PREVALENCE AND MOLECULAR ANALYSIS OF
MYCOPLASMA GENITALIUM STRAINS ISOLATED
FROM PREGNANT WOMEN AT DR GEORGE
MUKHARI ACADEMIC HOSPITAL.

By

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DECLARATION

I ………………………………………………………………………..declare that this thesis is my own original work and, to the best of my knowledge and belief, contains no material previously published or written by another person except where due reference is made. It has not been previously submitted to meet requirements for an award at this or any higher institution.

____________________________________  ____ day of _____ 20 _____

Signature of candidate
ACKNOWLEDGEMENTS

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ABSTRACT

Background: *Mycoplasma genitalium* is a sexually transmitted pathogen causing cervicitis, abnormal vaginal discharge, urethritis, pelvic inflammatory disease (PID) and infertility in women. As this organism cannot be cultured on standard laboratory media, nucleic acid amplification tests (NAATs) have become the gold standard of detection. Patients with symptoms are treated syndromically, regardless of the detection of *C. trachomatis* or *M. genitalium*, with tetracyclines, fluoroquinolones or macrolides, depending on local recommendations. Data is scanty regarding prevalence and antimicrobial resistance of *M. genitalium* in South Africa due to the syndromic treatment approach adopted in this country.

Aim: The study was aimed at determining the prevalence, antimicrobial resistance and the molecular characteristics of *M. genitalium* strains from pregnant women attending the termination of pregnancy (TOP) and antenatal clinics (ANC) at Dr George Mukhari Academic Hospital (DGMAH).

Methods: Vaginal swabs were collected from 100 consenting women attending the TOP clinic (50) and ANC (50) at DGMAH between June and December 2015. Following DNA extraction, all the specimens were tested for the presence of *M. genitalium* by two molecular methods: i.e. conventional polymerase chain reaction (PCR), and a commercial real-time polymerase chain reaction (q-PCR). Amplification of the *gyrA, parC* and V region of the *M. genitalium* 23S rRNA gene were performed on all *M. genitalium*-positive isolates and the amplicons were sequenced. All the *Mycoplasma genitalium* isolates were typed using both the MG191 (mgpB) single nucleotide polymorphism (SNP) and MG309 variable number tandem repeat (VNTR) analysis methods.
Results: Of the 100 specimens, 6.0% were positive with 4.0% from the TOP and 2.0% from the ANC using conventional PCR. The q-PCR assay detected *M. genitalium* in two more samples than the conventional PCR (8; 8.0%). Of these, 4.0% (4) were from the TOP and 4.0% (4) from the ANC. None of the *M. genitalium* isolated strains harbored mutations in the *gyrA* gene associated with fluoroquinolone resistance. Five of the 6 strains had a silent mutation (C234T) in the *parC* gene while one strain had the G248T mutation (Ser→Ile 80) which has been previously described for fluoroquinolone resistance. A third (2/6) *M. genitalium* strains were resistant to macrolides, based on the presence of the resistance associated mutation, A2059G. Four different sequence types (STs) were detected following MG191 SNP typing and four different VNTR patterns were seen after typing with MG-309-VNTR. With both typing methods, two major clusters were seen with the resistant strains grouping together in one cluster. The strains from the TOP and ANC clinics clustered together.

Conclusion: *Mycoplasma genitalium* is prevalent in women attending both the TOP and ANC clinics at DGMAH. Macrolide resistance was reported in this study. Fluoroquinolone resistance of *M. genitalium* was for the first time seen in South Africa. Resistance against macrolides and fluoroquinolones in this and studies all over the world continues to stress the need for alternative treatment options especially against macrolide resistant *M. genitalium* genotypes. Epidemiological surveillance of circulating *M. genitalium* strains and their resistance patterns is important in order to continue adapt treatment to *M. genitalium* infections.
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LIST OF ABBREVIATIONS

A  Adenine
ANC  Antenatal Clinic
ATTC  American Type Culture Collection
Bp  Base pair
C  Cytosine
CDC  Centers for disease control
CE  Communauté Européenne
CI  Confidence Interval
Ct  Threshold cycle
DGMAH  Dr George Mukhari Academic Hospital
DLST  Dual locus sequence type
DNA  Deoxyribose Nucleic Acid
dNTP  Deoxyribose nucleotide triphosphate
DOH  Department of Health
E. coli  Escherichia coli
Eg.  Exempli gratia (for example)
Et al  And others
FDA  Food and Drug Administration
FVU  First void urine
G  Guanidine
GUT  Genitourinary Infections
His  Histidine
HIV  Human Immune Virus
ICSB  International Committee on Systematic Bacteriology Subcommittee
kbp  Kilo base pair
kDa  Kilo daltons
LAMP  Loop mediated isothermal amplification
Met  Methionine
MgCl₂  Magnesium chloride
MG  Mycoplasma genitalium
M. genitalium  Mycoplasma genitalium
MgPa  Mycoplasma genitalium adhesion gene
MLVA  Multi-locus VNTR analysis
MSM  Men who have sex with men
NAAT  Nucleic acid amplification test
ND  Not done
NGU  Non-gonococcal urethritis
NIH  National Institutes of Health
P  Primary
PCR  Polymerase chain reaction
PID  Pelvic inflammatory disease
q-PCR  Real-time polymerase chain reaction
QRDR  Quinolone resistance determining region
RFLP  Restriction fragment length polymorphism
rRNA  Ribosomal ribonucleic acid
SARA  Sexually acquired reactive arthritis
SEM  Scanning electron microscopy
Ser  Serine
SMUREC  Sefako Makgatho University Research and Ethics Committee
SNP  Single nucleotide polymorphism
SM  Size marker
SN  Semi-nested
SP  Sucrose phosphate
ST(s)  Sequence type(s)
STI(s)  Sexually transmitted infection(s)
STR  Short tandem repeat
T  Thymine
TBE  Tris borate EDTA
TEM  Transmission electron microscope
T_m  Melting temperature
TMA  Transcription mediated amplification
TOP  Termination of pregnancy
tRNA  Transfer ribonucleic acid
UK  United Kingdom
USA  United States of America
VNTR  Variable-number tandem-repeat
LIST OF SYMBOLS

& And
\( \beta \) Beta
\( ^\circ \text{C} \) Degrees Celsius
\( g \) Gram / gravity
> Greater than
< Less than
L Liter
\( \mu \) Micro
m Meters
mg Milligram(s)
min Minutes
ml Milliliter(s)
mM Millimolar(s)
n Nano
-ve Negative
% Percentage
pH Potential of hydrogen
pmole Picomole(s)
+ve Positive
S Svedberg unit
Sec Second(s)
U Unit
CHAPTER 1: INTRODUCTION AND PROPOSAL

1.1 INTRODUCTION

*Mycoplasma genitalium* was first isolated in 1980 from men with non-gonococcal urethritis (NGU) (Tully *et al.*, 1981) and has since been established as a sexually transmitted infection (Svenstrup *et al.*, 2005) responsible for 20–35% of non-chlamydial NGU, as well as cervicitis. Studies have also shown the role of *M. genitalium* in other conditions such as endometritis, pelvic inflammatory disease (PID) and tubal factor infertility in women (Cohen *et al.*, 2002; Deguchi *et al.*, 2002; Jensen *et al.*, 2004) and balanoposthitis in men (Horner & Taylor-Robinson, 2011). As *M. genitalium* is a fastidious organism, it is difficult to make a diagnosis with conventional culture techniques and diagnosis requires the use of sensitive nucleic acid amplification tests (NAATs).

*M. genitalium* is not usually recommended among organisms for routine testing in sexually transmitted infection screening and treatment follows syndromic guidelines (Workowski *et al.*, 2015). The current recommended treatment for *M. genitalium* infection is the macrolide, azithromycin (Workowski *et al.*, 2015) however, treatment failure with single-dose azithromycin is increasingly evident (Bradshaw *et al.*, 2006; Jensen *et al.*, 2008). Moxifloxacin is used as a second-line treatment for *M. genitalium*-associated NGU and cervicitis (Twin *et al.*, 2012). Fluoroquinolone resistance–associated mutations have been detected in *M. genitalium* from patients exhibiting fluoroquinolone treatment failure (Hamasuma *et al.*, 2011). For the development of effective treatment, reliable typing of pathogenic microorganisms is required. Since *M. genitalium* is very difficult to culture from clinical specimens classical microbiological typing methods are not readily applicable. Typing of *M.*
*M. genitalium* strains relies on DNA sequence data. The majority of *M. genitalium* clinical sequence data available today is based upon the gene *mgpB* (locus MG191) of sequenced reference strain G37 (Musatovova & Baseman, 2009).

### 1.2 PROBLEM STATEMENT

*M. genitalium* is a sexually transmitted pathogen causing cervicitis, abnormal vaginal discharge, urethritis, PID and infertility (Manhart *et al*, 2003; Cohen *et al*, 2002) in women. As this organism cannot be cultured on standard laboratory media, NAATs have become the gold standard of detection. However, there is not a specific NAAT prescribed, and every laboratory uses their preferred test. By comparing some of these tests, one can find a test best suited for a specific laboratory and/or objective.

Patients with symptoms are treated syndromically, regardless of the detection of *C. trachomatis* or *M. genitalium*, with tetracyclines, fluoroquinolones or macrolides, depending on local recommendations. In this setting, the syndromic treatment approach, as prescribed by the South African Department of Health, is used. In February 2015, the recommended treatment has changed from a 7 day course of doxycycline to a single dose of azithromycin (DOH, 2015). Studies have indicated treatment failures with doxycycline, as well as azithromycin and fluoroquinolones in various parts of the world (Mroczowski *et al*, 2006; Bradshaw *et al*, 2006). Since *M. genitalium* cannot be cultured on standard laboratory media, quantifying PCR techniques have been used to monitor antimicrobial resistance, by detecting bacterial loads before and after treatment (Deguchi *et al*, 2002). The detection of resistance genes is therefore of clinical significance as mutations of these genes may confirm resistance to antimicrobial drugs (Jensen *et al*, 2008).
Within any bacterial species there is a lot of genetic variability. Sometimes this affects pathogenicity or antimicrobial resistance but not always. This variability is important to track down a particular strain of a species which might be the cause of an infection. As *M. genitalium* cannot be cultured, typing of strains relies on DNA sequence data. It was shown that although there is high intra-strain stability, high levels of sequence variability between clinical isolates are seen (Musatovova & Baseman, 2009). It may be that a specific sequence type may be associated with antimicrobial resistance. Comparison of the prevalence and strain variability of *M. genitalium* isolated from high risk pregnant women (termination of pregnancy clinic attendees) and normal pregnant women (women attending the antenatal clinic) will contribute to the knowledge of transmission, pathogenicity and management of this organism locally. Due to the syndromic treatment approach, limited data on the prevalence and antimicrobial resistance of *M. genitalium* in South African women is available (Mhlongo *et al*, 2010; Hay *et al*, 2015).

### 1.3. PURPOSE OF THE STUDY

#### 1.3.1 Research question

What are the prevalence and molecular characteristics of *M. genitalium* strains isolated from pregnant women attending the termination of pregnancy (TOP) and antenatal clinics (ANC) at Dr George Mukhari academic hospital?

**Secondary questions:**

- What is the prevalence of *M. genitalium* in pregnant women attending DGMAH?
- What is the prevalence of mutations in genes associated with resistance to fluoroquinolones (*gyrA*, *parC*, v-region of 23S rRNA)?
• Which *M. genitalium* strains are circulating in area served by DGMAH and what is the prevalence of these strains?
• What is the association between the presence of resistance genes (*gyrA*, *parC*, v-region of 23S rRNA) and *M. genitalium* genotype?

1.3.2 Aim of the study

To determine the prevalence and the molecular characteristics of *M. genitalium* strains from pregnant women attending the termination of pregnancy and antenatal clinics at DGMAH.

1.3.3 Objectives

i. To determine the prevalence of *M. genitalium* in pregnant women attending DGMAH using conventional and real-time PCR (q-PCR) assays.

ii. To compare the assays as detection method for *M. genitalium* from vaginal isolates.

iii. To detect genotypic resistance markers of macrolide and fluoroquinolones by sequence analysis of the 23S ribosomal RNA, *gyrA*, and *parC* genes.

iv. To genotype isolated strains using the two main *M. genitalium* molecular typing methods: mgpB single nucleotide polymorphism (SNP) typing and an analysis of a variable number tandem repeat (VNTR) marker in MG309.

v. To determine an association between the prevalence of resistance genes and the different *M. genitalium* genotypes.
CHAPTER 2: LITERATURE REVIEW

2.1 BACKGROUND

*M. genitalium* was first isolated in 1980 from two of 13 men with non-gonococcal urethritis (NGU) (Tully *et al*, 1981). *M. genitalium* is the smallest existing self-replicating prokaryote, lacks a cell wall and has a genome consisting of only 580 kbp (Taylor-Robinson, 1995). It is believed to have evolved from gram-positive bacteria through a process of degenerative evolution, leading to the loss of many ancestral genes and the reduction of its genome (Fraser *et al*, 1995). *M. genitalium* is one of fourteen mycoplasma species of human origin. It is an extremely slow-growing and fastidious bacterium, and its role as a pathogen in human disease was not established until the first diagnostic PCRs were developed in the early 1990’s (Jensen *et al*, 1991; Palmer *et al*, 1991). Male NGU was the first syndrome unequivocally associated with *M. genitalium* infection (Jensen *et al*, 1993; Horner *et al*, 1993). In women, several studies have demonstrated the association between *M. genitalium* and urethritis, cervicitis, endometritis, and PID (Cohen *et al*, 2002; Manhart *et al*, 2003; Cohen *et al*, 2005; Anagrius *et al*, 2005; Falk *et al*, 2005).

