. *et al.* Prevalence and antimicrobial susceptibility of Ureaplasma urealyticum in asymptomatic women in Northern Greece. *Hippokratia* **17,** 319–321 (2013).

394. Kletzel, H. H., Rotem, R., Barg, M., Michaeli, J. & Reichman, O. Ureaplasma urealyticum: the Role as a Pathogen in Women's Health, a Systematic Review. *Current Infectious Disease Reports* **20,** (2018).

395. Ondondo, R. O., Whittington, W. L. H., Astete, S. G. & Totten, P. A. Differential association of ureaplasma species with non-gonococcal urethritis in heterosexual men. *Sex. Transm. Infect.* **86,** 271–275 (2010).

396. Wetmore, C. M. *et al.* Ureaplasma urealyticum is associated with nongonococcal urethritis among men with fewer lifetime sexual partners: A case-control study. *J. Infect. Dis.* **204,** 1274–1282 (2011).

397. Gooren, L. J. & Toorians, A. W. Significance of oestrogens in male (patho)physiology. *Ann Endocrinol* **64,** 126–135 (2003).

398. Zheng, X., Lau, K., Frazier, M., Cassell, G. H. & Watson, H. L. Epitope mapping of the variable repetitive region with the MB antigen of Ureaplasma urealyticum. *Clin. Diagn. Lab. Immunol.* **3,** 774–8 (1996).

399. Xiao, L. *et al.* Extensive horizontal gene transfer in ureaplasmas from humans questions the utility of serotyping for diagnostic purposes. *J. Clin. Microbiol.* **49,** 2818–2826 (2011).

400. Waites, K. B., Schelonka, R. L., Xiao, L., Grigsby, P. L. & Novy, M. J. Congenital and opportunistic infections: Ureaplasma species and Mycoplasma hominis. *Semin. Fetal Neonatal Med.* **14,** 190–199 (2009).

401. Triantafilou, M. *et al.* Synergic Activation of Toll-Like Receptor (TLR) 2/6 and 9 in Response to Ureaplasma parvum & urealyticum in Human Amniotic Epithelial Cells. *PLoS One* **8,** (2013).

402. Hirschfeld, M. *et al.* Signaling by Toll-Like Receptor 2 and 4 Agonists Results in Differential Gene Expression in Murine Macrophages. *Infect. Immun.* **69,** 1477–1482 (2001).

403. Lesmeister, M. J., Jorgenson, R. L., Young, S. L. & Misfeldt, M. L. 17beta-estradiol suppresses TLR3-induced cytokine and chemokine production in endometrial epithelial cells. *Reprod. Biol. Endocrinol.* **3,** 74 (2005).

404. Fahey, J. V *et al.* Estradiol selectively regulates innate immune function by polarized human uterine epithelial cells in culture. *Mucosal Immunol.* **1,** 317–325 (2008).

405. Dasgupta, S. & Eudaly, J. Estrogen receptor-alpha mediates Toll-like receptor-2 agonist-induced monocyte chemoattractant protein-1 production in mesangial cells. *Results Immunol.* **2,** 196–203 (2012).

406. Abele-Horn, M., Wolff, C., Dressel, P., Pfaff, F. & Zimmermann, A. Association of Ureaplasma urealyticum biovars with clinical outcome for neonates, obstetric patients, and gynecological patients with pelvic inflammatory disease. *J. Clin. Microbiol.* **35,** 1199–202 (1997).

407. MAEDA, S.-I. *et al.* Detection of Mycoplasma genitalium, Mycoplasma hominis, Ureaplasma parvum (biovar 1) and Ureaplasma urealyticum (biovar 2) in patients with non-gonococcal urethritis using polymerase chain reaction-microtiter plate hybridization. *Int. J. Urol.* **11,** 750–754 (2004).

408. Povlsen, K., Bjørnelius, E., Lidbrink, P. & Lind, I. Relationship of Ureaplasma urealyticum biovar 2 to nongonococcal urethritis. *Eur. J. Clin. Microbiol. Infect. Dis.* **21,** 97–101 (2002).

