EVALUATION OF TRANSCRIPTION MEDIATED AMPLIFICATION AND POLYMERASE CHAIN REACTION ASSAYS FOR DETECTION OF *MYCOPLASMA GENITALIUM* IN URINE SPECIMENS OF MEN WITH URETHRITIS

by

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DECLARATION

I, **Magdeline Raesibe Ramoncha,** hereby declare that the work on which this dissertation is based, is original (except where acknowledgement is indicated otherwise) and that neither the whole work, nor any part of it has been, is being, or is to be submitted for another degree at this or other university or tertiary education, institution or examination body.

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Signature of candidate

______day of ________________________ 20 ___

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"Everyone has the power for greatness, not for fame…" **Dr Martin Luther King Jr.**

ABSTRACT

Mycoplasma genitalium, a human mycoplasma species has been established as a cause of nongonococcal urethritis (NGU) in men, particularly in *Chlamydia trachomatis*-negative patients. It was also shown to play a role in cervicitis and pelvic inflammatory disease (PID) in women. Due to difficulty in culturing, and the lack of routine molecular diagnostic tests, many *M. genitalium* infections are undetected.

The purpose of this study was to evaluate three nucleic acid amplification tests (NAATs) i.e. a recently developed Gen-Probe research only transcription mediated amplification (TMA) assay, a conventional polymerase chain reaction (PCR) assay and a real-time PCR (q-PCR) assay for the detection of *M. genitalium* in urine specimens of men with symptoms of urethritis. To evaluate the three assays, 300 urine specimens were collected between June 2007 and July 2008 from sexually active male patients presenting with discharge (N=94) and/or burning on micturition (N=206) to a private medical practitioner in Silverton, Pretoria. A specimen was considered positive by extension of the gold standard i.e. if any two of the three assays were positive. This was used to calculate the sensitivity and specificity of each method.

TMA detected *M. genitalium* in 62 (21%), PCR in 43 (14%) and q-PCR in 48 (16%) of the 300 patients. The sensitivities of the assays were 100% (TMA), 92% (q-PCR) and 78% (PCR), with specificities of 90% (TMA), 95% (q-PCR) and 97% (PCR). The sensitivity of the TMA assay was higher than that of the q-PCR and PCR assays. The lower sensitivity obtained by the q-PCR assay might have been due to inhibition and limitations in the amount of the DNA template. However, the q-PCR assay was easy to perform as it combines amplification and detection thus eliminating further handling of PCR products. The PCR, although with a higher specificity, was the least desirable in terms of testing time and problems with subjectivity when reading agarose gels.

We concluded that the Gen-Probe TMA assay is a highly sensitive method for detection of *M. genitalium* in urine specimens of men. The use of Gen-Probe TMA and the q-PCR assay, will increase the detection of *M. genitalium* in clinical specimens at this catchment area.

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ABBREVIATIONS

AIDS	**:**	Acquired immune deficiency syndrome
AST	**:**	Antimicrobial susceptibility test
ATCC	**:**	American Type Culture Collection
ATP	**:**	Adenosine triphosphate
C	**:**	Cytosine
CDC	**:**	Centers for Disease Control and Prevention
CT	**:**	*Chlamydia trachomatis*
C. trachomatis	**:**	*Chlamydia trachomatis*
dNTPs	**:**	Deoxynucleotide triphosphates
DNA	**:**	Deoxyribonucleic acid
DNase	**:**	Deoxyribonuclease
dH_2O	**:**	Distilled water
EDTA	**:**	Ethylenediaminetetraacetic acid
EIA	**:**	Enzyme Immunoassay
ELISA	**:**	Enzyme-Linked Immunosorbent Assay
EM	**:**	Electron microscope
FDA	**:**	Food and Drug Administration
Fig	**:**	Figure
FSW	**:**	Female sex workers
FVU	**:**	First-void urine
G	**:**	Guanine

g	**:**	Gram
G-37	**:**	*Mycoplasma genitalium* strain 37
gap	**:**	Glyceraldehyde-3-phosphate
gap	**:**	Glyceraldehyde-3-phosphate gene
GAPDH	**:**	Glyceraldehyde-3-phosphate dehydrogenase
GUM	**:**	Genitourinary medicine
GUS	**:**	Genital ulcer syndrome
***gyr*A**	**:**	Gyrase A gene
HEPES	**:**	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic acid
HIV	**:**	Human immunodeficiency virus
hpf	**:**	High power field
Ig	**:**	Immunoglobulin
kb	**:**	Kilobase
kbp	**:**	Kilobase pair
kDa	**:**	Kilo dalton
LAMP	**:**	Lipid-antigen membrane protein
LC	**:**	LightCycler
$MgCl_2$	**:**	Magnesium chloride
ml	**:**	Milliliter
MG	**:**	*Mycoplasma genitalium*
MgPa	**:**	*Mycoplasma genitalium* adhesion protein
mgpB	**:**	*Mycoplasma genitalium* protein B
M. genitalium	**:**	*Mycoplasma genitalium*

MIC	**:**	Minimum inhibitory concentration
mM	**:**	Millimolar
M. pneumoniae	**:**	*Mycoplasma pneumoniae*
MSM	**:**	Men who have sex with men
MSW	**:**	Men who have sex with women
MsrA	**:**	Methionine sulphate reductase A
MTRT PCR	**:**	Multi-target real-time polymerase chain reaction
MUS	**:**	Male urethritis syndrome
MW	**:**	Molecular weight
NAATs	**:**	Nucleic acid amplification tests
nm	**:**	Nanometer
NCNGU	**:**	Nonchlamydial Nongonococcal urethritis
NG	**:**	*Neisseria gonorrhoeae*
N. gonorrhoeae	**:**	*Neisseria gonorrhoeae*
NGU	**:**	Nongonococcal urethritis
ORF	**:**	Open reading frame
OD	**:**	Optical density
P	**:**	Phosphate
***par*C**	**:**	Parkin-like protein C
PBS	**:**	Phosphate buffered saline
PCR	**:**	Polymerase chain reaction
PMNL	**:**	Polymorphonuclear leukocyte
q-PCR	**:**	Real-time polymerase chain reaction