There are no estimates of the global burden of disease. Prevalence estimates are variable as a wide variation in the sensitivity of detection assays is present and there is no agreed gold standard. In sexually transmitted infection (STI) patients, the prevalence is usually from 60 to 85% of that of *C. trachomatis*, but in the general population, the ratio is generally significantly lower (Andersen *et al*, 2007; Manhart *et al*, 2007). *M. genitalium* is under-researched in South Africa and other developing countries, mainly because the organism
cannot readily be cultured due to its slow cell replication and fastidious growth requirements (Taylor-Robinson & Horner, 2001).

Resistance has developed against the treatment of choice such as tetracyclines, macrolides and fluoroquinolones (Björnelius et al, 2008). Azithromycin is currently the treatment of choice when treating *M. genitalium* infections on account of its long half-life, excellent tissue penetration, and the fact that it can be administered as a single-dose treatment (Ross & Jensen, 2006). However, resistance against azithromycin has become widespread since first reported in Australia in 2006 (Bradshaw et al, 2006; Taylor-Robinson, 2014).

Within any bacterial species there is a lot of genetic variability. Sometimes this affects pathogenicity or antimicrobial resistance but not always. Typing of *M. genitalium* strains relies on DNA sequence data and recently, high levels of sequence variability between clinical isolates have been observed (Musatovova & Baseman, 2009). Typing may also provide a way of predicting antimicrobial resistance. Pond *et al* (2014) used *M. genitalium* typing of clinical strains to assign macrolide resistance to two sequence types.

As *M. genitalium* is linked to cervicitis, the acquisition of other STIs, and infertility, efforts to detect and treat these infections could influence the sexual health of a great number of women worldwide.

### 2.2 CHARACTERISTICS OF MYCOPLASMA GENITALIUM

Most of the characteristics of *M. genitalium* are known through the findings from its thoroughly studied, genetically close relative, *Mycoplasma pneumoniae* (Dalio *et al*, 1989; Jensen, 2006).
2.2.1 Taxonomy

For many years *Mycoplasmas* were considered as viruses as they were capable of passing through filters meant to trap bacteria (Freundt *et al*, 1979). They were classified as bacteria later in the 1930s following a clearer understanding of viral concepts (Prescott *et al*, 2005). The International Committee on Systematic Bacteriology Subcommittee (ICSB) differentiate new mycoplasma species based on key defining characteristics such as their filterability and their inability to form a cell wall after incubation in a medium without antibiotics (ICSB, 1995).

According to the established taxonomy, *Mycoplasmas* belong to the Class Mollicutes [*mollis* (soft); *cutis* (skin)] which encompasses bacteria without a cell wall which are also commonly referred to as ‘naked bacteria’ (Jensen, 2006). The order Mycoplasmatales has a single family, Mycoplasmataceae. Both *Mycoplasma* and *Ureaplasma* genera belong to this family.

All species of *Mycoplasmas* belong to either one of these two genera: *Mycoplasma* and *Ureaplasma* (Taylor-Robinson & Furr, 1997). Mycoplasma contains more than 100 species but *M. genitalium* is one of only 14 *mycoplasmas* of human origin. Most of these species are known to be commensals either in the respiratory or urogenital tracts, and three of these species (*M. genitalium*, *U. parvum*, and *U. urealyticum*) are associated with genitourinary infections (GUT) (Schwartz & Hooton, 1998; Taylor-Robinson & Furr, 1997).
2.2.2 Morphology

The genus *Mycoplasma* contains very small bacteria, with sizes ranging from 0.2 to 0.7 micrometers (µm) depending on the shape of the species (Taylor-Robinson, 1995). The shape depends on the particular mycoplasma species, which may be spherical, filamentous or flask/pear-like (Stein & Baseman, 2005). *M. genitalium* and *M. pneumoniae* have the characteristic pear/flask-like morphology with a terminal/apical tip organelle (Figure 2.1) (Taylor-Robinson, 1995).

*M. genitalium* can be viewed under the transmission electron microscope (TEM) since it is too small to be visible under an ordinary light microscope (Tully et al, 1983), however there are possibilities to observe coccoid bodies of this organism with immunofluorescence, phase-contrast or a dark-field microscopy as its small size is believed to be just on the threshold of light microscopy (Taylor-Robinson, 1995).

The two wild type strains of *M. genitalium* (G-37 and M-30) were found by electron microscopy to be 0.6 - 0.7 µm in length, 0.3 - 0.4 µm wide near the base while it is only 0.06 - 0.08 µm wide at the terminal tip (Le Roux & Hoosen, 2010). The core of the tip has dense parallel tracts called a nap or terminal organelle. The nap protrudes from the main cell and is the structure responsible for giving these bacteria a pear-like appearance (Tully et al, 1983). The terminal organelle is responsible for attachment which facilitate adherence of *M. genitalium* to epithelia of eukaryotic host cells including other types of cells such as red blood cells, Vero monkey kidney cells and even object surfaces like plastic. This terminal tip is also responsible for locomotion of this organism as it enables it to glide along moist/mucous surfaces (Tully et al, 1981; Stein & Baseman, 2005).
M. genitalium does not have a peptidoglycan cell wall and therefore lacks cell surface markers. The absence of a cell wall also means that this bacterium has less osmotic stability in the host environment and is therefore prone to changes in its flask-like shape. This lack of a cell wall is a feature that is largely responsible for the two biologic properties of M. genitalium namely, no Gram stain reaction and non-susceptibility to common antimicrobials of the β-lactam class that inhibit bacterial cell wall synthesis (Taylor-Robinson, 1995).

### 2.2.3 Genomic organisation

In 1995 the first complete genome sequence of a free-living organism (Haemophilus influenzae) was published (Fleischmann et al, 1995) and this was closely followed by the M. genitalium genome sequence (Fraser et al, 1995). M. genitalium has the smallest genome of the Mollicutes and at only 580 kbp is the pathogenic bacterium with the smallest genome (Fraser et al, 1995).

The small genome of M. genitalium gives a good indication of the minimal set of genes
needed to sustain bacterial life. The minimum set of genes, also called essential genes, in both prokaryotes and eukaryotes, are those described as indispensable for the survival of an organism and are therefore the basis of life for a particular organism. Glass et al. (2006) identified 382 of the 482 *M. genitalium* protein-coding genes as essential. A more recent study (Zhang & Lin, 2009) showed that *M. genitalium* needed only 381 essential genes compared to the 642 required by *H. influenzae*. This highlights how the very small *M. genitalium* is capable to survive on its own. It has also been found that *M. genitalium*, unlike other bacteria, uses UGA to code for tryptophan instead of a stop codon, suggesting that expression of its genes is complicated since it would synthesize truncated proteins (Seto et al, 2001).

The Guanidine plus Cytosine (G+C) content in the DNA of most *mycoplasmas* is low, ranging from 24% to 33%, with *M. genitalium* at 32% (Jensen, 2006). The significance of the low G+C content is that *M. genitalium* would have a lower melting temperature (T_m) during the double-stranded DNA denaturation stage of PCR assays, making it convenient for DNA amplification (Bizarro & Schuck, 2007). However, in the Mollicutes, genes coding for rRNA and tRNA have a higher G+C content than the rest of the genome with an average of 44% and 52% respectively. This is to help maintain their secondary structures (Fraser et al, 1995; Razin, 1997).

### 2.3 CLINICAL MANIFESTATIONS OF *MYCOPLASMA GENITALIUM*

*M. genitalium* is a well-known sexually transmitted pathogen capable of causing a wide range of symptom-pathologies in women and men (Taylor-Robinson & Jensen, 2011).
2.3.1 Transmission

Transmission of *M. genitalium* is primarily by direct genital-genital mucosal contact with inoculation of infected secretions as illustrated by a high concordance rate of identical DNA types in sexual partners (Hjorth *et al.*, 2006). Genital-anorectal transmission has been shown (Edlund *et al.*, 2012) and may play a role as *M. genitalium* is commonly found in the anal mucosa (Soni *et al.*, 2010; Lillis *et al.*, 2011). Oral-genital contact is less likely to contribute to any significant extent, as carriage of *M. genitalium* in the oro-pharynx is low. Mother-to-child transmission at birth has not been systematically studied, but *M. genitalium* has been detected in the respiratory tract of new-born children (Luki *et al.*, 1998). The risk of contracting *M. genitalium* per sexual encounter has not been determined, but because *M. genitalium* is present in lower concentration in genital tract specimens than *C. trachomatis* (Walker *et al.*, 2013), it could be considered slightly less contagious than chlamydia.

2.3.2 *Mycoplasma genitalium* infection in women

*M. genitalium* is an emergent sexually transmissible pathogenic agent associated with several inflammatory reproductive tract syndromes in women (Taylor-Robinson & Jensen, 2011). Several studies have demonstrated the association between *M. genitalium* and urethritis, cervicitis, endometritis, and PID (Cohen *et al.*, 2002; Manhart *et al.*, 2003; Cohen *et al.*, 2005; Anagrius *et al.*, 2005; Falk *et al.*, 2005). This organism can infect different parts of the endometrium, cervix as well as the fallopian tubes and the extent of infection might be determined by the anatomic site involved (McGowin & Anderson-Smits, 2011). Although 40 – 75% of STD clinic attendees are asymptomatic, symptoms observed are related to cervical and urethral infections and include increased or altered vaginal discharge (<50%), dysuria or urgency (30%) and, rarely, inter-menstrual or post coital bleeding or menorrhagia (Falk *et al.*, 2005).
2005; Anagrius et al, 2005; Bjartling et al, 2012). Complications in women may include PID (endometritis, salpingitis), miscarriage (Edwards et al, 2006), tubal factor infertility (probably) and sexually acquired reactive arthritis (SARA) (Taylor-Robinson & Keat, 2015).

While there are less data in pregnancy, *M. genitalium* has been associated with preterm birth, and spontaneous abortion, but the prevalence of *M. genitalium* in pregnant women has generally been low in many settings (Oakeshott et al, 2004; Peuchant et al, 2015) and therefore, the relative importance of *M. genitalium* as a cause of adverse pregnancy outcome is probably rather small.

### 2.3.3 *Mycoplasma genitalium* infection in men

Male NGU was the first syndrome unequivocally associated with *M. genitalium* infection (Jensen et al, 1993; Horner et al, 1993) and confirmed in a meta-analysis including 37 studies up to 2010 (Taylor-Robinson & Jensen, 2011). *M. genitalium* is now commonly the second most frequent etiological agent in patients with NGU after *C. trachomatis*. Falk et al (2004) has shown that 70% of men infected with *M. genitalium* are symptomatic. Le Roux et al (2010) reported a *M. genitalium* prevalence of 17.3% among South African men with urethritis. Infection is characterised by urethritis (acute, persistent, and recurrent), dysuria and urethral discharge (Taylor-Robinson & Jensen, 2011).

Balanoposthitis has been associated with *M. genitalium* infection in one study (Horner & Taylor-Robinson, 2011) and it has been shown to cause rectal infections in men who have sex with men (MSM) (Reinton et al, 2013). *M. genitalium* may also play a role in male infertility. Sperm concentration significantly decreases in *M. genitalium* positive male patients (Gdoura et al, 2007) and as this pathogen has the ability to adhere to all parts of the
human spermatozoa it may result in sperm agglutination and immortality (Svenstrup et al, 2003; Rane et al, 2014).

2.4 EPIDEMIOLOGY OF MYCOPLASMA GENITALIUM

Before the development of the first PCR assay for detection of *M. genitalium*, prevalence studies have been scanty due to the fact that it is extremely difficult to culture the organism and there was no convenient test method to detect this illusive organism (Palmer et al, 1991). With the introduction of *M. genitalium* PCR assays the number of prevalence studies increased throughout the world, shedding light on its prevalence and pathogenesis (Daley et al, 2014). It was shown that prevalence patterns tend to differ between studies with *M. genitalium* prevalence rates ranging from 0% to as high as 47.5% in different population samples (Daley et al, 2014; McGowin & Anderson-Smits, 2011).

There are various reasons to explain the difference in prevalence found between studies. These include the specimen type as researchers may prefer swabs, urine or semen which may differ in terms of specificity and sensitivity (Daley et al, 2014). Furthermore, the manner in which the specimens are collected (self-collected or collected by the clinician) and processed can also influence this difference in prevalence reported (McGowin & Anderson-Smits, 2011). In addition, the study population targeted by researchers differs vastly as many studies targets the so-called ‘high risk’ populations such as sex workers, men with urethritis, women with PID, HIV-positive people, young participants or men and women with infertility. These include settings such as sexual health clinics, primary care, chlamydia screening programmes and further education colleges (Andersen et al, 2007; Svenstrup et al, 2014). Consequently, this always skews the outcome prevalence of this organism to be very high as compared to the so-called ‘low risk’ populations and thus rendering such studies biased (Daley et al,
Like any other STI, there are several risk factors which help perpetuate the prevalence of these bacteria. Data collected from various studies concerning the risk factors for *M. genitalium* infections are somehow inconsistent and contradictory and this is mainly due to the fact that populations studied to obtain these data are different (Daley *et al*, 2014).