409. K., P., P., T. & I., L. Relationship of Ureaplasma urealyticum biovars to the presence or absence of bacterial vaginosis in pregnant women and to the time of delivery. *Eur. J. Clin. Microbiol. Infect. Dis.* **20,** 65–67 (2001).

410. Borges, V. *et al.* Chlamydia Trachomatis in vivo to in vitro transition reveals mechanisms of phase variation and down-regulation of virulence factors. *PLoS One* **10,** e0133420 (2015).

411. Abdelsamed, H., Peters, J. & Byrne, G. I. Genetic variation in Chlamydia trachomatis and their hosts: Impact on disease severity and tissue tropism. *Future Microbiology* **8,** 1129–1146 (2013).

412. Anjuwon-Foster, B. R. & Tamayo, R. Phase variation of Clostridium difficile virulence factors. *Gut Microbes* **9,** 1–8 (2017).

413. Bradshaw, C. S. *et al.* Etiologies of Nongonococcal Urethritis: Bacteria, Viruses, and the Association with Orogenital Exposure. *J. Infect. Dis.* **193,** 336–345 (2006).

414. Katz, B. *et al.* Characterization of ureaplasmas isolated from preterm infants with and without bronchopulmonary dysplasia. *J. Clin. Microbiol.* **43,** 4852–4854 (2005).

415. Robertson, J. A. *et al.* Proposal of Ureaplasma parvum sp. nov. and emended description of Ureaplasma urealyticum (Shepard et al. 1974) Robertson et al. 2001. *Int. J. Syst. Evol. Microbiol.* **52,** 587–597 (2002).

416. Xiao, L. *et al.* Suppression of antimicrobial peptide expression by ureaplasma species. *Infect. Immun.* **82,** 1657–65 (2014).

417. Bjartling, C., Osser, S., Johnsson, A. & Persson, K. Clinical manifestations and epidemiology of the new genetic variant of chlamydia trachomatis. *Sex. Transm. Dis.* **36,** 529–535 (2009).

418. Unemo, M. *et al.* The Swedish new variant of Chlamydia trachomatis: Genome sequence, morphology, cell tropism and phenotypic characterization. *Microbiology* **156,** 1394–1404 (2010).

419. Verteramo, R. *et al.* An epidemiological survey of Mycoplasma hominis and Ureaplasma urealyticum in gynaecological outpatients, Rome, Italy. *Epidemiol. Infect.* **141,** 2650–2657 (2013).

420. Imudia, A. N., Detti, L., Puscheck, E. E., Yelian, F. D. & Diamond, M. P. The prevalence of ureaplasma urealyticum, mycoplasma hominis, chlamydia trachomatis and neisseria gonorrhoeae infections, and the rubella status of patients undergoing an initial infertility evaluation. *J. Assist. Reprod. Genet.* **25,** 43–46 (2008).

421. Choi, J. B. *et al.* Prevalence and Antimicrobial Susceptibility of Ureaplasma spp. and Mycoplasma hominis in Asymptomatic Individuals in Korea. *Microb. Drug Resist.* **24,** mdr.2017.0431 (2018).

422. Tibaldi, C. *et al.* Vaginal and endocervical microorganisms in symptomatic and asymptomatic non-pregnant females: Risk factors and rates of occurrence. *Clin. Microbiol. Infect.* **15,** 670–679 (2009).

423. Wang, Q. Y., Li, R. H., Zheng, L. Q. & Shang, X. H. Prevalence and antimicrobial susceptibility of Ureaplasma urealyticum and Mycoplasma hominis in female outpatients, 2009–2013. *J. Microbiol. Immunol. Infect.* **49,** 359–362 (2016).

424. Furr, P. M. & Taylor-Robinson, D. Prevalence and significance of Mycoplasma hominis and Ureaplasma urealyticum in the urines of a non-venereal disease population. *Epidemiol. Infect.* **98,** 353–359 (1987).