RNA	**:**	Ribonucleic acid
RNase	**:**	Ribonuclease
rRNA	**:**	Ribosomal ribonucleic acid
RSA	**:**	Republic of South Africa
R-T PCR	**:**	Real-time polymerase chain reaction
STD	**:**	Sexually transmitted disease
STI	**:**	Sexually transmitted infection
SP4	**:**	Sucrose phosphate 4
Taq	**:**	Thermus aquaticus
TBE	**:**	Tris borate ethylenediaminetetraacetic acid
TCS	**:**	Target capture system
TMA	**:**	Transcription mediated amplification
TTU	**:**	Ten-Tube unit
T. vaginalis	**:**	*Trichomonas vaginalis*
µm	**:**	Micrometer
U. urealyticum	**:**	*Ureaplasma urealyticum*
USA	**:**	United States of America
UV	**:**	Ultraviolet
VCT	**:**	Voluntary counseling and testing
WHO	**:**	World Health Organisation

CHAPTER 1: LITERATURE REVIEW

1.1 HISTORICAL BACKGROUND

The organism *Mycoplasma genitalium* (*M. genitalium*) was discovered in 1980 by Tully *et al* in Maryland, United States of America (USA) (Tully *et al*, 1981). The anatomical site of isolation for this organism was the genitourinary tract of men diagnosed with inflammation of the urethra (urethritis). Furthermore, this organism was observed to exist in urethritis patients in the absence of other known pathogens causing urethritis such as *Neisseria gonorrhoeae* (*N. gonorrhoeae*) and *Chlamydia trachomatis* (*C. trachomatis*) (Black *et al*, 2008; Pépin *et al*, 2001).

Two strains of *M. genitalium*, namely, G-37 and M-30, have been isolated (although very difficult and time consuming) by Tully *et al* (1981) from the urethral material of two men who were attendees of a genitourinary medicine (GUM) clinic at St Mary's hospital, Paddington, London (Jensen, 2006, Anagrius *et al*, 2005, Deguchi *et al*, 2002). These isolates have since been implicated as one of the causal agents of nongonococcal urethritis (NGU) in men, and still in recent times, it was shown by Manhas *et al* (2009) that *M. genitalium* constitutes one of the most important causes of NGU besides other genital mycoplasmas.

According to Jensen *et al* (1996), subsequent attempts by Taylor-Robinson *et al* (1985) to isolate this organism by culture and serology were unsuccessful as was also experienced by Samra *et al* (1988). In the few studies that followed, they reported that it took several weeks to months with several passages to grow this human mycoplasma species (Ross & Jensen, 2006; Jensen *et al*,

1996). Therefore, due to the lack of reliable culture and serological tests it was difficult to establish the role of *M. genitalium* in nonchlamydial NGU (NCNGU) (Jensen *et al*, 1993; Jurstrand *et al*, 2005).

The development and utilization of nucleic acid amplification tests (NAATs) such as polymerase chain reaction (PCR) specific for *M. genitalium* by Jensen *et al* in 1991 and the later developed real-time PCR assay by Yoshida *et al* in 2002, has made it possible to detect *M. genitalium* in clinical specimens. Recently Gen-Probe, Inc. (USA) has introduced a research-only transcription mediated amplification (TMA) assay for the detection of *M. genitalium* (Wroblewski *et al*, 2006). These tests have helped to establish *M. genitalium*'s role in male urethritis.

1.2 CHARACTERISTICS OF *M. GENITALIUM*

Most of the known characteristics of *M. genitalium* are based on findings from its thoroughly studied, genetically close relative, *Mycoplasma pneumoniae* (*M. pneumoniae*) (Jensen, 2006). While *M. genitalium* can cause genitourinary disease, *M. pneumoniae* is an established pathogen of the human respiratory tract and causes atypical pneumonia (Stein & Baseman, 2005; Jensen, 2006).

1.2.1 Taxonomic classification

Mycoplasmas are prokaryotes of the family *Mycoplasmataceae* within the order Mycoplasmatales (Prescott *et al*, 2005). According to Taylor-Robinson (2007) there are eight genera within the Mollicutes [*mollis* (soft); *cutis* (skin)] class that comprises 200 species, with most of these species belonging to the genus *Mycoplasma*, which encompasses bacteria without a

cell wall (Jensen, 2006; Prescott *et al*, 2005).

The phylogenetic tree of evolution has revealed that mycoplasmas may be descendants of Gram-positive bacteria, presumably of clostridial origin (Stein & Baseman, 2005; Jensen, 2006). This transformation is suspected to have occurred through a genome reduction process resulting in *M. genitalium* having the smallest bacterial genome, close to the size of a virus (Bizarro & Schuck, 2007). The phylogenetic tree, following the 16S ribosomal ribonucleic acid (rRNA) gene sequence has revealed that *M. genitalium* and *M. pneumoniae* belong to the same cluster within the *Mycoplasma* genus thus making the two organisms closely related (Jensen, 2006). This close relationship was confirmed by the many similar morphological and antigenic characteristics including lipid components that the two bacteria share (Svenstrup *et* al, 2005; Hyman *et al*, 1987).

1.2.2 Morphological properties and metabolism

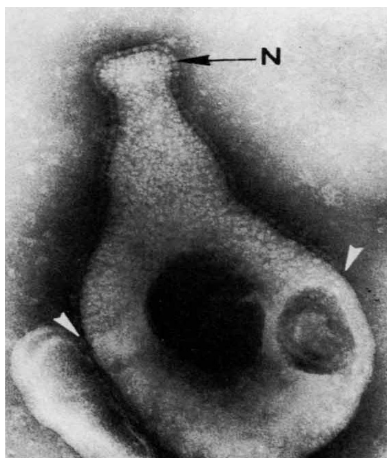
The genus *Mycoplasma* generally contains a very small type of bacteria, with the size ranging from 0.2 to 0.3 micrometers (µm) depending on the shape (Taylor-Robinson, 1995), which may be spherical, filamentous or flask/pear-like (Jensen, 2006; Stein & Baseman, 2005) depending on the species.