In a study at a TOP clinic in Denmark, *M. genitalium* was detected in less than 1% of the 102 participants (Baczynska *et al*, 2008), while Huppert *et al* (2008) detected *M. genitalium* in 22.4% of the 331 women recruited from an urban medical centre in Cincinnati, USA. The prevalence of *M. genitalium* in the general population is very low, as was shown by Andersen *et al* (2007) where 2.3% women aged 21 - 23 years tested positive for the organism. Sonnenberg *et al* (2015) conducted a national survey in Britain where urine samples were collected from 4507 sexually experienced participants, aged 16–44 years, and tested for *M. genitalium*. The prevalence was 1.2% in men and 1.3% in women. There were no positive tests in men aged 16–19, and prevalence peaked at 2.1% (1.2–3.7%) in men aged 25–34 years. In women, the prevalence of *M. genitalium* was the highest in 16–19 year olds, at 2.4% and decreased with age.

Few studies on the prevalence of *M. genitalium* in South African women have been done. In a study by Mhlongo *et al* (2010) a total of 300 samples were collected from women with vaginal discharge syndrome (Cape Town, 94; Johannesburg, 206). It was shown that patients in Johannesburg had statistically significantly more *M. genitalium* than those in Cape Town (12.2% vs 2.1%). A similar prevalence (10.0%) was seen in a study in 2010 among 88 TOP clinic attendees at the DGMAH in Ga-Rankuwa (De Villiers *et al*, 2010). The same infection rate (10.8%) was also reported by Hay *et al* (2015) among 601 woman visiting primary health care clinics across Mopani District, Limpopo province, South Africa.
2.5 LABORATORY DIAGNOSIS

Before the introduction and dominance of molecular techniques which are based on molecular analysis of genomic DNA, ribosomal RNAs, cell proteins and lipids, identification and laboratory diagnosis of *Mycoplasmas* was difficult, and was based on conventional culture and microscopical methods (Razin, 2005). The type of specimen (eg. urine, endourethral swabs, endocervical swabs) taken also plays a role in the identification of the pathogen (Jensen, 2006).

2.5.1 Conventional methods for detecting *Mycoplasma genitalium*

The lack of a cell wall and the fact that *M. genitalium* is very small, make it impossible to view under an ordinary light microscope. Gram stain or other staining techniques are not useful as *M. genitalium* has no peptidoglycan layer in its membrane structure. The organism can only be clearly observed under the TEM or the scanning electron microscope (SEM) (Tully et al, 1983; Prescott et al, 2005).

In 1979, Tully and others designed a sucrose phosphate based culture medium (SP4) at the National Institutes of Health (NIH), Maryland, USA to isolate *mycoplasmas* and *spiroplasmas* (Tully et al, 1979). This medium played a major breakthrough in the discovery of *M. genitalium* as it was used to isolate *M. genitalium* for the very first time from two men with NGU at St. Mary’s Hospital, London, United Kingdom (Tully et al, 1981).

With the knowledge that *M. genitalium* is a highly fastidious bacteria, the SP4 medium was comprised of mycoplasma broth base supplemented with glutamine, bovine serum, yeast extract, and penicillin as all *Mycoplasmas* bare natural resistance against β-lactam antimicrobials. Phenol
red was included as the indicator and a colour change from red to yellow, as well as the increase in turbidity, was a confirmation of Mycoplasmal growth. Although there have been several attempts following the initial successful isolation of two *M. genitalium* strains using SP4 medium by Tully and co-workers (Tully *et al*, 1979), subsequent isolation of this organism proved to be difficult, even in the recent years where cutting edge diagnostic techniques are available. Most laboratories that study *M. genitalium*, have adapted the organism to grow on cell culture lines. It has been successfully isolated using cell cultures such as Vero cell lines extracted from monkey kidney cells (Jensen *et al*, 1996; Totten *et al*, 2001, Hamasuna *et al*, 2007; Mondeja *et al*, 2016), although this approach is extremely time-consuming and requires dedicated laboratories.

Due to the antigenic similarities between *M. genitalium* and *M. pneumoniae*, there is a significant rate of cross-response between the two species. This has essentially hampered the utilization of specific serology for the analysis and epidemiological investigations of *M. genitalium*. Various serological procedures have been utilized to distinguish *M. genitalium*; however in most studies the diagnostic performance has not been validated (Jensen, 2006).

### 2.5.2 Specimens used for diagnosis

Although many studies ignored the fact that the type of specimen collected might influence the optimal detection of *M. genitalium*, some studies have reported preferences in the type of specimens collected. These include: first void urine, endourethral- and endocervical swabs (Jensen *et al*, 2004a; Wroblewski *et al*, 2006; Carlsen & Jensen, 2010). The processing of specimens before freezing, in particular those from women, appears to be important in maintaining a high detection rate (Carlsen & Jensen, 2010). Jensen *et al* (2004a) demonstrated that first void urine (FVU) specimens from either men or women had a higher
sensitivity compared to vaginal swab specimens which is commonly used. However, contrasting results were reported by Wroblewski et al (2006), who observed that vaginal specimens provided a greater sensitivity compared to both cervical or urine specimens.

### 2.5.3 Molecular techniques

The development of molecular technology had a major impact on the detection of *M. genitalium* in clinical specimens. After the initial studies using DNA probes (Hooton et al, 1988), the development of conventional PCR assays allowed for the first comprehensive studies on *M. genitalium* (Jensen et al, 1991). A real-time PCR assay was developed in 2002 by Yoshida et al. Gen-Probe® has developed a research transcription mediated amplification (TMA) assay for the detection of *M. genitalium* that was first evaluated in a clinical study in 2006 (Hardick et al, in 2006). In 2015, Edwards et al published a loop mediated isothermal amplification (LAMP) assay for the detection of *M. genitalium*.


The initial PCR assays targeted the 95kD adhesin gene (encoding the major surface protein MgPa) of *M. genitalium*. When these assays were compared, it was found that although they targeted in rather conserved parts of the MgPa gene, a mutation sometimes occurred, leading to the misdiagnosis of *M. genitalium* (Deguchi et al, 1995). A modification of the MgPa-1/MgPa-3 assay of Jensen et al (1991) was published and has been used on most studies, eg. on male urethritis (Totten et al, 2001), mucopurulent cervicitis (Manhart et al, 2003), and
endometritis (Cohen *et al*, 2002). This primer pair has also been used as confirmatory assay in the development of 16S rRNA PCR assays (Jensen, 2006). This primer pair is also often used in multiplex PCR assays to detect *M. genitalium* simultaneously with other urethritis-causing STI-bacteria such *C. trachomatis*, *Neisseria gonorrhoeae* and *Trichomonas vaginalis* (Mahoney *et al*, 1997; Le Roy *et al*, 2012) and in typing studies (Hjorth *et al*, 2006; Cazanave *et al*, 2012; Pond *et al*, 2014).

The first real-time PCR assay for *M. genitalium* was developed by Yoshida *et al*. in 2002, based on the detection of the *M. genitalium* 16S rRNA gene. The conventional and real-time assays were compared by Edberg *et al* (2008). They reported real-time MgPa gene PCR to be more sensitive than conventional PCR, and to have a considerably increased sensitivity compared with real-time 16S rRNA gene PCR for the detection of *M. genitalium*. Müller and colleagues developed a quantitative real-time Rotor-Gene PCR (q-PCR) assay targeting the *pdhD* gene of *M. genitalium* (Müller *et al*, 2012). New insights in *M. genitalium* disease could be gained by the use of quantitative techniques (Jensen, 2006). Quantitative PCR assays have been used to assess antimicrobial efficacy as bacterial loads during the treatment, could be monitored (Hamasuna *et al*, 2009).

Several real-time PCR assays have been described for the molecular detection of *M. genitalium* (Taylor-Robinson & Jensen, 2011; Cazanave *et al*, 2012); however, none of these commercial kits has been approved by the USA Food and Drug Administration (FDA). Several companies (Bio-Rad, PathoFinder, and Seegene) have commercialized multiplex PCR for the detection of sexually transmitted pathogens, including *M. genitalium* (Le Roy *et al*, 2012; Lee *et al*, 2012). The TIB MOLBIOL LightMix kit (Roche Diagnostics) that targets the *mg219* gene and Communauté Européenne (CE)-marked Diagenode kit (Diagenode,
Belgium) targeting the gap gene, are the most recently developed monoplex real-time PCR kits for the detection of *M. genitalium* (Le Roy *et al*, 2014). However, the sensitivity and specificity of these tests are not typically published as many have not been compared and validated alongside other molecular-based or culture-based methods of *M. genitalium* (Waites *et al*, 2012).

In an effort to establish a simpler and streamlined protocol for *M. genitalium* detection, Takanashi and colleagues developed a PCR test using InvaderPlus® technology, carrying out both the endonuclease and PCR in the same simple step (Takanashi *et al*, 2015). This approach would require less genetic material and would be less labour and time consuming. The approach was tested with first-void urine samples and the PCR target was the 16S rRNA gene of *M. genitalium*. The InvaderPlus assay was comparable to typical hybridization microtiter PCR, able to detect as few as 10 DNA copies per reaction.

In terms of molecular diagnosis of *M. genitalium* for clinical reasons, various authors subscribe to the so-called “The double Positive Rule” stressing the importance of confirmation of positive results by repeating and/or use of different primers (Manhart *et al*, 2003). Preferably, the adhesin gene based PCR assay should be confirmed with a 16S rRNA gene based assay to improve reliability (Manhart *et al*, 2003). Gaydos *et al* (2009) accepted and regarded their patients as truly infected only after the *M. genitalium* positive results were confirmed with two different set of primers targeting both MgPa and 16S rRNA genes.

### 2.6 TREATMENT OF MYCOPLAMA GENITALIUM
Like all *mycoplasmas*, *M. genitalium* has natural resistance against all beta-lactamases and other antibiotics targeting peptidoglycan assembly since they lack a cell wall. Treatment options are tetracyclines, macrolides, and fluoroquinolones (Renaudin et al, 1992).

Although tetracyclines, in particular doxycycline, have been used to treat NGU for many years, the efficacy of this antimicrobial class is relatively poor and isolates with reduced susceptibility have been reported (Falk et al, 2003; Hamasuna et al, 2009). Tetracycline treatment failure was associated with persisting NGU (Jensen, 2006; Wikstrom & Jensen, 2006).

Azithromycin, a macrolide, is now preferred for the treatment of NGU and related clinical syndromes on account of its long half-life, excellent tissue penetration (Bissessor et al, 2015), and the fact that it can be administered as a single-dose treatment (Stamm et al, 1995). A single dose of azithromycin was proven to be as effective as 100 mg of doxycycline twice daily for 7 days (Björnelius et al, 2008).

However, as with most antimicrobials, reduced efficacy and/or resistance have been observed, which has led to research efforts focussing on studying the effectiveness of the single 1 g dose of azithromycin (Schwebke et al, 2011; Manhart et al, 2013; Bissessor et al, 2015). It has been proposed that switching to a higher dose and longer course of azithromycin (1.5 g administered over a period of 5 days) improved efficacy (Bradshaw et al, 2008; Terada et al, 2012; Bissessor et al, 2015). The controversy over the optimal dosage of azithromycin continues to exist as there is yet to be a randomised controlled trial comparing the single dose azithromycin with extended azithromycin (Horner et al, 2014; Jensen & Bradshaw, 2015). In 2008, Bjornelius and co-workers published a non-randomized clinical trial study focusing on
the efficacy of *M. genitalium* therapy on patients with urethritis or cervicitis. In their study, when comparing the single dose efficacy against the extended 1.5 g regimen of azithromycin following doxycycline treatment failure, they reported that the extended azithromycin regimen was more effective in eradicating *M. genitalium* than the initial single 1 g dose (Bjornelius *et al*, 2008). A study done by Anagrius *et al* reported a similar outcome putting enough weight on the fact that an extended dosage (1.5 g dose) of azithromycin therapy is superior than a single dose and it is good enough not to developed macrolide resistance (Anagrius *et al*, 2013). Furthermore, it is feared that should the clearance of *M. genitalium* fail during the 1 g single dose of azithromycin therapy, it is more likely to result in the selection of resistant strains unlike in the case of the extended regimen (Bjornelius *et al*, 2008). The lack of appropriate and efficient methods to remove such rapid selection of resistant strains from the population perpetuates macrolide resistance, especially in high-risk populations where the use of 1 g azithromycin is prevalent due to syndromic management of STIs (Jensen & Bradshaw, 2015). Several studies have reported the efficacy of higher doses and longer courses of azithromycin, particularly the extended 1.5 g course, given as 500 mg on day 1 and then 250 mg daily on days 2–5, or less often, two 1 g doses given 5–7 days apart (Jernberg *et al*, 2008; Terada *et al*, 2012).