425. Gdoura, R. *et al.* Assessment of Chlamydia trachomatis, Ureaplasma urealyticum, Ureaplasma parvum, Mycoplasma hominis, and Mycoplasma genitalium in semen and first void urine specimens of asymptomatic male partners of infertile couples. *J. Androl.* **29,** 198–206 (2008).

426. Zozaya, M. *et al.* Bacterial communities in penile skin, male urethra, and vaginas of heterosexual couples with and without bacterial vaginosis. *Microbiome* **4,** 16 (2016).

427. Mändar, R. *et al.* Complementary seminovaginal microbiome in couples. *Res. Microbiol.* **166,** 440–447 (2015).

428. Turnbaugh, P. J. *et al.* The Human Microbiome Project. *Nature* **449,** 804–810 (2007).

429. Mändar, R. Microbiota of male genital tract: Impact on the health of man and his partner. *Pharmacological Research* **69,** 32–41 (2013).

430. Baumann, L. *et al.* Prevalence of Mycoplasma genitalium in different population groups: systematic review andmeta-analysis. *Sex. Transm. Infect.* **94,** 255–262 (2018).

431. Shkarupeta, M. M., Lazarev, V. N. & Govorun, V. M. Experimental Mycoplasma hominis infection of the genital tract in BALB/c mice. *Bull. Exp. Biol. Med.* **137,** 53–5 (2004).

432. Cruickshank, R. & Sharman, A. The Biology of the Vagina in the Human Subject. *BJOG An Int. J. Obstet. Gynaecol.* **41,** 369–384 (1934).

433. Boskey, E. R., Telsch, K. M., Whaley, K. J., Moench, T. R. & Cone, R. A. Acid production by vaginal flora in vitro is consistent with the rate and extent of vaginal acidification. *Infect. Immun.* **67,** 5170–5 (1999).

434. Boskey, E. R., Cone, R. A., Whaley, K. J. & Moench, T. R. Origins of vaginal acidity: high D/L lactate ratio is consistent with bacteria being the primary source. *Hum. Reprod.* **16,** 1809–13 (2001).

435. Paavonen, J. Physiology and ecology of the vagina. *Scand. J. Infect. Dis. Suppl.* **40,** 31–5 (1983).

436. Pybus, V. & Onderdonk, A. B. Microbial interactions in the vaginal ecosystem, with emphasis on the pathogenesis of bacterial vaginosis. *Microbes and Infection* **1,** 285–292 (1999).

437. Dover, S. E., Aroutcheva, A. A., Faro, S. & Chikindas, M. L. NATURAL ANTIMICROBIALS AND THEIR ROLE IN VAGINAL HEALTH: A SHORT REVIEW. *Int. J. Probiotics Prebiotics* **3,** 219–230 (2008).

438. Kovachev, S. Defence factors of vaginal lactobacilli. *Critical Reviews in Microbiology* **44,** 31–39 (2018).

439. Wylie, J. G. & Henderson, A. Identity and glycogen-fermenting ability of lactobacilli isolated from the vagina of pregnant women. *J. Med. Microbiol.* **2,** 363–366 (1969).

440. Martín, R. *et al.* Characterization of indigenous vaginal lactobacilli from healthy women as probiotic candidates. *Int. Microbiol.* **11,** 261–266 (2008).

441. Spear, G. T. *et al.* Human α-amylase Present in Lower-Genital-Tract Mucosal Fluid Processes Glycogen to Support Vaginal Colonization by Lactobacillus. *J. Infect. Dis.* **210,** 1019–1028 (2014).

442. Smith, R. W., Brittingham, A. & Wilson, W. A. Purification and identification of amylases released by the human pathogen Trichomonas vaginalis that are active towards glycogen. *Mol. Biochem. Parasitol.* **210,** 22–31 (2016).

443. Wilson, W. A., Pradhan, P., Madhan, N., Gist, G. C. & Brittingham, A. Glycogen synthase from the parabasalian parasite Trichomonas vaginalis: An unusual member of the starch/glycogen synthase family. *Biochimie* **138,** 90–101 (2017).