The species *M. genitalium* was first mistaken for a virus as it could not be visualized with a light microscope due to its size (Jensen, 2006). It has the ability to self-reproduce and does not have a peptidoglycan cell wall therefore lacks cell surface markers (Tully *et al,* 1981; Taylor-Robinson, 1995). The absence of a cell wall also indicates that this bacterium has less osmotic stability in

the host environment and is therefore prone to changes in shape. Like other mycoplasmas, the entire cytoplasm of *M. genitalium* is enclosed by a cell membrane only (Jensen, 2006). 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).

Among the different shapes of mycoplasmas, both *M. genitalium* and *M. pneumoniae* have characteristic pear/flask-like morphology with a terminal/apical tip organelle (Taylor-Robinson, 1995; Jensen, 2006). In order to identify these species based on shape, a negatively stained specimen has to be viewed under a scanning electron microscope (SEM) or transmission electron microscope (TEM) (Tully *et al*, 1981; Tully *et al*, 1983; Jensen, 2006).

The electron micrograph of both the G-37 and M-30 *M. genitalium* strains revealed an organism of 0.6-0.7μm in length, 0.3-0.4μm wide near the base and 0.06-0.08μm wide at the terminal tip (Tully *et al,* 1981). The core of the tip has dense parallel tracts called a nap at the neck-like structure that protrudes from the main cell thus giving it a pear-like appearance (Tully *et al* 1981; Tully *et al* 1983; Taylor-Robinson, 1995) (Fig. 1.1).



Where:

N: nap/neck

►: base

Figure 1.1 TEM of negatively stained intact *M. genitalium* cell. (Adopted from Tully *et al*, 1983).

According to Lind *et al* (1984), the neck-like region of *M. genitalium* is shorter than that of *M. pneumoniae*. The terminal tip organelle is specialized to give *M. genitalium* a gliding motility along moist/mucous surfaces, as well as adherence to other surfaces such as plastic, red blood cells, Vero monkey kidney cells, and epithelia of eukaryotic host cells (Stein & Baseman, 2005, Tully *et al*, 1981).

Unlike other mycoplasmas that utilize arginine (*M. hominis*) or urea (*U. urealyticum*), *M. genitalium* metabolizes glucose resulting in production of an acid (Taylor-Robinson, 1995; Stein & Baseman, 2005). *M. genitalium* lacks the metabolic citric acid cycle and as a Mollicute it is assumed to survive by relying on substrate (glucose) phosphorylation that is associated with glycolytic kinase enzymes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate kinase and phosphoglycerate kinase for the synthesis of essential nucleotriphosphates (NTPs) for its genome (Pollack *et al*, 2002). It was observed that the phosphorylation process in *M. genitalium* is comparable to that of other common bacteria (Su *et al*, 2007). *M. genitalium* exclusively makes use of GAPDH, which it has retained in its small genome, during the process of glycolysis (Svenstrup *et al* 2005; Stein & Baseman, 2005).The glycolytic process catalyzed by GAPDH, among other enzymes, generates energy for this organism (Stein & Baseman, 2005).

1.2.3 Genetic make-up

When comparing the genomes of *M. genitalium* and *M. pneumoniae,* it was found that the *M. genitalium* genome is smaller (580 kilobases (kb)) than that of *M. pneumoniae* (816 kb) (Taylor-

Robinson, 1995; Su *et al*, 2007). While having the smallest genome size among all bacteria (Taylor-Robinson & Horner, 2001; Jensen, 2006; Bizarro & Schuck, 2007), it is surprising that *M. genitalium* contains both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) genomes (Taylor-Robinson, 1995).

In 1995, the genome of *M. genitalium* G-37 followed that of *Haemophilus influenzae* (*H. influenzae*) to be fully sequenced and published and has become an American Type Culture Collection (ATCC 33530) strain (Jensen, 2006; Ueno *et al*, 2008; Zhang & Lin, 2009). It is from this *M. genitalium* G-37 sequence that a minimum set of genes required to sustain life was determined (Jensen, 2006; Zhang & Lin, 2009). 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 the particular organism (Zhang & Lin, 2009). According to the authors, *H. influenzae* has 642 essential genes whereas *M. genitalium* has only 381. This shows how the small *M. genitalium* is still capable to survive on its own. An earlier study by Glass *et al* (2006) found 382 essential genes in *M. genitalium*, a value similar to that found by Zhang & Lin (2009).

Taylor-Robinson (1995) and Su *et al* (2007) have identified only 480 protein coding regions in the *M. genitalium* genome while a later report by Ueno *et al* (2008) found 484 coding regions which is still a small number compared to other bacteria. All these identified coding regions include genes for DNA replication, transcription, translation, DNA repair, cellular transport and energy metabolism.

The genetic make-up of the terminal tip organelle shows that it is a protein complex. Himmelreich *et al* (1997) and Jensen (2006) among other authors made an observation with comparison to the counterpart tip structure of *M. pneumoniae*. The 140 kilodalton (kDa) *M. genitalium* protein (P140/MgPa or MG191) that is the major adhesion protein at the tip, closely resembles the 170 kDa main adhesion protein 1 (P1) of *M. pneumoniae* whereas the 32 kDa *M. genitalium* protein (P32 or MG318) resembles P30 of *M. pneumoniae* (Baseman *et al*, 1988; Stein & Baseman, 2005; Jensen, 2006). In a later study, Burgos *et al* (2007) identified a structural protein, MG312 of the *M. genitalium* operon, as another component of the tip essential for the assembly and movement of this terminal instrument.

M. genitalium falls under the so called "low G+C" mycoplasmas because its DNA genome typically has fewer guanine (G) and cytosine (C) DNA bases than adenine (A) and thymine (T) bases as compared to other bacteria (Mombach *et al*, 2006; Bizarro & Schuck, 2007). The G+C content in the DNA of mycoplasmas in general ranges 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. However, *M. genitalium* has a significantly higher G+C content (44%) in its ribosomal rRNA gene (Jensen, 2006). *M. genitalium* has several genes that are used as targets in the laboratory for its detection, including the *gap* gene, *MgPa* gene and rRNA (Jensen *et al*, 1991; Svenstrup *et* a*l*, 2005).