Fluoroquinolones such as moxifloxacin, gatifloxacin, and sitafloxacin remain highly active against most macrolide-resistant *M. genitalium* isolates (Deguchi *et al*, 2012). Second generation fluoroquinolones such as ciprofloxacin and ofloxacin have poor eradicating efficiency against *M. genitalium* and their cure rate can only reach as high as 59 % at most (Jernberg *et al*, 2008). Equally so, the third generation quinolone levofloxacin also have a low cure rate of about 54 % despite the fact that this drug produced promising results when tested *in vitro* (Hamasuna *et al*, 2009; Takahashi *et al*, 2011; Terada *et al*, 2012). The newer
fluoroquinolones, including gemifloxacin, sparfloxacin, grepafloxacin, trovafloxacin, and garenoxacin have demonstrated to have high activity against *M. genitalium* in vitro, but have not yet been evaluated in clinical trials (Deguchi *et al.*, 2012). Moxifloxacin (400 mg once daily for 7–10 days) generally cures *M. genitalium* infections that have failed azithromycin therapy (Jensen *et al.*, 2008). As a result, moxifloxacin is currently the treatment of choice for macrolide-resistant *M. genitalium* infections (Deguchi *et al.*, 2012).

Given the diagnostic challenges, treatment of most *M. genitalium* infections will occur in the context of syndromic management for urethritis, cervicitis, and PID. While evidence-based guidelines for treatment of *M. genitalium* infections is yet to be made, treatment guidelines include 1 g single dose of azithromycin with a longer course of azithromycin (an initial 500-mg dose followed by 250 mg daily for 4 days) in case of treatment failure (Centers for Disease Control (CDC), 2015). In settings where validated *M. genitalium* testing is available, persons with persistent urethritis, cervicitis, or PID accompanied by persistent detection of *M. genitalium* might be treated with moxifloxacin (400 mg daily x 7, 10 or 14 days) (CDC, 2015). South Africa employed a syndromic treatment approach which used to include treating with a 7 day course of doxycycline as drug of choice (Lewis & Maruma, 2009). This was changed in 2015 to a single 1g dose of azithromycin (DOH, 2015).

### 2.7 Antimicrobial Resistance in *Mycoplasma genitalium*

#### 2.7.1 Tetracyclines

*In vivo* antimicrobial susceptibility testing of clinical isolates has demonstrated the emergence of some strains with decreased susceptibility to doxycycline and tetracycline (Hamasuma *et al.*, 2009). Although tetracycline resistance-associated mutations have not so
far been identified in *M. genitalium*, *tetM* gene mutations conferring tetracycline resistance have been identified in *M. hominis* and *U. urealyticum* isolated from genital specimens (Deguchi *et al*, 2012).

### 2.7.2 Macrolides

Macrolide antibiotics, including azithromycin, prevent bacterial replication by binding to the 50S ribosomal subunit, inhibiting translation of mRNA and thus interfering with protein synthesis. Resistance to macrolides is believed to be due to either several point mutations occurring in region 5 (referred to as ‘V region’) of the 23 S-rRNA (Shimada *et al*, 2011) or point mutations occurring within L4 and L22 ribosomal proteins (Diner & Hayes, 2009).

Three mutations at positions 2058 and 2059 (*E. coli* numbering) in region V of the 23S rRNA gene have been reported (Jensen *et al*, 2008). These alter ribosomal structure, thereby preventing macrolide binding, and have been associated with macrolide resistance in a number of pathogenic bacteria, including *M. genitalium* and two other sexually acquired pathogens, *N. gonorrhoeae* and *Treponema pallidum* (Jensen *et al*, 2008; Vester & Douthwaite, 2001). While the latter two sexually transmitted pathogens have multiple copies of 23S rRNA genes, *M. genitalium* has only a single rRNA gene operon encoding for the 23S, 16S, and 5S rRNA subunits. It has been hypothesized that this relative deficiency in the number of 23S rRNA gene copies may increase the susceptibility of *M. genitalium* to develop high-level macrolide resistance (Kikuchi *et al*, 2014). Point mutations occurring within L4 and L22 ribosomal proteins were already associated with macrolide resistance in other *Mollicutes* (Jensen *et al*, 2008).
The first study to demonstrate macrolide resistance in azithromycin treatment failure in *M. genitalium* urethritis was reported in 2006 (Bradshaw *et al*, 2006). Since then, numerous studies around the world have shown an increase in azithromycin resistance. In Melbourne, Australia, azithromycin efficacy has declined from 84% between 2005 and 2007, to 69% from 2007 to 2009 (Twin *et al*, 2012). In the USA only 40% of infections were cured by single-dose azithromycin 1 g in 2011, compared with 87% in 2002–2004 (Mena *et al*, 2009; Manhart *et al*, 2013). A Danish national survey reported a 38% prevalence of macrolide resistance-associated mutations in first *M. genitalium* test samples from 2007 to 2010 (Salado-Rasmussen & Jensen, 2014). The only reported macrolide resistance data from Africa has been laboratory-based using remnant specimens collected over 4 months in 2011–2012 from women attending primary health care clinics across Mopani District, Limpopo province of rural South Africa (Hay *et al*, 2015). A prevalence of 23S rRNA gene mutations in four (9.8%) of 41 DNA extracts screened was reported. The most recent data on macrolide resistance is from a prospective cohort of *M. genitalium*-infected patients with NGU, cervicitis, or pelvic inflammatory disease, as well as their sexual contacts, enrolled in Melbourne, Australia, between June 2012 and July 2013 (Bissessor *et al*, 2015). A high azithromycin 1 g treatment failure rate (39%) was reported in this study.

### 2.7.3 Fluoroquinolones

Following treatment failure by macrolides, patients with macrolide-resistant strains of *M. genitalium* are usually treated with fluoroquinolones particularly moxifloxacin (Weinstein & Stiles, 2012). This fourth generation quinolone drug is bactericidal and generally well tolerated although in some unlikely cases, it may show some serious adverse events such as hepatotoxicity on patients which consequently see moxifloxacin to be restricted as a second line drug in Europe (Kaye *et al*, 2014).
Fluoroquinolone antibiotics bind to the DNA gyrase and topoisomerase IV enzymes, blocking DNA replication. Mutations in defined regions of the DNA gyrase genes, \textit{gyrA} and \textit{gyrB}, and the topoisomerase IV genes, \textit{parC} and \textit{parE}, have been linked to high-level fluoroquinolone resistance in various bacteria, including \textit{N. gonorrhoeae} and \textit{M. genitalium} (Deguchi \textit{et al}, 2001; Tagg \textit{et al}, 2013). Mutations in quinolone resistance determining regions (QRDR) of the \textit{parC} gene, more precisely in amino acid positions S83 and D87 (\textit{M. genitalium} numbering) which are regarded as hotspot regions (Deguchi \textit{et al}, 2001), are more likely to trigger resistance against moxifloxacin as well other fourth generation quinolones (Shimada \textit{et al}, 2010).

The first clinical reports of \textit{M. genitalium} infection failing therapy with moxifloxacin as a result of fluoroquinolone-associated resistance mutations emerged in 2013 (Couldwell \textit{et al}, 2013). Subsequently, fluoroquinolone resistance was also reported from a London clinic (Pond \textit{et al}, 2014), as well as from Japan where approximately one-third of 51 Japanese men with NGU were infected with \textit{M. genitalium} had fluoroquinolone resistance-associated mutations in \textit{parC} (Kikuchi \textit{et al}, 2014).

Moxifloxacin treatment failure, especially from patients with \textit{M. genitalium} strains showing concurrent macrolide resistance (Bissessor \textit{et al}, 2015) is posing a serious threat to the availability of treatment options as current treatment failures is reported to be around 30% (Gundevia \textit{et al}, 2015).

\subsection*{2.8 GENOTYPING OF \textit{MYCOPLASMA GENITALIUM}}

Genotyping is the process of determining differences in the genetic make-up (genotype) of an organism by examining its DNA sequence and comparing it to another organism’s sequence.
or a reference sequence. *M. genitalium* remain extremely difficult to culture, making it difficult to fully understand the pathogenesis and epidemiology of this elusive pathogen. However, using different typing methods individually and in combination has made it possible to conduct several epidemiological studies. In order to understand transmission, pathogenesis and drug resistance of *M. genitalium* it has become important to type the different strains.

Since the whole genome of *M. genitalium* is fully sequenced and available (Fraser *et al*, 1995), this sequence data enable researchers to search and identify various *M. genitalium* genetic markers for typing purposes which affords an insight and breakthrough as far as STI studies are concerned. This also enables scientists to compare and relate different genotypes of this organism using all this various typing methods.

After the first PCR assay (Jensen *et al*, 1991), several molecular methods have been reported that could be useful for strain typing. These include short tandem repeat (STR) analysis of putative lipoprotein genes (MG309) (Ma & Martin, 2004); SNPs in the rRNA genes (Ma *et al*, 2008); restriction fragment length polymorphisms (RFLP) of the MG192 (*mgpC*) gene (Musatovova *et al*, 2006); and SNPs in the MG191 (*mgpB*) conserved gene (Hjorth *et al*, 2006).

Ma & Martin (2004) characterized the SNPs in the rRNA operon and variable numbers of tandem repeats in the lipoprotein gene MG309 among *M. genitalium* strains from clinical specimens by PCR and sequencing. Analysis of 31 *M. genitalium*-infected patient specimens and 7 American Type Culture Collection strains (ATCC) identified six types of rRNA sequences and 11 different numbers of MG309 repeats. SNPs are DNA sequence variations
occurring when a single nucleotide (A, T, C, or G) in the genome differs between members of a species or paired chromosomes in an individual. They concluded that using a combination of these typing methods (the variability in the rRNA sequences and MG309 STR) is more useful than using a single marker for studying sexual transmission patterns of this organism (Ma & Martin 2004).

Hjorth and colleagues (2006) developed a typing assay based on an amplification and sequencing regions of the \textit{mgpB} gene, which encodes the MgPa adhesion. PCR products were obtained with the MgPa-1/MgPa-3 primer set and sequenced. The sequences were aligned and compared with the 281-bp sequence located between MgPa-1 and MgPa-3 in the genome of \textit{M. genitalium} strain G37. They were able to evaluate and apply this system directly to urogenital specimens. The assay had a low limit of detection and hence a high typeability. Sequences of isolates from 52 unrelated patients were divided into 29 different sequence types, giving a discriminatory index of 0.95. This typing system could be used to trace strains and compare strains relatedness as well as identifying and documenting the uniqueness of newly isolated \textit{M. genitalium} strains (Hjorth \textit{et al}, 2006). This MgPa primer based SNP typing assay was used to prove sexual transmission between Texan couples (Musatovova & Baseman, 2009).

As a follow up to their study done in 2004, Ma and co-workers did a study aimed at further identifying potential genetic markers using STR genotyping (Ma \textit{et al}, 2008). Their extensive computerized analyses of the \textit{M. genitalium} type strain G37 genome afford them to identify 18 loci containing STRs. In addition to previously studied loci, MG309 and MG191, they included two others, MG307 and MG338, for further study. A combination of MG309-STRs and MG191-SNPs yielded optimal discrimination of 0.9894. This typing system afforded
them a platform to confirm the sexual transmission of *M. genitalium* as high infection concordance rates among couples was observed (Ma *et al*, 2008). It was proposed by them that a combination of the MG309-STRs and MG191-SNPs is efficient for general epidemiological studies and addition of MG307-STRs and MG338-STRs is potentially useful for sexual network studies of *M. genitalium* infection. By combining the results of Hjorth *et al* (2006), Musatovova & Baseman (2009) and Ma *et al* (2008; 2012) MG191 sequence type (ST) types was assigned and numbered 1–80.

In a study to compare molecular typing methods on French and Tunisian *M. genitalium*-positive specimens, Cazanave *et al* (2012) used the two main *M. genitalium* molecular typing methods, the mgpB SNP typing method and the combination analysis of a VNTR marker in MG309 and mgpB SNP. Furthermore, they tried to develop a multiple-locus VNTR analysis (MLVA) method. The genome of *M. genitalium* G37 was analysed for VNTRs and four VNTRs were used in the MLVA. Comparison of the typing results obtained with the three methods showed that the MLVA assay seemed too discriminatory to be used. According to the discriminatory power and the feasibility of each mgpB-based method, they also recommended that the mgpB analysis be used for general epidemiological studies and that the combination of MG309-STR and mgpB SNP methods should be used for sexual-network studies of *M. genitalium* infection (Cazanave *et al*, 2012).

Pond *et al* (2014) used this dual-locus typing system to establish whether prevalence of *M. genitalium* and resistance was local only to their population in London (UK) or could be applicable more generally. Typing assigned *M. genitalium* double locus sequence type (DLSTs) to 2 major clusters, broadly distributed among previously typed international strains. Genotypic macrolide resistance was spread within these 2 clusters.
Accumulation and analysis of additional sequence data should further enhance studies of *M. genitalium* infections, evaluation of treatment effectiveness, and understanding of its epidemiology, transmission, pathogenicity, and phylogeny.
CHAPTER 3: MATERIALS AND METHODS

3.1 ETHICAL CONSIDERATIONS

The protocol was approved by the Sefako Makgatho Health Sciences University Research and Ethics Committee (SMUREC) (SMUREC/P/138/2015: PG) (Appendix 1). Prospective subjects were informed about the purpose of the project, methods to protect confidentiality, and that joining was voluntary before asking them to sign an “informed consent form” (Appendix 2). The laboratory numbers of the patients were delinked from any personal identifiers to ensure confidentiality. Permission to conduct this study was also obtained from the chief executive officer of the Dr George Mukhari Academic Hospital.