444. Global incidence and prevalence of selected curable sexually transmitted infections: 2008. *Reprod. Health Matters* **20,** 207–208 (2012).

445. Poole, D. N. & McClelland, R. S. Global epidemiology of Trichomonas vaginalis. *Sexually Transmitted Infections* **89,** 418–422 (2013).

446. Huppert, J. S. *et al.* Use of an immunochromatographic assay for rapid detection of Trichomonas vaginalis in vaginal specimens. *J. Clin. Microbiol.* **43,** 684–687 (2005).

447. Van Der Pol, B., Kraft, C. S. & Williams, J. A. Use of an adaptation of a commercially available PCR assay aimed at diagnosis of chlamydia and gonorrhea to detect Trichomonas vaginalis in urogenital specimens. *J. Clin. Microbiol.* **44,** 366–73 (2006).

448. McClelland, R. S. *et al.* Infection with *Trichomonas vaginalis* Increases the Risk of HIV-1 Acquisition. *J. Infect. Dis.* **195,** 698–702 (2007).

449. Martinotti, M. G. & Savoia, D. [Effect of some steroid hormones on the growth of Trichomonas vaginalis]. *G. Batteriol. Virol. Immunol.* **78,** 52–9 (1985).

450. Sugarman, B. & Mummaw, N. The Effect of Hormones on Trichomonas vaginalis. *Microbiology* **134,** 1623–1628 (1988).

451. Sena, A. C. *et al. Trichomonas vaginalis* Infection in Male Sexual Partners: Implications for Diagnosis, Treatment, and Prevention. *Clin. Infect. Dis.* **44,** 13–22 (2007).

452. Schwebke, J. R. & Hook III, E. W. High Rates of Trichomonas vaginalis among Men Attending a Sexually Transmitted Diseases Clinic: Implications for Screening and Urethritis Management. *J. Infect. Dis.* **188,** 465–468 (2003).

453. Hirt, R. P. & Sherrard, J. Trichomonas vaginalis origins, molecular pathobiology and clinical considerations. *Current Opinion in Infectious Diseases* **28,** 72–79 (2015).

454. Koch, A., Bilina, A., Teodorowicz, L. & Stary, A. Mycoplasma hominis and Ureaplasma urealyticum in patients with sexually transmitted diseases. *Wien. Klin. Wochenschr.* **109,** 584–9 (1997).

455. Fiori, P. L., Rappelli, P., Addis, M. F. & Carta, F. Mycoplasma hominis parasitism of

Trichomonas vaginalis. *Lancet* **352,** 1286 (2002).

456. Dessì, D. Mycoplasma hominis and Trichomonas vaginalis: a unique case of symbiotic relationship between two obligate human parasites. *Front. Biosci.* **11,** 2028 (2007).

457. Vancini, R. G., Pereira-Neves, A., Borojevic, R. & Benchimol, M. Trichomonas vaginalis harboring Mycoplasma hominis increases cytopathogenicity in vitro. *Eur. J. Clin. Microbiol. Infect. Dis.* **27,** 259–267 (2008).

458. Darroch, J. E., Landry, D. J. & Oslak, S. Age Differences Between Sexual Partners in the United States. *Fam. Plann. Perspect.* **31,** 160 (2006).

459. Manlove, J., Terry-Humen, E. & Ikramullah, E. Young Teenagers and Older Sexual Partners: Correlates and Consequences for Males and Females. *Perspect. Sex. Reprod. Health* **38,** 197–207 (2006).

460. Zdrodowska-Stefanow, B., Kłosowska, W. M., Ostaszewska-Puchalska, I., Bułhak-Kozioł, V. & Kotowicz, B. Ureaplasma urealyticum and Mycoplasma hominis infection in women with urogenital diseases. *Adv. Med. Sci.* **51,** 250–3 (2006).

461. Njunda, A. L. *et al.* Genital mycoplasmas in women attending the Yaoundé University Teaching Hospital in Cameroon. *J. Public Health Africa* **2,** 68–71 (2011).