Despite its exceptionally small genome, reduced number of essential genes, lack of a rigid cell wall around its cytoplasm and the low nucleic acid G+C content, *M. genitalium* has the ability to

survive independently, self-reproduce and cause disease.

1.3 ROLE OF *M. GENITALIUM* IN URETHRITIS

1.3.1 Case definition of urethritis

The 2006 Canadian Guidelines on Sexually Transmitted Infections (STIs) (Public Health Agency of Canada, 2006) define urethritis as inflammation of the urethra with or without discharge that is mucoid, mucopurulent or purulent. The urethral inflammation is categorized into gonococcal urethritis caused by *N. gonorrhoeae* and nongonococcal urethritis (NGU) caused by pathogens other than *N. gonorrhoeae*.

The National Network of STD/HIV Prevention Training Centers (NNPTC, 2007) under the Centers for Disease Control and Prevention (CDC) has stated that an inflammatory response in the urethra is reflected microscopically by the presence of an excess number (≥5) of polymorphonuclear leukocytes (PMNLs) per high power field in urethral smears obtained from the anterior urethra or ≥10 PMNLs per high power field of first void urine sediment. In addition to the PMNL count, urethritis caused by *M. genitalium* can also be shown by a positive leukocyte esterase test (Shahmanesh *et al*, 2009) or by an electron micrograph of this bacterium (Tully *et al*, 1981; Ross & Jensen, 2006).

1.3.2 Signs and symptoms of urethritis

According to the Canadian Guidelines on STIs (Public Health Agency of Canada, 2006), the inflammation may be manifested by dysuria (painful urination), pruritis (itching) or meatal

erythema (reddening of the urinary canal). In many men with a low grade urethral inflammation, no known pathogen is detected and about one third of men who have *M. genitalium* or *C. trachomatis* as a pathogen may not show an excess of PMNLs (Shahmanesh *et al*, 2009).

1.4 PATHOGENESIS

M. genitalium is an obligate intracellular parasite primarily of the lower genital tract of humans (Taylor-Robinson & Horner, 2001; Bizarro & Schuck, 2007). Although the primary tissue infected by *M. genitalium* is the human urogenital tract (Tully *et al*, 1981, Jensen, 2006) the organism has been detected at other sites such as the synovial joints (Jensen, 2006; Baseman *et al*, 1988).

1.4.1 Transmission

According to Shahmanesh *et al* (2009), the European Guidelines have emphasized that the causative agents (pathogens) of urethritis are mainly sexually transmitted, with the common pathogen being *N. gonorrhoeae*, followed by *C. trachomatis*. *M. genitalium* is grouped among other urethritis pathogens such as *U. urealyticum*, *M hominis, T. vaginalis* and some viruses such as herpes simplex virus type-2 (HSV-2) (Black *et al*, 2008; Pépin *et al*, 2001; Sturm *et al*, 2004).

Like other STIs, *M. genitalium* infection is transmitted between heterosexual partners during unprotected coital activity (Ross & Jensen, 2006; Manhart *et al*, 2007b). The ability of *M. genitalium* to adhere to spermatozoa aids in the sexual transmission (Svenstrup *et al*, 2003).

Oral sex has been shown to play an insignificant role in transmission of this organism (Bradshaw *et al,* 2009). In their study, they have not detected any *M. genitalium* in the pharyngeal swabs of 521 men who have sex with men (MSM) who met at male-only saunas. The organism was present in the urethral and rectal swabs of these men. Further investigation is needed to find out if the rectal presence of this organism could cause proctitis or if it is only asymptomatic carriage. In a previous study by Francis *et al* (2008), it was stated that although it is likely that *M. genitalium* infects the rectum, it is unclear if it contributes to clinical symptoms.

Very little is known on vertical transmission and subsequent colonization of newborn infants by *M. genitalium* (Jensen, 2006). However, Waites *et al* (2005) have mentioned that *Mycoplasma hominis* and *Ureaplasma* species, both belonging to the same family (*Mycoplasmataceae*) as *M. genitalium*, can be transmitted from an infected female to the fetus or neonate by (1) gaining access to the amniotic sac through ascending intrauterine infection, (2) hematogenous route through placental infection where umbilical vessels are involved, or (3) perinatal route during passage of the neonate through the infected maternal birth canal with the resultant colonization of the skin, mucosal membranes and respiratory tract of the neonate.

1.4.2 Virulence factors of *M. genitalium*

M. genitalium has several virulence factors that are responsible for its pathogenicity. These include:

1.4.2.1 Attachment and entry

In the absence of a cell wall and its associated attachment structures such as fimbriae, *M.*

genitalium utilizes an attachment organelle at the tip that is a complex protein structure, to mediate adhesion (Jensen, 2006; Burgos *et al*, 2006). This cell membrane bound protein complex is required for intimate adherence to host target cells.

The attachment mediated by adhesins is the prerequisite for the organism to gain entry into the host cell. Specific *M. genitalium* adhesins, although surface-exposed, are linked to an internal cytoskeleton that constitutes the tip organelle (Ueno *et al*, 2008) The major adhesin in the attachment protein complex is the MgPa that encodes the reciprocally stabilized P140 (MG191) and P110 (MG192) cytadherence proteins (cytadhesins) (Jensen, 2006; Burgos *et al*, 2006). MgPa is highly immunogenic (Sventrup *et al*, 2005; Burgos *et al,* 2006) and its adherence to target cells is anchored by these cytadherence proteins. Loss of either P140 or P110 results in loss of motility and adherence properties of the entire MgPa attachment organelle (Burgos *et al*, 2006; Stein & Baseman, 2005) thus showing the importance of these proteins in attachment. It was later discovered by Burgos *et al* (2007) that another protein component of the tip designated MG312 assists in the assembly and gliding movement of the attachment organelle for successful colonisation.