3.2 STUDY DESIGN

This was a prospective, cross-sectional, quantitative study, to determine the prevalence and molecular characteristics of *M. genitalium* in pregnant women attending the termination of pregnancy and ante-natal clinics at the Dr George Mukhari Academic Hospital.

3.3 SAMPLE SIZE

Epi Info version 7.1.5. (Centres for Disease Control and Prevention, USA) was used to calculate the sample size. Assuming an expected frequency of 50% to give the largest sample size at 80% study power and 95% confidence, a sample size of 96 was calculated which was rounded off to 100 samples.
3.4 STUDY POPULATION

The study population were consenting women attending the TOP clinic and ANC at DGMAH. Fifty vaginal swabs were collected from women attending the TOP clinic and the ANC respectively. These swabs were stored at -70°C in the post-graduate laboratory of the Department of Microbiological Pathology.

3.5 SPECIMEN COLLECTION

Vaginal swab specimens were collected under direct vision during a speculum examination. Any vaginal discharge and/or abnormal lesions around the genital area and the cervix were noted. This information as well as the participant’s age, and if available, HIV status were recorded. Dacron tipped swabs were used to collect secretions from the posterior fornixes’ of the participants.

3.5.1 Inclusion/Exclusion criteria

Inclusion: All consenting pregnant women.

Exclusion: Women who had antimicrobial therapy within 2 weeks prior of the sampling.

3.6 LABORATORY METHODS

3.6.1 Control Strains

American type culture collection *M. genitalium* DNA (ATCC G-37, [33530D]) was used as positive control in all molecular techniques and PCR grade water (Bioline) was used as negative control.
3.6.2 Extraction of *Mycoplasma genitalium* deoxyribose nucleic acid (DNA).

DNA was extracted from all 100 specimens (50 TOP and 50 ANC) with the ZR Genomic DNA™-Tissue MiniPrep kit (Zymo Research Corporation, USA) according to the manufacturer’s instructions. The protocol for biological liquids and cell suspensions was followed.

Briefly, while swabs were allowed to reach room temperature, 260 µl of proteinase K storage buffer was added to each Proteinase K tube prior to use (the final concentration of proteinase K was 20 mg/ml). After the required number of sterile polypropylene tubes was labeled, 500 µl of sterile distilled water was added to each tube. By using a pair of scissors, each swab was cut into the corresponding tube, the tube was closed and then vortexed for 3 to 5 seconds. The pair of scissors was decontaminated between each sample using 70 % ethanol. A new set of tubes was labeled, including a negative control of extraction. One hundred microliters of sample was transferred to the corresponding tubes using aerosol barrier pipet tips. To prepare the negative control, 100 µl of sterile distilled water was added to the control tube. Ninety-five microliters of 2X digestion buffer and 5 µl of proteinase K were added to each tube, mixed and incubated in a thermomixer (Eppendorf, Germany) at 55°C for 20 minutes with gentle agitation. Seven hundred microliters of genomic lysis buffer was added to the tube and mixed thoroughly by vortexing. The vortexed mixture was transferred to a Zymo-Spin™ IIC column that was placed into a collection tube and centrifuged in a microcentrifuge (Hettich, Germany) at 10000 x g for 1 minute. The spin column was placed into a new collection tube and 200 µl of DNA pre-wash buffer added to the filter of the spin column. After the tube was centrifuged at 10 000 x g for 1 minute, 400 µl of g-DNA wash buffer was added to the spin column. The tube was centrifuged at 10 000 x g for 1 minute and the spin column was
transferred to a sterile polypropylene tube. One hundred microliters of DNA elution buffer was added to the spin column and incubated for 2 – 5 minutes at room temperature. The tube was centrifuged at top speed for 30 seconds to elute the DNA. The eluted DNA was stored at -70°C until PCR analysis was performed.

3.6.3 *Mycoplasma genitalium* detection

PCR was performed in separate rooms: for mastermix preparation, amplification and detection. DNA stored at -70°C was thawed at room temperature and mixed well by vortexing.

3.6.3.1 Conventional polymerase chain reaction (PCR)

The extracted DNA was amplified in a PCR assay targeting a 281bp region of the 140 kDa adhesion gene (*MgPa*) of *M. genitalium* (Jensen et al, 1991). This is the major adhesin mediating the attachment of mycoplasma to the ciliated epithelium. The primers used were synthesized by Inqaba Biotechnologies, Pretoria as follows:

Forward Primer/ sense:  *MgPa-l*: 5’-TGA AAC CTT AAC CCC TTG G-3’

Reverse Primer/ antisense: *MgPa-3*: 5’-AGG GGT TTT CCA TTT TTG C-3’.

Positive (ATCC G-37, [33530D]) and negative (water) controls were included in each PCR run. MyTaq™ HS DNA Polymerase (Bioline, UK) was used in the PCR reactions. It is a high performance enzyme powered by antibody mediated hot-start PCR. The buffer contains dNTPs, MgCl₂ and enhancers at optimal concentrations. For each sample, a 45 µl master mix solution was prepared using 32.5 µl distilled water, 10 µl 5x buffer (5 mM dNTPs, 15 mM MgCl₂), 1 µl (20 pmole) of each of the 2 primers and 0.5 µl Taq DNA polymerase (5 U/µl).
Five microliters of extracted DNA was added to 45µl of the master mix in the PCR reaction. The amplification tubes were placed into a thermocycler (GeneAmp PCR System 2700, Applied Biosystems). Amplification was performed as follows: an initial hold cycle at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 10 seconds, primer annealing at 55°C for 50 seconds and extension at 72°C for 45 seconds, followed by a final extension cycle at 72°C for 5 minutes.

Amplicons were detected on a 2.0% agarose gel with 0.5x Tris Borate EDTA buffer; pH 8.3 (Crystal TBE, Bioline, UK) as running buffer. The gels contained ethidium bromide (5 µl/100ml of a 10 mg/ml stock solution). For each gel, a size marker was included (Hyperladder IV, Bioline, UK). Gels were viewed and photographed (Gel Doc™ EZ System, BioRad, USA). The expected amplicon size was 281bp for *M. genitalium*. Results were recorded and *M. genitalium* positive specimens were used in antimicrobial resistance analysis and serotyping assays.

3.6.3.2 Real-time polymerase chain reaction (q-PCR)

The *M. genitalium* Real-TM kit from Sacace (Italy) was used following the manufacturer’s instructions. The kit employs taqman probe technology where the amplification product is detected using fluorescent dyes linked to the oligonucleotide probes which bind specifically to the amplified product during thermocycling.

A master mix was prepared by pipetting 10 µl of PCR -mix-1-FRT (primers and molecular beacons), 5 µl of PCR-mix-2-FRT (buffer, dNTPs and MgCl₂) and 0.5µl DNA Taq polymerase into a 200 µl PCR tube. The contents were mixed to obtain a homogenous mixture before addition of 10 µl of sample DNA or controls to make a final volume of 25 µl.
per reaction. For every amplification reaction, a negative control (water) and positive control (DNA from the ATCC *M. genitalium* G37 strain [33530D] were included.

Amplification was done using SA Cycler-96 RUO (Sacace, Italy) with the following cycling conditions: An initial cycle at 95 °C for 15 minutes; 5 cycles of 95°C for 5 seconds, 60°C for 20 seconds and 72°C for 15 seconds; 40 cycles of 95°C for 5 seconds, 60°C for 20 seconds and 72°C for 15 seconds, and a final hold at 4°C.

Results were interpreted with the software of the SA Cycler-96 through the presence of the crossing of the fluorescence curve with the threshold line (Ct). Specimens with a Ct <33 were considered positive.

### 3.6.4 Antimicrobial resistance analysis

#### 3.6.4.1 Fluoroquinolone resistance

To detect fluoroquinolone resistance, the *gyrA* and *parC* genes in all *M. genitalium*-positive isolates were amplified using primers targeting a 230bp region of the *M. genitalium gyrA* gene, and a 220bp region of the *parC* gene (Pond *et al*, 2014). The primers used were synthesized by Inqaba Biotechnologies, Pretoria as follows:

- MG-gyrAPoF 5’-CCTGATGCTAGAGATGGACTTAAA-3’
- MG-gyrAPoR 5’-AAGTTCTGCTGCAAGTTTAGATAAT-3’
- MG-parCPoF 5’-GTGCTGTTGGGAGAGATCAT-3’
- MG-parCPoR 5’-CCATGGATAGAAACAGTTGTTCA-3’
The MyTaq™ HS DNA Polymerase kit (Bioline, United Kingdom) as described in 3.6.3.1 was used. The same cycling parameters were used for both the gyrA and parC assays namely an initial denaturation at 95°C for 2 minute followed by 30 cycles of 15 seconds at 94°C, 1 minute at 60°C and 30 seconds extension at 72°C. This was followed by a final extension at 72°C for 10 minutes. Amplicons were detected by agarose gel electrophoresis as described in 3.6.3.1. All amplified genes were sent to Inqaba Biotechnologies, Pretoria for nucleic acid sequencing.

BLAST technology was used to compare sequences with DNA gyrase subunit A [M. genitalium G37; ID: L43967.2]; the whole genome [M. genitalium MG6320; ID:CP003772.1] and topoisomerase IV, A subunit [M. genitalium strain LA107; ID: HF947096.1].

3.6.4.2 Macrolide resistance

Macrolide resistance was determined by PCR of a unique 147bp region of the M. genitalium 23S rRNA gene. The primers (below) flanked the mutations usually associated with macrolide resistance found in the V region of the 23S rRNA (Jensen et al, 2008).

Mg23S-F  5’-CCATCTCTTGACTGTCTCGGCTAT-3’
Mg23S-R  5’-CCTACCTATTCTCTACATGGTGGTGT-3’

PCR and amplicon detection was performed as described in 3.6.3.1 with the following cycling conditions: an initial denaturation at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60 seconds at 60°C (annealing and extension combined). This was followed by a final extension of 10 minutes at 72°C. All amplified genes were sent to Inqaba Biotechnologies, Pretoria for nucleic acid sequencing.
BLAST technology was used to compare sequences with the *M. genitalium* G37 complete genome [L43967.2] and the MG 23S rRNA gene of G37 [HF572950.1]. Sequences of strains with known mutations were also used in the analysis: LA141 [HF572938.1] (A2058G); LA088 [HF572933.1] (A2059G) and LA202 [HF572946.1] (A2059C).

### 3.6.5 *Mycoplasma genitalium* typing

#### 3.6.5.1 MG191 (*mgpB*) SNP typing method

PCR targeting the adhesion gene was performed on all *M. genitalium* positive specimens, as described in 3.6.3.1 using the primers described by Jensen *et al* (1991). The PCR products were purified using a QiaQuick PCR purification kit (QIAGEN, Hilden, Germany). Sequencing was performed by Inqaba Biotek, Pretoria. Both strands of the amplified fragments were sequenced. The sequences were aligned and compared with the 281-bp sequence located between MgPa-1 and MgPa-3 on the genome of *M. genitalium* strain G37 [L43967.2], as well as sequence types 1 to 55 as described by Hjorth *et al* (2006).

#### 3.6.5.2 MG309 variable number tandem repeat analysis

This methodology relies upon the enumeration of tri-nucleotide repeat units occurring within the MG309 gene of *M. genitalium* and the numbers of copies identified and, when combined with mgpB SNP typing, can be used to further assess the relatedness of strains. Primers targeting a locus on the MG309 gene with VNTRs were used in a semi-nested PCR reaction (Cazanave *et al*, 2012). The primers (sequenced by Inqaba Biotechnologies, Pretoria) are described in Table 3.1.
Table 3.1 Primers used in semi-nested MG309 PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG309-STR-F</td>
<td>5’-GTGCTAGAGAAGTGTTTCTAGGATC</td>
<td>P</td>
</tr>
<tr>
<td>MG309-STR-R</td>
<td>5’-AACTAGCAGAAACGTAACCAACC</td>
<td>P, SN</td>
</tr>
<tr>
<td>MG309-STR-F-Nest</td>
<td>5’-TATTGCGGTGAGGGAATTAC</td>
<td>SN</td>
</tr>
</tbody>
</table>

P: Primary PCR
SN: Semi–nested PCR

PCR was performed using the MyTaq™ HS DNA Polymerase kit (Bioline, United Kingdom) as described in 3.6.3.1 on all the *M. genitalium* positive samples. For the primary PCR, a touchdown cycling protocol was used in the thermocycler (GeneAmp PCR System 2700, Applied Biosystems, US) as follows: the initial hold at 95°C for 8 minutes was followed by 10 cycles of 95°C for 60 seconds, 65°C for 120 seconds (decrease 1.5 °C every cycle to a final temperature of 50°C) and 72°C for 120 seconds. This was followed by 30 cycles of 95°C for 60 seconds, 50°C for 120 seconds and 72°C for 120 seconds. Five microliters of the primary PCR amplicons were used in the semi-nested PCR as follows: an initial denaturation at 95°C for 9 minutes, followed by 30 cycles of 45 seconds at 95°C, 60 seconds at 50°C and 120 seconds at 72°C.