462. Andersen, B. *et al.* Mycoplasma qenitalium: Prevalence and behavioural risk factors in the general population. *Sex. Transm. Infect.* **83,** 237–241 (2007).

463. Zhou, Y. H., Ma, H. X., Yang, Y. & Gu, W. M. Prevalence and antimicrobial resistance of Ureaplasma spp. and Mycoplasma hominis isolated from semen samples of infertile men in Shanghai, China from 2011 to 2016. *Eur. J. Clin. Microbiol. Infect. Dis.* **37,** 729–734 (2018).

464. Kwak, D. W., Hwang, H. S., Kwon, J. Y., Park, Y. W. & Kim, Y. H. Co-infection with vaginal Ureaplasma urealyticum and Mycoplasma hominis increases adverse pregnancy outcomes in patients with preterm labor or preterm premature rupture of membranes. *J. Matern. Neonatal Med.* **27,** 333–337 (2014).

465. Edwards, J. L., Brown, E. J., Ault, K. A. & Apicella, M. A. The role of complement receptor 3 (CR3) in Neisseria gonorrhoeae infection of human cervical epithelia. *Cell. Microbiol.* **3,** 611–622 (2001).

466. Edwards, J. L. & Apicella, M. A. The molecular mechanisms used by Neisseria gonorrhoeae to initiate infection differ between men and women. *Clinical Microbiology Reviews* **17,** 965–981 (2004).

467. Batra, S. C. & Iosif, C. S. Female urethra: a target for estrogen action. *J. Urol.* **129,** 418–20 (1983).

468. D'Inzeo, T. *et al.* Comparison of Mycoplasma IES, Mycofast Revolution and Mycoplasma IST2 to detect genital mycoplasmas in clinical samples. *J. Infect. Dev. Ctries.* **11,** 98–101 (2017).

469. Mondeja, B., Jensen, J. S., Blanco, O. & Rodríguez, N. Evaluation of a new accelerated commercial micro-method for the presumptive diagnosis of Mycoplasma genitalium infections. *Int. J. Infect. Dis.* **73,** 337 (2018).

470. Beeton, M. L. & Spiller, O. B. Antibiotic resistance among Ureaplasma spp. isolates: Cause for concern? *J. Antimicrob. Chemother.* **72,** 330–337 (2017).

471. Ibrahim-Elmagboul, I. B. & Livermore, D. M. Sensitivity testing of ciprofloxacin for Pseudomonas aeruginosa. *J. Antimicrob. Chemother.* **39,** 309–317 (1997).

472. Bowie, W. R. *et al.* Etiology of nongonococcal urethritis. Evidence for Chlamydia trachomatis and Ureaplasma urealyticum. *J. Clin. Invest.* **59,** 735–742 (1977).

473. KATOH, N. Significance of Ureaplasma urealyticum and Clostridium difficile in Nongonococcal Urethritis. *J. Japanese Assoc. Infect. Dis.* **59,** 687–700 (2014).

474. Viarengo, J., Hebrant, F. & Piot, P. Ureaplasma urealyticum in the urethra of healthy men. *Sex. Transm. Infect.* **56,** 169–172 (2008).

475. Shimada, Y. *et al.* Bacterial loads of Ureaplasma urealyticum contribute to development of urethritis in men. *Int. J. STD AIDS* **25,** 294–298 (2014).

476. Strauss, M. *et al.* Detection of ureaplasma species by a semi-quantitative pcr test in urine samples: Can it predict clinical significance? *Isr. Med. Assoc. J.* **20,** 9–13 (2018).

477. Frølund, M. *et al.* The bacterial microbiota in first-void urine from men with and without idiopathic urethritis. *PLoS One* **13,** (2018).

478. Tsai, C. C. & Li, C. C. Nonchlamydial nongonococcal urethritis in men. *Urological Science* **24,** 73–77 (2013).

479. Abele-Horn, M., Scholz, M., Wolff, C. & Kolben, M. High-density vaginal ureaplasma urealyticum colonization as a risk factor for chorioamnionitis and preterm delivery. *Acta Obstet. Gynecol. Scand.* **79,** 973–978 (2000).