According to Jensen (2006), some studies have shown that *M. genitalium* can also attach to erythrocytes, Vero monkey kidney cells and ciliated epithelium of human fallopian tubes using this tip organelle.

1.4.2.2 Enzyme-mediated attachment

Alvarez *et al* (2003) have found that the activity of the glycolysis enzyme glucose-3-phosphate

dehydrogenase (GAPDH) brings about attachment of *M. genitalium* to human vaginal and cervical mucin. Thus GAPDH, among other binding proteins, acts as a ligand to receptors mucin and fibronectin, particularly in vaginal and cervical disease (Alvarez *et al*, 2003).

Like *M. penetrans* and *M. fermentans*, *M. genitalium* penetrates host epithelial cells after attachment (Waites *et al*, 2005; Jensen, 2006). The target cell membrane then invaginates in a manner similar to the clathrin-coated pit mechanism of endocytosis observed in *C. trachomatis* (Jensen, 2006). Clathrin is a large protein that helps in the formation of a coated pit on the inner surface of the plasma membrane of a cell. The pit later buds into the cell to form a coated vacuole in the cytoplasm of the cell through which it delivers the infecting organism into the cell. Following entry into the target cell, the organism appears to reside in the membrane-bound vacuoles closer to the target cell nucleus (Jensen, 2006; Ueno *et al*, 2008). According to these authors, the internuclear localization may take place 30 minutes after entering the human host.

1.4.2.3 Enzymes

M. genitalium has the ability to translocate its cytoplasmic enzymes to the cell membrane surfaces to enhance host tissue colonization (Blaylock *et al*, 2004; Ueno *et al*, 2008). In addition to GADPH, another enzyme, methionine sulfoxide reductase (MsrA) which is an antioxidant repair enzyme, can be released to enhance the pathogenicity (Alvarez *et al*, 2003; Dhandayuthapani *et al*, 2001). MsrA restores proteins that have lost their biological activity due to the oxidation of their methionines thereby protecting the bacterium protein structure from the host oxidative damage (Dhandayuthapani *et al*, 2001).

1.4.2.4 Antigenic variation

In order to escape the host immune attack, proteins P140 and P110 of the MgPa has the ability to undergo antigenic variation thus altering the entire genetic sequence of the MgPa with subsequent generation of variants that are not recognizable by the host immune system on subsequent encounters (Jensen, 2006; Ueno *et al*, 2008). This is a limitation when using this gene as target in PCRs. Other survival mechanisms of this organism may be the ability to mimic host cell antigens and the intracellular location within professional macrophages (Jensen, 2006).

1.5 EPIDEMIOLOGY

In general, prior to the use of molecular techniques the information on the epidemiology of *M. genitalium* was scanty due to the absence of routine and effective diagnostic methods (Taylor-Robinson & Horner, 2001); furthermore most prevalence studies have been published on results from men (Ross & Jensen, 2006), with limited available data from females.

Hooton *et al* (1988) studied the prevalence of *M. genitalium* using a DNA probe technique, on male genitourinary medicine (GUM) clinic attendees presenting with urethritis and reported that in the group with nongonococcal urethritis (NGU), 10% (3/30) of the men were positive for *M. genitalium* in conjunction with other pathogens, whereas 13% (4/31) were positive for *M. genitalium* only. This was an indication that *M. genitalium* could cause urethritis independent of *C. trachomatis*.

Taylor-Robinson & Horner (2001) did a review study on published data on *M. genitalium* prevalence and reported that *M. genitalium* was detected significantly more often in the urethra

of men with clinical signs of urethritis than in those without. When comparing men with nonchlamydial NGU (NCNGU) to those who were asymptomatic, the Taylor-Robinson & Horner (2001) study found *M. genitalium* to be present in 23.5% of men with NCNGU, which falls within the European Guidelines (2009) range of 9-25%. Mena *et al* (2002) also found a comparable prevalence of 25% in men with NCNGU. A large literature review conducted by Deguchi & Maeda (2002) found *M. genitalium* to be between 18.4 and 45.5% in all cases of male NCNGU.

Using real-time PCR (q-PCR), Yoshida *et al* (2002) have detected *M. genitalium* in 19.8% of NCNGU male patients in Japan, a finding confirmed by Ross & Jensen (2006) who found *M. genitalium* in 20% of Danish men with NCNGU.

Studies performed in Norway and Sweden, have also shown *M. genitalium* rates of between 3.2-7.1% in men with symptoms of urethritis (Moi *et al*, 2009b; Edberg *et al*, 2008).

The results from all these prevalence studies implicate *M. genitalium* as a highly possible pathogen of NGU among other known NGU pathogens such as *C. trachomatis*, *T. vaginalis* or *U. urealyticum.*

1.5.1 In Africa

Not many studies addressing *M. genitalium* infection in Africa have been done; however, studies are in progress.

In a study done in West Africa by Pépin *et al* (2001), *M. genitalium* together with *N. gonorrhoeae*, *T. vaginalis* and *C. trachomatis*, was found more frequently in men with urethritis than in asymptomatic individuals. *M. genitalium* was present at 10.0% (66/659) in the urethritis group (Pépin *et al*, 2001).

1.5.2 In South Africa

Few studies on *M. genitalium* and its association with NGU, using molecular diagnostic techniques have been done in South Africa. In the first study on black South African men with urethritis, Taylor-Robinson *et al* (2002) enrolled 182 men with signs and symptoms of urethritis presenting to a family practitioner in Johannesburg. Urine specimens were collected from these men as well as from 150 asymptomatic men seen at the same time. Specimens were sent to Copenhagen for *M. genitalium* testing by 16S rRNA PCR. *M. genitalium* was detected in 16.7% 96 men with NGU, and 8.9% of men without symptoms.

Ballard *et al*, (2002) studied the coexistence of urethritis with genital ulcer diseases in South African men, and recruited 186 mine workers with genital ulcers in Carletonville. Although none of the men complained of urethritis symptoms, through microscopy (>5 PMNLs/hpf), 99 (53%) of the men were diagnosed with the disease. *M. genitalium* infection was confirmed by PCR. In the men with NGU, 18% harbored either *C. trachomatis* or *M. genitalium.*

Sturm *et al* (2004) investigated the aetiology of male urethritis in a high HIV prevalent population in Durban. They enrolled 335 men with reported symptoms of urethral discharge or dysuria (confirmed with microscopy) and 100 asymptomatic men in their study. *M. genitalium* was detected by an in-house PCR test in 5% of patients and in 3% of controls.