Amplicons were separated on gel electrophoresis to detect the difference, if any, of the number of repeats. The amplification products were further evaluated by sequencing (Inqaba Biotechnologies, Pretoria). Sequences were aligned with the partial MG309 gene of *M. genitalium* strain G37 [KC445182.1] which contains 12 copies of the VNTR (AGT or AAT), MG Strain LA227 [KC445181.1] containing 11 copies, and MG Strain LA197 [KC445178.1] with 10 copies of the VNTR.
3.7 DATA ANALYSIS

Data was imported from Microsoft Excel for analysis using Epi Info version 7.1.4.0 (CDC, 2014). Descriptive statistical analysis was performed. Measures of central tendency and dispersion was calculated for continuous data (e.g. age), while frequencies and proportions of categorical data was calculated. To compare the diagnostic tests used, sensitivity, specificity, positive and negative predictive values were determined.

Sequences obtained were edited using Chromas Lite and BioEdit. Sequences were aligned with MAFFT and MEGA 6 was used to edit sequences and draw phylogenetic trees. Branch support values were generated from 1000 bootstrap replicates. Values of 70% or higher was considered statistically significant for the cluster grouping.

3.8 RELIABILITY, VALIDITY & OBJECTIVITY

All tests were reliable and valid as they were performed according to recognized accredited standard operating procedures as well as to the instructions of the manufacturers in the case where commercially available kits were used. Negative (water) and positive (ATCC \textit{M. genitalium} DNA 33530D) controls was included in all PCR reactions. Molecular size markers were used during agarose gel electrophoresis. Objectivity was ensured by using laboratory numbers, and by conducting tests blindly i.e. without knowledge of results using different tests.
CHAPTER 4: RESULTS

The results are tabulated in Appendix 3.

4.1 STUDY POPULATION

One hundred specimens collected from consenting women attending the TOP and ANC clinics at DGMAH between June and December 2015 were included in this study. Fifty vaginal swabs were collected from women attending the TOP clinic the ANC respectively. The median age of the women was 28.5 years (ANC) and 23 years (TOP) clinics with their age ranging from 15 – 42 years. Abnormal lesions, vaginal discharge and HIV status (if known) were recorded (Appendix 3).

4.2 MYCOPLASMA GENITALIUM DETECTION

Following DNA extraction, all the specimens were tested for the presence of *M. genitalium* by two molecular methods: i.e. conventional polymerase chain reaction (PCR) and Real-time polymerase chain reaction (q-PCR).

4.2.1 Conventional polymerase chain reaction

Of the 100 specimens (50 TOP, 50 ANC), 6 (6.0%) were positive with 4.0% (4) from the TOP and 2% (2) from the ANC (Table 4.1). A 281 bp band represented *M. genitalium* positive specimens (Figure 4.1).
Figure 4.1  Representative Agarose gel electrophoresis of *M. genitalium* MgPa PCR amplicons

Lane +ve: Positive control;  Lane -ve: Negative control; Lanes 1-15: Clinical isolates; SM: 100bp Size marker

4.2.2 *Mycoplasma. genitalium* Real-time polymerase chain reaction

The q-PCR assay detected *M. genitalium* in two more samples than the conventional PCR (8; 8.0%). Of these, 4.0% (4) were from TOP and 4.0% (4) from ANC (Table 4.1). Ct values < 33 were deemed positive (Figure 4.2).

Figure 4.2  Representative results from *M. genitalium* q-PCR (Sacace).

Red curves (FAM channel): *M. genitalium* positive samples

Green curve (Cy5 channel): Internal control
Table 4.1  *M. genitalium* detection by PCR

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Clinic</th>
<th>Conventional PCR</th>
<th>q-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>T30</td>
<td>TOP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T63</td>
<td>TOP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T75</td>
<td>TOP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T76</td>
<td>TOP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M25</td>
<td>ANC</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M73</td>
<td>ANC</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>M84</td>
<td>ANC</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M102</td>
<td>ANC</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

The q-PCR detected all the *M. genitalium* positives detected by the conventional PCR with an additional 2 (M73 and M102), both from the ANC. Comparing q-PCR to conventional PCR, the sensitivity and specificity of the conventional PCR was 100% and 75.00% respectively (95% CI).

4.3  ANTIMICROBIAL RESISTANCE ANALYSIS

4.3.1 Fluoroquinolone resistance

Amplification of the *gyrA* and *parC* genes was performed on the *M. genitalium* isolates and the amplicons sequenced.

4.3.1.1 *GyrA* gene analysis

The *gyrA* gene of two of the *M. genitalium* positive isolates (M73 and M102) did not amplify (Figure 4.3). Sequences obtained for the remaining 6 isolates were aligned with the DNA gyrase subunit A [*M. genitalium* G37; ID: L43967.2] as well as the whole genome [*Mycoplasma genitalium* MG6320; ID:CP003772.1] (Figure 4.4). None of the strains
harboured mutations in the *gyrA* gene (G285C; Met→Ile 83) associated with fluoroquinolone resistance as reported previously (Tagg *et al.*, 2013).

![Figure 4.3 Agarose gel electrophoresis of *M. genitalium* MG-*gyrA* PCR amplicons](image)

Lane Pos: Positive control; Lane Neg: Negative control; SM: 100bp Size marker

Clinical isolates: Lane 1: M25; Lane 2: T30; Lane 3: M102; Lane 4: T63; Lane 5: M73; Lane 6: T75; Lane 7: T76; Lane 8: M84

![Figure 4.4 Amino acid alignment of the *M. genitalium* *gyrA* gene of clinical isolates with reference strains](image)

**: Amino acid where mutation is associated with resistance (G285C; Met→Ile)**
4.3.1.2 ParC gene analysis

The parC gene of six of the 8 M. genitalium strains could be amplified (Figure 4.5). Amplicons could not be obtained for M73 and M102. Topoisomerase IV, A subunit [M. genitalium strain LA107; ID: HF947096.1]. Five of the 6 strains had a silent mutation (C234T) in the parC gene (Figure 4.6). One strain (T30) had the G248T mutation (Ser→Ile 80) (Figure 4.7) which has been previously described for M. genitalium associated with resistance (Gruson et al, 2005; Tagg et al, 2013).

![Agarose gel electrophoresis of M. genitalium MG-parC PCR amplicons](image)

Figure 4.5  Agarose gel electrophoresis of M. genitalium MG-parC PCR amplicons

Lane +ve: Positive control; Lane -ve: Negative control; SM: 100bp Size marker

Clinical isolates: Lane 1: M25; Lane 2: M102; Lane 3: T30; Lane 4: T63; Lane 5: T75; Lane 6: T76; Lane 7: M73; Lane 8: M84
Figure 4.6 Nucleic acid sequence alignment of the *M. genitalium* parC gene of clinical isolates with reference strains

- Silent mutation (C234T; His 75)
- G248T mutation (Ser → Ile 80) associated with resistance

Figure 4.7 Amino acid sequence alignment of the *M. genitalium* parC gene of clinical isolates with reference strains

- Mutation (Ser → Ile 80) associated with resistance
4.3.2 Macrolide resistance

The V region of the *M. genitalium* 23S rRNA gene could be amplified for 6 isolates (Figure 4.8). The sequences obtained from these amplicons were compared to reference strains and strains with mutations associated with macrolide resistance. Two of the 6 strains (33.3%) contained A2059G resistance mutation (Figure 4.9). These two strains (M25 and T30) were deemed resistant to macrolides.

![Agarose gel electrophoresis of M. genitalium MG-23S rRNA PCR amplicons](image)

Figure 4.8  Agarose gel electrophoresis of *M. genitalium* MG-23S rRNA PCR amplicons

Lane +ve: Positive control; Lane 1: Negative control; SM: 100bp Size marker

Clinical isolates:  Lane 2: M25; Lane 3: T30; Lane 4: M73; Lane 5: T63; Lane 6: T75; Lane 7: T76; Lane 8: M84
Figure 4.9 Nucleic acid sequence alignment of the *M. genitalium* 23S rRNA gene of clinical isolates with reference strains

 Mutations associated with macrolide resistance:

LA141: A2058G (Pond *et al*, 2014)
LA202: A2059C (Pond *et al*, 2014)
M6320: A2059G (Tagg *et al*, 2013)
M25: A2059G
T30: A2059G

4.4 MYCOPLASMA GENITALIUM TYPING

All the *M. genitalium* isolates were typed using both the MG191 (mgpB) SNP and MG309 VNTR analysis methods.

4.4.1 MG191 (mgpB) SNP typing

MG191 sequence type (ST) type was assigned on the basis of previously described types (Hjorth *et al*, 2006). STs could be obtained for 6 of the 8 *M. genitalium* isolates. Two isolates, T76 and M84 were ST1, T63 was ST2; T30 and T75 were ST4 and one isolate (M25) was ST39 (Figure 4.10). Phylogenetic analysis revealed a tree divided into 2 major clusters,
designated Major Cluster A and B (Figure 4.11). Three isolates (T63, T76 and M84) fell in the same cluster as the wild type strain G37. The remaining isolates fell in cluster B.

<table>
<thead>
<tr>
<th>L43967.2 MG G37</th>
<th>ggagagaaaACggagatctttggattagaagaaacaaatgaacaacttaaatattcaagtgttacaa</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgPa[1]</td>
<td></td>
</tr>
<tr>
<td>M84 MgPa-1</td>
<td></td>
</tr>
<tr>
<td>T76_MgPa-1</td>
<td></td>
</tr>
<tr>
<td>M2341[2]</td>
<td></td>
</tr>
<tr>
<td>T63_MgPa-1</td>
<td></td>
</tr>
<tr>
<td>LF-p6486[4]</td>
<td></td>
</tr>
<tr>
<td>M25 MgPa-1</td>
<td></td>
</tr>
<tr>
<td>T30_MgPa-1</td>
<td></td>
</tr>
<tr>
<td>T75_MgPa-1</td>
<td></td>
</tr>
<tr>
<td>129-0[39]</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>L43967.2 MG G37</th>
<th>agaattctagtgtgataatctcaagtatctcaatgtgtagaaatacttgatggtcagcaaaactt</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgPa[1]</td>
<td></td>
</tr>
<tr>
<td>M84 MgPa-1</td>
<td></td>
</tr>
<tr>
<td>T76_MgPa-1</td>
<td></td>
</tr>
<tr>
<td>M2341[2]</td>
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</tr>
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<td>M25 MgPa-1</td>
<td></td>
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<tr>
<td>T30_MgPa-1</td>
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<tr>
<td>T75_MgPa-1</td>
<td></td>
</tr>
<tr>
<td>129-0[39]</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.10 MG191 nucleic acid sequence alignment of reference and clinical strains

MG G37: Wild type strain (ST1); M2341: ST2; LF-p6486: ST4; 129-0: ST39

Figure 4.11 Phylogenetic tree of MG191 ST types of M. genitalium from clinical and reference strains
4.4.2 MG309 variable number tandem repeat analysis

Seven of the 8 isolates were successfully typed using MG-309-VNTR (Figure 4.12). Four different VNTR patterns were seen. The most commonly seen pattern was 10 repeats (M25; T30 and T75), followed by the wild type pattern of 12 repeats (T76 and T84). One isolate (M73) had 11 repeats and one isolate (T63) had 14 repeats. Phylogenetic analysis revealed a tree clearly divided into 2 major clusters, designated Major Cluster A and B (Figure 4.13). As with MG191 SNP analysis, T76 and M84 clustered with the wild type. Its nearest neighbour is T63 that also fell in Cluster A with MG191 SNP typing.

![Figure 4.12 Nucleic acid sequence alignment of MG309 VNTR region](image)

Lines indicate repeat sequences *agt* and *aat*
G37 (Wildtype): 12 repeats; LA227: 11 repeats; LA197: 10 repeats; T30; M25; T75: 10 repeats; T63: 14 repeats; T84; T76: 12 repeats; M73: 11 repeats

![Figure 4.13 Phylogenetic tree of MG309 VNTR types of M. genitalium from clinical and reference strains](image)
4.5 **SUMMARY OF RESULTS OBTAINED**

The demographic, genotypic resistance and typing data for *M. genitalium* positive clinical samples is given in Table 4.2. No genotypic results could be obtained for M102, while only VNTR analysis was obtained for M73.