480. Kasper, D. C. *et al.* The bacterial load of Ureaplasma parvum in amniotic fluid is correlated with an increased intrauterine inflammatory response. *Diagn. Microbiol. Infect. Dis.* **67,** 117–121 (2010).

481. Husslein, P. *et al.* Increased intrauterine frequency of Ureaplasma urealyticum in women with preterm labor and preterm premature rupture of the membranes and subsequent cesarean delivery. *Am. J. Obstet. Gynecol.* **193,** 1663–1669 (2005).

482. Witt, A. *et al.* IL-8 concentration in maternal serum, amniotic fluid and cord blood in relation to different pathogens within the amniotic cavity. *J. Perinat. Med.* **33,** 22–26 (2005).

483. Andreoli, M. A. *et al.* Co-infection of sexually transmitted pathogens and Human Papillomavirus in cervical samples of women of Brazil. *BMC Infect. Dis.* **17,** 769 (2017).

484. Claus, H. *et al.* Ureaplasma Species Differentially Modulate Pro- and Anti-Inflammatory Cytokine Responses in Newborn and Adult Human Monocytes Pushing the State Toward Pro-Inflammation. *Front. Cell. Infect. Microbiol.* **7,** 484 (2017).

485. Glaser, K. *et al.* Ureaplasma isolates stimulate proinflammatory CC chemokines and matrix metalloproteinase-9 in neonatal and adult monocytes. *PLoS One* **13,** e0194514 (2018).

486. Vancutsem, E., Faron, G., Foulon, W. & Naessens, A. Genital tract colonization with Ureaplasma spp. And its association with abnormal vaginal flora. *Journal of Medical Microbiology* **64,** 654–656 (2015).

487. Merenstein, G. B. & Weisman, L. E. Premature rupture of the membranes: Neonatal consequences. *Semin. Perinatol.* **20,** 375–380 (1996).

488. Higgins, R. D. *et al.* Evaluation and Management of Women and Newborns with a Maternal Diagnosis of Chorioamnionitis: Summary of a Workshop. *Obstet. Gynecol.* **127,** 426–436 (2016).

489. Turner, M. A. *et al.* Ureaplasma, bronchopulmonary dysplasia, and azithromycin in European

neonatal intensive care units: a survey. *Sci. Rep.* **4,** 4076 (2014).

490. Jan, A. I., Ramanathan, R. & Cayabyab, R. G. Chorioamnionitis and Management of Asymptomatic Infants ≥35 Weeks Without Empiric Antibiotics. *Pediatrics* **140,** e20162744 (2017).

491. Xiaolei, C., Tao, H., Zongli, S. & Hongying, Y. The role of ureaplasma urealyticum infection in cervical intraepithelial neoplasia and cervical cancer. *Eur. J. Gynaecol. Oncol.* **35,** 571–575 (2014).

492. Bachmann, L. H. *et al.* Advances in the Understanding and Treatment of Male Urethritis. *Clin. Infect. Dis.* **61,** S763–S769 (2015).

493. Wetmore, C. M., Manhart, L. E. & Golden, M. R. Idiopathic Urethritis in Young Men in the United States: Prevalence and Comparison to Infections with Known Sexually Transmitted Pathogens. *J. Adolesc. Heal.* **45,** 463–472 (2009).

494. Allsworth, J. E. & Peipert, J. F. Prevalence of bacterial vaginosis: 2001-2004 National Health and Nutrition Examination Survey data. *Obstet. Gynecol.* **109,** 114–120 (2007).

495. Mohammadzadeh, F., Dolatian, M., Jorjani, M. & Alavi Majd, H. Diagnostic Value of Amsel's Clinical Criteria for Diagnosis of Bacterial Vaginosis. *Glob. J. Health Sci.* **7,** 8–14 (2014).