In a recent study done in Johannesburg by Black *et al* (2008), 430 men with male urethritis syndrome (MUS) attending a STI clinic in Hillbrow were recruited. The diagnosis was confirmed with microscopy, and urine specimens were collected for laboratory testing. *M. genitalium* was detected by an in-house real-time PCR assay in 14.4% of the specimens.

1.6 LABORATORY DIAGNOSIS

1.6.1 Specimen type

Various specimen types such as urine, endourethral swabs, cervical swabs and vaginal swabs can be used to detect *M. genitalium* infection in males and females. The advantages of urine are that it is easy to obtain, non-invasive and allows for rapid and direct detection when using assays such as transcription mediated amplification (TMA). However, the cytotoxic effects of urine may hamper results if the specimens are not properly prepared or if it is stored for very long periods (Hamasuna *et al,* 2007). Fresh urine specimens are therefore preferred for good quality results. Jensen *et al* (2004) compared fist-void urine (FVU) and urogenital swabs for the detection of *M. genitalium* and *C. trachomatis* in males and females by PCR. FVU was found to yield better results. However, Moi *et al* (2009a) have recently found cervical swabs to give a higher sensitivity (86%) than FVU (62%) when they tested the presence of *M. genitalium* in female participants in Norway, Oslo.

It is important to note that due to the absence of a cell wall, *M. genitalium* is sensitive to drying and heat and therefore specimens of all types require proper transport from time of collection to time of arrival in the laboratory. A cold chain should be maintained and specimens have to be

delivered to the laboratory within 24 hours of collection to preserve quality of both the specimen and the results. For longer storage of specimens, a temperature of -70^{0}C is recommended (Murray *et al*, 2003).

1.6.2 Microscopy and staining

The detection of *M. genitalium* using an ordinary light microscope is not possible as this organism is very small (0.22-0.45µm) (Jensen, 2006; Hamasuna *et al*, 2007). The organism can only be clearly observed under the transmission electron microscope (TEM) or scanning electron microscope (SEM) (Tully *et al*, 1981; Jensen, 2006; Prescott *et al*, 2005).

Due to the absence of a peptidoglycan cell wall in *M. genitalium,* the use of the conventional Gram or other staining techniques has not been successful. However, Shankar *et al* (2005) have reported that they were able to observe the colonies of *M. genitalium* under an inverted microscope at 100X objective by making use of the Diane's stain, which contains a methylene blue dye, maltose and azure II.

1.6.2.1 Transmission electron microscopy (TEM)

Electron microscopy (EM) is a type of microscopy where electrons, instead of light, are passed through a sample to view the organism's internal structure by means of the transmission EM (TEM) or the surface structure by scanning EM (SEM). This type of microscopy makes it possible to get a better resolution than the light microscope by using a lower wavelength to observe viruses, flagella, biological membranes or proteins, and very small bacteria (<0.45µm) such as *M. genitalium* that cannot be seen when using an ordinary light microscope (Jensen,

2006).

According to Hayat & Miller (1990) and Razin & Tully (1983), EM uses a negative staining technique that is an established method in diagnostic microscopy for contrasting a thin specimen with an electron dense stain opaque for the electrons. The fluid stain is usually a heavy metal salt, the most suitable and common type being phosphotungstic acid. Other suitable stains include ammonium molybdate, uranyl acetate, uranyl formate etc. The heavy metal salts scatter electrons well and can adsorb to biological matter of structures that are much smaller than those studied with the light microscope. For *M. genitalium* the specimen such as urine is spread on a glass slide. This is mixed with the salt stain and allowed to dry. When viewed with the EM the bacterial cells appear light against the dark surrounding background and an electron micrograph can be taken.

1.6.3 Culture

Laboratory culture methods to isolate *M. genitalium* have been challenging (Taylor-Robinson & Horner, 2001; Jensen, 2006). For other mycoplasmas such as *M. penetrans*, culture in SP-4 and Friis liquid broth and solid agar media has been the gold standard (Shankar *et al*, 2005; Hussain *et al,* 1999). Tully *et al* (1981) developed a sucrose-phosphate (SP-4) medium to isolate mycoplasmas and spiroplasmas at the National Institute of Health (NIH), Maryland, USA. This medium consists of mycoplasma broth base (tryptone, peptone, glucose, deionized water), supplemented with a sterile tissue culture component containing glutamine, 25% yeast extract, 2% TC Yeastolate, fetal bovine serum, penicillin and 0.1% phenol red, and with the final pH

adjusted to 7.5. This was mixed with *M. genitalium* strains G-37 and M-30 and incubated horizontally in tissue culture flasks at 37^0C.

Evidence of mycoplasmal growth was found to be an increase in turbidity as well as an acidic pH change from red to yellow as indicated by the phenol red due to glucose fermentation by the organism (Tully *et al*, 1981; Hussain *et al,* 1999). The layer of cells adhering to the container surface was scraped off to inoculate the solid agar medium. Prior to inoculation, the solid medium was prepared by adding 0.6% agarose or 0.8% Noble agar to the broth base and incubation was anaerobic at 37^0C.

Evidence of growth on agarose was the presence of very small "fried-egg" colonies (Fig 1.2) on the agar plate surface (Tully *et al*, 1981; Hussain *et al,* 1999). The "fried-egg" appearance is due to the growth pattern of mycoplasmas on the solid SP-4 medium (Tully *et al*, 1981), where the mycoplasmas grow deep into the agar while spreading superficially outwards, thus forming a dense centre and a lighter periphery (Prescott *et al*, 2005; Shankar *et al*, 2005; Taylor-Robinson, 1995).