**Table 4.2** Demographic and genotypic characteristics of patients with *M. genitalium* infection

<table>
<thead>
<tr>
<th>Spec</th>
<th>Clinic</th>
<th>Age</th>
<th>Discharge</th>
<th>HIV</th>
<th>Mutant Fluoroquinolone QRDR: Amino Acid Change</th>
<th>23S rRNA Mutation</th>
<th>mgpB SNP Type</th>
<th>MG309 VNTR Copy Number</th>
<th>Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>T30</td>
<td>TOP</td>
<td>28</td>
<td>None</td>
<td>Neg</td>
<td>WT S80I WT*</td>
<td>A2059G ST4</td>
<td>10</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>T63</td>
<td>TOP</td>
<td>23</td>
<td>3+</td>
<td>Neg</td>
<td>WT WT*</td>
<td>WT ST2</td>
<td>14</td>
<td>A/B#</td>
<td></td>
</tr>
<tr>
<td>T75</td>
<td>TOP</td>
<td>21</td>
<td>3+</td>
<td>Pos</td>
<td>WT WT*</td>
<td>WT ST4</td>
<td>10</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>T76</td>
<td>TOP</td>
<td>19</td>
<td>2+</td>
<td>Neg</td>
<td>WT WT*</td>
<td>WT ST1</td>
<td>12</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>M25</td>
<td>ANC</td>
<td>24</td>
<td>2+</td>
<td>Pos</td>
<td>WT WT</td>
<td>A2059G ST39</td>
<td>10</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>M73</td>
<td>ANC</td>
<td>27</td>
<td>1+</td>
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<td>ND ND</td>
<td>ND ND</td>
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</tr>
<tr>
<td>M84</td>
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<td>Neg</td>
<td>WT WT*</td>
<td>WT ST1</td>
<td>12</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>M102</td>
<td>ANC</td>
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<td>Neg</td>
<td>ND ND</td>
<td>ND ND</td>
<td>ND</td>
<td>ND ND</td>
<td></td>
</tr>
</tbody>
</table>

TOP: Termination of pregnancy clinic  
ANC: Ante natal clinic  
QRDR: Quinolone resistant determinant region  
WT: Wild type  
WT*: Silent mutation  
ND: Not done  
#: Cluster A with MG191 SNP and Cluster B with MG309 VNTR
CHAPTER 5: DISCUSSION

*M. genitalium* has become an established sexually transmitted pathogen causing cervicitis, abnormal vaginal discharge, urethritis, PID and infertility (Manhart *et al*, 2003; Cohen *et al*, 2002). *M. genitalium* is not usually recommended among organisms for routine testing in sexually transmitted infection screening and treatment follows syndromic guidelines (Workowski *et al*, 2015). Comparison of the prevalence and strain variability of *M. genitalium* isolated from high risk pregnant women (termination of pregnancy clinic attendees) and normal pregnant women (women attending the antenatal clinic) will contribute to the knowledge of transmission, pathogenicity and management of this organism locally.

In this study, 100 specimens were collected from consenting women attending the TOP and ANC clinics at DGMAH. The median age of the women was 28.5 years (ANC) and 23 years (TOP) clinics with their ages ranging from 18 – 42 years. The prevalence of *M. genitalium* in these women, the prevalence of mutations in genes associated with antimicrobial resistance as well as data concerning *M. genitalium* strains circulating in area served by DGMAH was determined.

5.1 PREVALENCE OF *MYCOPLASMA GENITALIUM*

In this study, both conventional PCR and q-PCR were used to determine the prevalence of *M. genitalium* among the participants. With the conventional PCR assay, a prevalence of 6% (6/100) was found. Of these, 4.0% (4) were from the TOP and 2% (2) from the ANC. The q-PCR assay was able to detect *M. genitalium* in two more samples than the conventional PCR with a prevalence of 8.0% (8/100). *M. genitalium* was detected in 4 patients each from the
TOP and ANC. A *M. genitalium*-positive sample was collected from a patient with HIV from both the TOP clinic and ANC.

The *M. genitalium* prevalence of 8.0% found in this study was in line with that of a study previously conducted in the same setting. De Villiers et al reported a *M. genitalium* prevalence of 10.0% among TOP attendees in 2009 (de Villiers et al, 2010). However, a similar study conducted at this setting among the same target group the following year, reported a relatively higher *M. genitalium* prevalence of 17.1% (Mavhunga et al, 2010). In the present study, only 50% of participants were from the TOP which are regarded as a ‘high risk’ group compared to those participants from the ANC. This may account for the difference in prevalence.

When comparing the prevalence of *M. genitalium* detected in this study to studies published around the world, there are huge variations between studies which may depend among other factors, on the target population. Lawton et al (2008) detected *M. genitalium* in 8.7% of women attending a TOP clinic in New Zealand, while a study done in Denmark in a TOP clinic detected *M. genitalium* in less than 1% of the 102 participants (Baczynska et al, 2008). Sonnenberg et al (2015) conducted a national survey in Britain where urine samples were collected from 4507 sexually experienced participants, aged 16–44 years, and tested for *M. genitalium*. The prevalence documented was 1.2% in men and 1.3% in women. A Swedish study aimed at determining the prevalence, clinical significance as well as transmission of *M. genitalium*, conducted amongst 445 female patients attending STD clinic reported a prevalence of 6.3% (Anagrius et al, 2005). Huppert et al (2008) reported a much higher prevalence of *M. genitalium* of 22.4% among 331 women were recruited from an urban medical centre in Cincinnati, USA. These different figures contributed to the conclusion derived by Daley et al (2014) that the prevalence of *M. genitalium* varies greatly with different geographical regions.
Although there are only a few *M. genitalium* prevalence studies reported in South Africa, the findings are similar to this present study. In a study by Mhlongo *et al* (2010) a total of 300 samples were collected from women with vaginal discharge syndrome (94 from Cape Town; 206 from Johannesburg) and this pathogen was detected in 2.1% and 11.2% of these women from the two cities respectively. This amounted in an overall prevalence of 13.3%. Lewis *et al* (2012) reported a slightly lower infection rate of 6.1% after screening 558 HIV infected women attending an HIV treatment centre in Johannesburg for *M. genitalium*. Hay *et al* (2015) reported an infection rate of 10.8% amongst 601 woman visiting primary health care clinics across the Mopani District, Limpopo province.

### 5.2 LABORATORY DETECTION OF *MYCOPLASMA GENITALIUM*


In this study, all the specimens were tested for the presence of *M. genitalium* by two molecular methods: i.e. conventional PCR, and real-time polymerase chain reaction (q-PCR) following DNA extraction. The conventional PCR assay targeted a 281bp region of the 140 kDa adhesion gene (*MgPa*) of *M. genitalium* while the commercial q-PCR assay targeted the 16S rRNA gene. The q-PCR detected all the *M. genitalium* positives detected by the conventional PCR with an additional 2 (M73 and M102) isolates, both from the ANC. Comparing q-PCR to conventional PCR, the sensitivity and specificity of the conventional PCR was 100% and
75.00% respectively (95% CI). These findings were in correlation with other studies that has previously compared the two PCR techniques (Svenstrup et al, 2005; Edberg et al, 2008).

In 2005, Svenstrup et al demonstrated that real time PCR assays were superior to conventional PCR. They used two q-PCR assays targeting different M. genitalium genes, the housekeeping gap gene as well as the MgPa gene, and a conventional PCR targeting the 16S rRNA gene to detect the pathogen in 246 urethral swab specimens. Both q-PCR assays showed better sensitivity. When comparing the q-PCR assays to each other, real-time MgPa gene PCR proved to be the most sensitive, detecting M. genitalium DNA in three specimens that were negative by real-time gap gene PCR (Svenstrup et al, 2005). Similar results were reported in a study done by Edberg et al (2008) where real-time MgPa gene PCR was more sensitive than conventional 16S rRNA gene PCR (97.4 % vs 80.3 %). Although q-PCR was more sensitive than conventional PCR, the target site also played a role in the successful detection of M. genitalium.

In our study, a low load of M. genitalium DNA was suspected as some of M. genitalium isolates were detected upon re-amplification, particularly with the conventional PCR. This was also apparent in subsequent sequencing reactions where results could not be obtained for all positive specimens. The low organism load could be attributed to the type of specimen used. Jensen et al (2004a) demonstrated that first void urine (FVU) specimens from either men or women had a higher sensitivity compared to vaginal swab specimens which is commonly used. Jensen and co-workers also demonstrated that M. genitalium DNA is found in a very low load in clinical specimens, highlighting the need for very sensitive assays. They demonstrated that 28% of urethral swab specimens and 14% of FVU specimens contained less than ten genome equivalents of M. genitalium DNA (Jensen et al, 2004a). This low DNA load is of considerable
concern as it begs the question of whether the prevalence of this organism is being underreported in various clinical specimens. Our study highlighted the importance of the so-called “Double Positive Rule” which various authors subscribe to as it stresses the importance of confirmation of positive results by repeating and/or use of different primers when detecting *M. genitalium* (Manhart *et al*, 2003). In addition to low bacterial loads, prolonged storage of the samples at -70°C may also have contributed to the lack of results for subsequent sequencing reactions. Carlsen & Jensen (2010) showed that *M. genitalium* DNA load as well as the detection rate decreased after storage. This was more pronounced in clinical specimens stored frozen than in stored DNA extracts, particularly in those with an initial low DNA load.

### 5.3 Antimicrobial Resistance Analysis

Like all *mycoplasmas*, *M. genitalium* has natural resistance against all beta-lactamases and other antibiotics targeting the peptidoglycan layer since they lack a cell wall. Treatment options are tetracyclines, macrolides, and fluoroquinolones (Renaudin *et al*, 1992). Patients with symptoms are treated syndromically, regardless of the detection of *C. trachomatis* or *M. genitalium*, with tetracyclines, fluoroquinolones or macrolides, depending on local recommendations. In this setting, the syndromic treatment approach, as prescribed by the South African Department of Health, is used (DOH, 2015). Studies have indicated treatment failures with doxycycline, as well as azithromycin and fluoroquinolones in various parts of the world (Mroczowski *et al*, 2006; Bradshaw *et al*, 2006). In February 2015, the South African Department of Health has changed their recommended syndromic treatment from a 7 day course of doxycycline to a single dose of azithromycin (DOH, 2015). This study sought to investigate macrolide-resistance inducing mutations as azithromycin is now the drug of choice in this country. Resistance to macrolides is believed to be due to either several point mutations
occurring in region 5 (referred to as the ‘V region’) of the 23 S-rRNA (Shimada et al, 2011) or point mutations occurring within L4 and L22 ribosomal proteins (Diner & Hayes, 2009). Identification of mutations in the 23S rRNA gene at nucleotides 2058 and 2059 (E. coli numbering), positions known to be linked to high-level azithromycin resistance (Jensen et al, 2008), allowed for the first estimate of the prevalence of azithromycin resistance in M. genitalium in Sydney, Australia (Tagg et al, 2013).

In this present study, the V region of the M. genitalium 23S rRNA gene could be amplified for 6 isolates. The sequences obtained from these amplicons were compared to a reference strain and strains with mutations associated with macrolide resistance (Tagg et al, 2013; Pond et al, 2014). Two of the 6 strains (33.3%) contained A2059G resistance mutations and these strains (M25 and T30) were deemed resistant to macrolides. In their Australian study, Tagg et al. (2013) reported an overall azithromycin resistance prevalence of 43% (62/143), with the A2059G resistance mutation accounting for 61% (38/62) of all mutations. Hay et al (2015) described for the first time macrolide resistance in M. genitalium–positive specimens from South Africa, with a prevalence of 9.8% among woman visiting primary health care clinics across the Mopani District, Limpopo province. Contrary to our study, all their mutated specimens harboured the A2058G mutation. It has to be noted that their study was done before the change in the national treatment guidelines to include azithromycin, while the present study was done after the change.

Azithromycin failure was initially reported by Bradshaw et al in 2006 where 28% of an Australian cohort of symptomatic men reported treatment failure attributable to resistance in vivo. Lau et al (2015) then conducted a systematic review and meta-analysis study of M. genitalium which included a total of 21 studies. These studies have concluded that the efficacy
of 1 g azithromycin for the treatment of urogenital *M. genitalium* has decreased from 85% prior to 2009 and is approaching 60% which is well below the 95% threshold recommended by the World Health Organization for STI treatment (Bradshaw *et al* 2006).

It is believed that the continued use of azithromycin 1 g therapy is driving the increase in macrolide antimicrobial resistance as result of frequent selection of resistant genotypes. Twin *et al* (2012) reported that selection over the course of treatment accounted for 55% of azithromycin treatment failure cases. Studies where the macrolide antimicrobial resistance status is known prior to treatment indicate that between 6%–25% of individuals treated with azithromycin 1 g develop macrolide antimicrobial resistance, with about 90% of treatment failures developing macrolide resistance (Horner, 2015; Lau *et al*, 2015).

Following the increase in azithromycin resistance in South Africa as reported by Hay *et al* (2015) and this current study, it may be important that the newly adopted guideline for syndromic management of NGU and cervicitis be revised from a single 1g dose to an extended dose of azithromycin as suggested by numerous studies (Anagrius *et al*, 2013; Terada *et al*, 2012). Treating with an extended azithromycin regimen (1.5 g over 5 days) has been shown to have over 95% efficacy and has a lower risk of inducing macrolide antimicrobial resistance.

Following treatment failure by macrolides, patients with macrolide-resistant strains of *M. genitalium* are usually treated with fluoroquinolones particularly moxifloxacin (Weinstein & Stiles, 2012). Treatment with 400 mg moxifloxacin for 7–14 days has been shown to be highly effective in the treatment of NGU, although patient compliance may be a concerning issue to guard against.
Mutations in defined regions of the DNA gyrase genes, \textit{gyrA} and \textit{gyrB}, and the topoisomerase IV genes, \textit{parC} and \textit{parE}, have been linked to high-level fluoroquinolone resistance in various bacteria, including \textit{M. genitalium} (Deguchi et al, 2001; Tagg et al, 2013). The first clinical reports of \textit{M. genitalium} infection failing therapy with moxifloxacin as a result of fluoroquinolone-associated resistance mutations emerged in 2013 (Couldwell et al, 2013). Subsequently, fluoroquinolone resistance was also reported from a London clinic (Pond et al, 2014), as well as from Japan where approximately one-third of 51 Japanese men with NGU were infected with \textit{M. genitalium} had fluoroquinolone resistance-associated mutations in \textit{parC} (Kikuchi et al, 2014).