496. Fernández, J., Karau, M. J., Cunningham, S. A., Greenwood-Quaintance, K. E. & Patel, R. Antimicrobial Susceptibility and Clonality of Clinical Ureaplasma Isolates in the United States. *Antimicrob. Agents Chemother.* **60,** 4793–4798 (2016).

497. Xiao, L. *et al.* Chromosomal mutations responsible for fluoroquinolone resistance in Ureaplasma species in the United States. *Antimicrob. Agents Chemother.* **56,** 2780–3 (2012).

498. Kawai, Y. *et al.* In Vitro Activity of Five Quinolones and Analysis of the Quinolone Resistance-Determining Regions of gyrA, gyrB, parC, and parE in Ureaplasma parvum and Ureaplasma urealyticum Clinical Isolates from Perinatal Patients in Japan. *Antimicrob. Agents Chemother.* **59,** 2358–2364 (2015).

499. Meygret, A., Pereyre, S., Renaudin, H., Bébéar, C. & Le Roy, C. Tetracycline and fluoroquinolone resistance in clinical Ureaplasma spp. and Mycoplasma hominis isolates in France between 2010 and 2015. *J. Antimicrob. Chemother.* **73,** 2696–2703 (2018).

500. Beeton, M. L., Chalker, V. J., Jones, L. C., Maxwell, N. C. & Spiller, O. B. Antibiotic resistance among clinical Ureaplasma isolates recovered from neonates in England and Wales between 2007 and 2013. *Antimicrob. Agents Chemother.* **60,** 52–56 (2016).

501. Takahashi, S. *et al.* Clinical efficacy of levofloxacin 500 mg once daily for 7 days for patients with non-gonococcal urethritis. *J. Infect. Chemother.* **17,** 392–396 (2011).

502. Messano, G. A., Cancrini, F. & Marsella, L. T. Antibiotic resistance and defensive medicine, a modern challenge for an old problem: the case of Ureaplasma urealyticum. *Ig Sanita Pubbl* **70,** 295–304 (2014).

503. Karabay, O. *et al.* Prevalence and antibiotic susceptibility of genital Mycoplasma hominis and Ureaplasma urealyticum in a university hospital in Turkey. *Clin. Exp. Obstet. Gynecol.* **33,** 36–38 (2006).

504. Taylor-Robinson, D. Antibiotic susceptibilities of mycoplasmas and treatment of mycoplasmal infections. *J. Antimicrob. Chemother.* **40,** 622–630 (2002).

505. Hall, J. V. *et al.* The multifaceted role of oestrogen in enhancing chlamydia trachomatis

infection in polarized human endometrial epithelial cells. *Cell. Microbiol.* **13,** 1183–1199 (2011).

506. Zariffard, M. R., Saifuddin, M., Sha, B. E. & Spear, G. T. Detection of bacterial vaginosis-related organisms by real-time PCR for Lactobacilli, Gardnerella vaginalis and Mycoplasma hominis. *FEMS Immunol. Med. Microbiol.* **34,** 277–281 (2002).

507. Colaizy, T. T., Kuforiji, T., Sklar, R. S. & Pillers, D. A. M. PCR methods in clinical investigations of human ureaplasmas: A minireview. *Molecular Genetics and Metabolism* **80,** 389–397 (2003).

508. S.A., M. *et al.* Urogenital Chlamydia trachomatis serovars in men and women with a symptomatic or asymptomatic infection: An association with clinical manifestations? *J. Clin. Microbiol.* **38,** 2292–2296 (2000).

509. Caliendo, A. M. *et al.* Real-time PCR improves detection of Trichomonas vaginalis infection compared with culture using self-collected vaginal swabs. *Infect. Dis. Obstet. Gynecol.* **13,** 145–150 (2005).

510. de Waaij, D. J., Ouburg, S., Dubbink, J. H., Peters, R. P. H. & Morré, S. A. Evaluation of Prestoplus assay and LightMix kit Trichomonas vaginalis assay for detection of Trichomonas vaginalis in dry vaginal swabs. *J. Microbiol. Methods* **127,** 102–104 (2016).