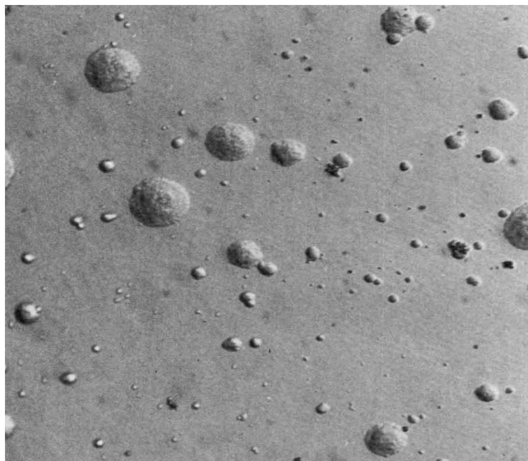


Figure 1.2 "Fried-egg" colonies of *M. genitalium*. (Adopted from Tully *et al*, 1983).

It took Tully *et al* (1981) more than 50 days to grow this organism through a series of passages. Further attempts to isolate the original strains and hopefully new strains from clinical specimens using the broth or the agar were not successful. Hence *M. genitalium* isolates have been rare and the organism still remains very difficult to culture (Edberg *et al*, 2008; Hamasuna *et al*, 2007).

Other attempts to obtain more strains of *M. genitalium* were made by exploring the property of *M. genitalium* to adhere to moist surfaces. In Denmark, Jensen *et al* (1996) could recover 5 new strains by co-culture of *M. genitalium* with Vero monkey kidney cells. In Japan, Hamasuna *et al* (2007) tried to improve the Vero cell co-culture method by monitoring growth of *M. genitalium* through the detection of DNA load of the organism using real-time PCR. The complete isolation of the organism took a period of 1 year for Hamasuna and colleagues.

Various improvements to the quality of the SP-4 medium, the use of different specimen types, as well as an extended incubation period from 14 days to 13 weeks, were not successful in recovering of any new isolates (Jensen *et al*, 2006).

1.6.4 Serology

The relatedness between *M. genitalium* and *M. pneumoniae* due to common or closely related antigens has brought about high rates of antisera cross-reactions. These cross-reactions have significant limitations for the diagnosis as well as epidemiological studies of *M. genitalium* based on serology (Lind *et al*, 1984; Jensen *et al*, 1996). The cross-reactivity was observed as early as 1984 by Lind *et al* when they used rabbit antisera and chloroform-methanol-extracted *M. genitalium* antigens in the compliment fixation test (CFT), indirect immunofluorescence, inhibition tests and immunoelectrophoresis. This cross-reactivity makes it very difficult to

separate the two mycoplasmas by means of serology from specimens of patients that may have a co-infection (Jensen, 2006).

Wang *et al* (1997) have studied the sero-prevalence of antibodies to *M. genitalium* in different populations using Lipid-Associated Membrane proteins (LAMPs) in an enzyme immunoassay (EIA) system. LAMPs are mycoplasma surface proteins that are Triton X- extracted and purified to be used as antigens (Perez *et al*, 1998). The Wang *et al* (1997) assay was validated using serum and urine specimens from 104 patients from whom 40 (38%) was LAMP-EIA positive. From the 40 positive patients, 38% gave positive PCR results. The LAMP-EIA method therefore appeared to be one of the most promising of the serological assays for the diagnosis of *M. genitalium*.

Jurstrand *et al* (2007) published a serological study done on female patients with PID and ectopic pregnancy using this LAMP-EIA. Antisera of the women were mixed with a purified fraction of *M. genitalium* cells containing the LAMP as antigen in the EIA. According to the authors, the LAMP antigen seemed to be specific for *M. genitalium* since no cross-reactivity was observed with other mycoplasmas. The assay was validated by using other mycoplasma species, anti-*M. hominis* or anti-*U. urealyticum* sera and high titre anti-*M. pneumoniae* sera. Although labour intensive, the LAMP-EIA seemed to have worked well in these studies. It is therefore surprising that the assay was discontinued as it appeared promising.

1.6.5 Molecular techniques

Different authors have independently amplified different fragments of the adhesion/attachment

organelle MgPa to improve the sensitivity and specificity of PCR based assays (Taylor-Robinson & Horner, 2001). The *MgPa* gene was thought to be conserved, based on its importance in the pathogenesis of mycoplasmal infections but research has shown that some variations have developed (Jensen, 2006). Molecular approaches that followed the first assays, targeted different genes such as the 16S rRNA (Jensen *et al* in 2003) and *gap* (Svenstrup *et al* in 2005).

1.6.5.1 DNA probes

In 1987 Göbel *et al* introduced the first molecular assay using radioactive labeled oligonucleotide probes targeting the 16S rRNA for the detection of *M. genitalium.* The results showed that the detection limit of this probe was low. Hyman *et al* (1987) used a ^{32}P-labeled cloned DNA probe, selected from genomic libraries prepared in plasmids, that was specific for *M. genitalium*. This probe also had a low detection limit. The first really successful probe was by Hooton *et al* in 1988. They used a nick-translated whole-genome DNA probe that was radioactively labeled to determine *M. genitalium* prevalence in men with urethritis. The ^{32}P-labelled DNA probe showed a good sensitivity that was 10-fold higher than the probe used by Hyman *et al* (1987), with a detection limit of 6 X 10^4–10^5 genome copies.