In this study, amplification of the \textit{gyrA} and \textit{parC} genes was performed on the \textit{M. genitalium}-positive isolates and the amplicons sequenced and analysed. The \textit{gyrA} gene of two of the 8 \textit{M. genitalium} positive isolates (M73 and M102) did not amplify. Sequences obtained for the remaining 6 isolates were aligned with the DNA gyrase subunit A as well as the whole genome. None of the strains harboured mutations in the \textit{gyrA} gene (G285C; Met→Ile 83) associated with fluoroquinolone resistance. Tagg et al (2013) previously reported one such mutation (G285C; Met→Ile 83) in the quinolone resistance determining region (QRDR) of \textit{gyrA} after aligning five \textit{M. genitalium} genomes with G37 (wild type) suggesting that this kind of mutation could not be that prevalent although this mutation occurred on a position that have been previously associated with fluoroquinolone resistance.

Mutations in the QRDR of the \textit{parC} gene, particularly in the hotspot regions (Deguchi et al, 2001), are more likely to trigger resistance against moxifloxacin as well other fourth generation quinolones (Shimada et al, 2010). In this study, the \textit{parC} gene of six of the 8 \textit{M. genitalium} strains could be amplified. Alignment of the sequences obtained with the corresponding gene
sequences in GenBank, revealed that 5 of the 6 strains had a silent mutation (C234T) (83.3%) in the \textit{parC} gene which is in line with the findings by Tagg \textit{et al} who also observed a high number (27/143) of C234T mutations in their specimens. The role of this mutation is still not known (Tagg \textit{et al}, 2013).

A single patient (T30) (16.6%) in our study had the G248T mutation (Ser$\rightarrow$Ile 80) associated with genotypic resistance to fluoroquinolones as previously described by Tagg \textit{et al} (2013). They observed G248T mutations in 7.7% of their isolates, although there was a huge difference in sample size between their study and this present study (143 vs 6). The presence of this G248T mutation (Ser$\rightarrow$Ile 80) in clinical strains of \textit{M. genitalium} is to our knowledge, the first found in South African isolates. It raises serious concerns about the potential for fluoroquinolone resistance to emerge in the community. This also raises concerns globally as several studies have recommended fluoroquinolones to be a drug of choice following the high rate of macrolide treatment failure.

Furthermore, this isolate was deemed to harbour a multidrug resistant strain (patient T30) as this strain was found to be resistant to macrolides as it harbours A2059G resistance mutation as well as the G248T mutation associated with genotypic resistance to fluoroquinolones. To our knowledge, this strain was the first to be reported in South Africa although it was of no surprise since there has been a sudden rise of multidrug-resistant \textit{M. genitalium} being reported in some parts of Europe, America, Japan and Australia (Braam \textit{et al}, 2017).

This follows an observation initially reported by Deguchi \textit{et al} (2016) of a staggering increase on the prevalence of \textit{M. genitalium} with both macrolide resistance -and fluoroquinolone resistance–associated mutations. These multidrug-resistant \textit{M. genitalium} strains have risen from 0% before 2013 to 16.7% in 2013 and 30.8% in 2014, raising a global concern (Deguchi \textit{et al}, 2016). It is thus imperative to initiate the awareness against the rising number of multidrug-resistant \textit{M. genitalium} strain as treatment options for multidrug-resistant \textit{M. genitalium} continued to be limited (Braam \textit{et al}, 2017). Development of promising new antibiotic regimens for \textit{M. genitalium} infections should be a direction to take and channeling our research effort on since it is only logical that there will be emergence of strains with clinically significant high-level resistance to all existing antibiotics (Deguchi \textit{et al}, 2016). Meanwhile, the treatment of \textit{M. genitalium} infections needs to be carefully managed in order to limit the increase of these multidrug-resistant \textit{M. genitalium} strains.
5.4 GENOTYPING OF *MYCOPLASMA GENITALIUM* ISOLATES

Typing of the different strains of *M. genitalium* using different typing methods individually and in combination continues to play a pivotal role in epidemiological studies as far as understanding the transmission, pathogenesis and drug resistance of *M. genitalium* and may shed light on possible treatment strategies. To our knowledge, this was the first study in South Africa to describe the *M. genitalium* strains circulating in the country.

In this study, all the *M. genitalium*-positive isolates were typed using both the MG191 (mgpB) SNP and MG309 VNTR analysis methods. MG191 sequence type (ST) type was assigned on the basis of previously described types (Hjorth et al, 2006). Hjorth co-workers were able to type 267 specimens which resulted in 56 different sequence types after aligning them with the *M. genitalium* G37 wild type strain (Hjorth et al, 2006).

In this study, STs could be obtained for 6 of the 8 *M. genitalium* isolates. The 6 isolates grouped into 4 different STs, with two isolates (T76 and M84) as ST1, T63 was ST2; T30 and T75 were ST4 and one isolate (M25) was ST39. The fact that 4 different STs (ST1, ST2, ST4 & ST39) were obtained from only 6 isolates indicated that there could be many different *M. genitalium* strains circulating in this setting. What the effect of ST is on resistance genotypes is unknown as the two isolates (T30, M25) that were deemed resistant to macrolides belonged to different STs; ST4 and ST39 respectively, and were collected from different clinics (TOP and ANC) within the hospital. The isolates with the silent fluoroquinolones mutation also belonged to different STs: ST1 (T76, M84); ST2 (T63); ST4 (T30, T75) and ST39 (M25) and are randomly distributed amongst those two clinics (TOP and ANC). This finding is in agreement with Pond et al (2014) where 11 of 22 *M. genitalium* infections were caused by 8 STs.
Using this typing method, Cazanave et al (2012) could obtain a single sequence variant for 73 out of 76 *M. genitalium* isolates from Tunisian patients. Sixteen different STs were detected which highlighted the diversity among the specimens, with distinct clusters corresponding to the more frequent STs 1, 2, and 4. Macrolide resistance was also spread amongst the STs in our study. However, phylogenetic analysis done revealed a tree divided into 2 major clusters. Three isolates (T63, T76 and M84) fell in the same cluster as the wild type strain G37 while the remaining isolates (T30, T75, M25 and M73) fell in cluster B. The two isolates with genotypic macrolide resistance clustered together unlike in the study reported by Pond et al (2014). In their study genotypic macrolide resistance was spread amongst the two major clusters found. The lower number of isolates in this present study may however have played a role in our findings. This typing system is simple and reproducible and may be useful in studies of sexual networks and for evaluation of transmission. However, the MG191 typing system alone is less discriminatory and more useful to describe broad epidemiological STs.

Using the MG309 VNTR analysis method, 7 of the 8 *M. genitalium*-positive isolates were successfully typed. Four different VNTR patterns were seen. The most commonly seen pattern was 10 repeats (M25; T30 and T75), followed by the wild type pattern of 12 repeats (T76 and T84). One isolate (M73) had 11 repeats and one isolate (T63) had 14 repeats. Phylogenetic analysis revealed a tree clearly divided into 2 major clusters. In the study by Pond et al (2014) the 20 *M. genitalium* isolates displayed 7 VNTR patterns (8, 9, 10, 11, 13, 14 and 15). Similar to the present study, the most common pattern was also 10 repeats (5/20). In their study, Ma et al (2008) reported 9 different VNTRs with 10 repeats also the most commonly seen. Phylogenetic tree analysis clearly demonstrated that the macrolide resistant strains, T30 and M25, were closely related, falling within the same cluster. As with MG191 SNP analysis, this
differs from the finding by Pond et al (2014) where macrolide resistant strains were spread amongst the two major clusters.

The results from this study demonstrate that using MG191 SNP and MG309 VNTR analysis in combination forms a reliable and vital tool to compare and analyse M. genitalium strains’ relatedness. The two typing methods were in concordance as they both yielded two distinct clusters, with the same strains in each cluster. Patients with M. genitalium infection, with or without genotypic resistance, separated into distinct genotypic clusters and were also related to a diverse population of international control sequence types. This indicated that our findings, rather than being a local clonal phenomenon that could have biased the data, were more likely to represent established infection and resistance rates in this patient population as a whole.

The study of M. genitalium presents several unique challenges. It remains extremely difficult to isolate the organism from clinical specimens, and thus, identification of infected individuals is dependent on the use of PCR tests. Low M. genitalium loads and human cell inhibitors in specimens have impeded progress in understanding the antimicrobial resistance, epidemiology and pathogenic role of the organism. The assays used in this study have been useful for detecting gene mutations associated with antimicrobial resistance and for strain typing.

5.5 STUDY LIMITATIONS

Due to the low prevalence of M. genitalium in the studied population, the sample number was low. Although antimicrobial resistance was present in these samples, no clear conclusions regarding resistance could be drawn from only 6 samples. Similarly, due to the low M. genitalium numbers, strain variation, especially between the two groups of women was not
clearly established. It may be of interest to follow concordant and discordant couples to investigate transmission.

5.6 CONCLUSION

In conclusion, *M. genitalium* is prevalent in women attending both the TOP and ANC clinics at Dr George Mukhari Academic Hospital. Macrolide resistance associated mutations were seen in two isolates, and for the first time in South Africa, a fluoroquinolones resistance associated mutation was seen in one isolate. The prevalence of macrolide resistance reported in this study, emphasises the importance of surveillance to adopt the optimal guidelines for syndromic management of NGU and cervicitis. In addition, resistance against fluoroquinolones as found in this study and other studies all over the world continues to stress the need for alternative treatment regimes. Combination analyses of both the MG191 (mgpB) SNP and MG309 VNTR successfully genotype isolated *M. genitalium* strains, grouping them into two major clusters. The resistant isolates clustered together. It is important to continue epidemiological surveillance of circulating *M. genitalium* strains to adapt treatment to *M. genitalium* infections, especially in countries where syndromic management is used.
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APPENDIX 1: ETHICAL APPROVAL

Sefako Makgatho Health Sciences University
Research & Postgraduate Studies Directorate
Sefako Makgatho University Research Ethics Committee
(SMUREC)

Molotlegi Street, Ga-Rankuwa 0208
Tel: (012) 521 5617/3698 | fax: (012) 521 3749
Email: lorato.phiri@smu.ac.za
P.O. Box 163 Medunsa 0204

APPROVAL NOTICE - NEW APPLICATION

04 June 2015

Mr M Mafurise
Department of Microbiology
P.O Box 264
Medunsa, 0204

MEETING:

05/2015

SMUREC Ethics Reference Number:
SMUREC/P/138/2015: PG

The New Application, received on 21 May 2015, was reviewed by members of Sefako Makgatho University Research Ethics Committee on 04 June 2015 and was approved on 04 June 2015.

Title:
Prevalence and molecular analysis of Mycoplasma genitalium strains isolated from pregnant women at Dr George Mukhari Academic Hospital

Researcher:
Mr M Mafurise

Supervisor:
Dr MC le Roux

Co-supervisor:
BE de Villiers

Hospital Superintendent:
Dr MC Holm (DGMAH)

Department:
Microbiological Pathology

Pathology & Pre-Clinical Sciences

School:
MSc (Med) Microbiology

Degree:

Please note the following information about your approved research protocol:


Please remember to quote your protocol number (SMUREC/P/138/2015: PG) on any documents or correspondence with the REC concerning your research protocol.

Please note that the REC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review: Please note a template of the progress report is obtainable in the Research Office and should be submitted to the Committee before the year has expired. The Committee will consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit. Translation of the consent document in the language applicable to the study participants should be submitted.

International Organisation (IORG0004319), Institutional Review Board (IRB00005122), Federal Wide Assurance (FWA00009419)
Expiry date: 11 October 2016 and NHREC No: REC 210408-003

Sincerely,

[Signature]

PROF GA OUNGBANJO
CHAIRPERSON SMUREC

[Stamp]
APPENDIX 2: CONSENT FORM

Statement concerning participation in a Research Project.

Name of Project

Prevalence and molecular analysis of *Mycoplasma genitalium* strains isolated from pregnant women at Dr George Mukhari Academic Hospital.

I have read the information and heard the aims and objectives of the proposed study and was provided the opportunity to ask questions and given adequate time to rethink the issue. The aim and objectives of the study are sufficiently clear to me. I have not been pressurized to participate in any way.

I understand that participation in this Project is completely voluntary and that I may withdraw from it at any time and without supplying reasons. This will have no influence on the regular treatment that holds for my condition neither will it influence the care that I receive from my regular doctor.

I know that this Project has been approved by the Sefako Makgatho Health Sciences University Research and Ethics (SMUREC), / Dr George Mukhari Academic Hospital. I am fully aware that the results of this project will be used for scientific purposes and may be published. I agree to this, provided my privacy is guaranteed.

I hereby give consent to participate in this Project.

-------------------------------------------  -------------------------------------------
Name of patient/volunteer                  Signature of patient or guardian.

-------------------------------------------  -------------------------------------------  -------------------------------------------
Place.                                      Date.                                     Witness

Statement by the Researcher

I provided verbal and/or written information regarding this Project

I agree to answer any future questions concerning the Project as best as I am able.

I will adhere to the approved protocol.

-------------------------------------------  -------------------------------------------  -------------------------------------------
Name of Researcher                          Signature                                  Date                          Place
### APPENDIX 3: RESULTS

#### Ante-natal Clinic

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