1.6.5.2 Polymerase chain reaction (PCR) based assays

The first PCR assay to detect *M. genitalium* in clinical specimens was developed by Jensen *et al* in 1991. The antigenic variations in the *MgPa* gene that might give false negative results prompted another molecular approach (Jensen, 2006). Palmer *et al* (1991) developed a hemi-nested PCR using two sets of primers i.e. the inner forward and reverse and the outer forward

and reverse primers to target the MgPa gene. A slight modification by designing a new outer forward primer was compared by Deguchi *et al* (1995) to the original Jensen *et al* (1991) PCR and was found to be in agreement. This primer pair (MgPa1 TGA AAC CTT AAC CCC TTG G forward/ MgPa3 CCG TTG AGG GGT TTT CCA TTT TTG C reverse) was widely used and forms the basis of the microwell-plate-based PCR assay developed by Dutro *et al* (2003). To evaluate its performance, Dutro *et al* (2003) compared the microwell-plate-based PCR assay with the Southern-blot-based PCR assay described by Totten *et al* (2001). They found the two assays to be about 90% in agreement when urine specimens were tested for *M. genitalium.*

1.6.5.3 Real-time Polymerase Chain Reaction

Real-time polymerase chain reaction (q-PCR), a development and modification of the traditional (conventional) PCR, combines amplification and detection of amplified PCR products (amplicons). It has several advantages over the earlier DNA hybridization probes or PCR based assays (Espy *et al*, 2006). It has improved the rapid detection of DNA and RNA of many organisms as it has shown to be more specific (99.5%) and sensitive (91.8%) than a conventional PCR assay (Hardick *et al*, 2006). The simultaneous detection eliminates the labour intensive post amplification agarose gel method of detection as in the conventional PCR assay, or probe hybridization with reduced risk of carryover contamination. Espy *et al* (2006) have shown that both the amplification and detection can be completed in an hour which is much faster than the three or more hours taken by conventional PCR assays or DNA probes.

There are a number of different fluorescent probe chemistries available in q-PCR. Among these are (1) dual fluorescence resonance energy transfer (FRET) probes (2) minor groove binding

(MGB) probes (3) molecular beacons (4) dual labeled probes, also called 5' Nuclease (TaqMan) probes, with a fluorescent dye on 5' end and a quenching dye on 3' end of the probe (5) intercalating dyes (SYBR green) that bind to double stranded DNA and (6) fluorescent labeled primers such as Scorpion, Sunrise or Lux (Gunson *et al*, 2006). However, all these chemistries rely on the transfer of light energy from the donor to the adjacent acceptor dye molecule, a process known as FRET as in technology (1) above (Espy *et al*, 2006). The principle of FRET is that when a high-energy dye (donor) is in close proximity to a low-energy dye (acceptor) there will be transfer of energy from high to low donor. The intensity of FRET increases as the distance between the adjacent dye molecules decreases i.e. the closer the molecules the more FRET. Therefore all types of q-PCR probes are generally referred to as FRET probes.

The dyes for FRET hybridization probes are attached separately to two probes that align in a head-to-tail manner on the nucleic acid target. The first dye is fluorescent while the second is a quencher that absorbs the fluorescent light transferred from the first dye and re-emits it at a different wavelength. For TaqMan probes and molecular beacons, the two dye molecules are attached to one probe as they are single probes assays. The TaqMan probes were the first fluorescent q-PCR probes developed (Espy *et al*, 2006).

Gunson *et al* (2006) have compared the different real-time PCR chemistries and found the FRET hybridization probes to be highly specific since they can detect single nucleotide differences in the nucleic acid as they require an exact match to the target nucleic acid for a light signal to be released. However, the design of these probes relies on strict optimization and they function on accurate denaturation temperature. The TaqMan probes are noted to have increased fluorescence

but because of probe mismatch may give false negative results. Molecular beacons although specific, have shown reduced fluorescence and are also susceptible to probe mismatch. Intercalating dyes are sensitive but less specific (Pillay *et al*, 2007; Gunson *et al*, 2006) as they can bind to any double stranded DNA and are prone to primer-dimer configurations; however, they are useful to detect larger DNA sequences and are the most inexpensive.

Yoshida *et al* (2002) were the first to publish a real-time q-PCR assay for the detection of *M. genitalium*. They used TaqMan probe chemistry to target the 16S rRNA *M. genitalium* gene in urine specimens of men with NCNGU and found the assay to be highly sensitive (98.8%) compared to the sensitivity (87.0%) found by Mena *et al* (2002) in the same specimen type using conventional PCR. Recently in Sweden, Edberg *et al* (2008) compared q-PCR to conventional PCR assays and found q-PCR to be highly sensitive (97.4%) compared to 80.3% of the conventional PCR assay.

1.6.5.4 Transcription mediated amplification (TMA)

Gen-Probe Inc. (USA) has developed first generation transcription mediated amplification (TMA) assays for the individual detection of the known urogenital pathogens *C. trachomatis* (CT) and *N. gonorrhoeae* (NG) (Stary *et al*, 1998). The manufacturers later noticed that the assays had limited performance due to cumbersome specimen processing and inhibition that yielded false negative results. To enhance its performance, Gen-Probe improved these areas for CT and NG NAATs by introducing technologies such as target capture to remove the inhibitors from clinical specimens as well as to change to the simultaneous detection of both pathogens. From this improvement the second generation APTIMA® Combo 2 CT/NG assay protocol was

established (Gaydos *et al*, 2003).

In 2006 Gen-Probe Inc. (USA) has developed a research TMA method for the detection of *M. genitalium* targeting the 16S rRNA of the organism. This is a modification of the second generation APTIMA® Combo 2 CT/NG assay protocol that has been reported to be more sensitive (98.1%) and more specific (98.1%) when compared to the first generation assay (Hardick *et al,* 2006). One of the reasons that the sensitivity of the TMA assay is higher than some of the PCR based assays in the detection of *M. genitalium*, is the fact that rRNA is present in a higher concentration than DNA in all cells and bacteria (Ikeda-Dantsuji *et al*, 2005).

The target capture procedure is based on the removal of nucleic acids from the solution. The rRNA targets are released in the transport medium and attach to specific capture oligomers that are coated with magnetic particles. The capture oligomers contain sequences complementary to specific regions of the target molecules as well as a string of deoxyadenosines (poly-dAs). Hybridization occurs between the poly-dAs and poly-deoxythymidines (poly-dTs). The captured rRNA targets are ready for amplification.

The amplification process of the target rRNA takes place at a constant temperature (isothermal). In this procedure, two primers and three enzymes (RNA polymerase, reverse transcriptase and RNase H) are used. The first primer has a promoter sequence that RNA polymerase recognizes and initiates amplification. The second primer binds to the DNA copy (intermediate). At the start of amplification, there is complementary binding of the first promoter primer with target rRNA at a specific site. Complementary (c) DNA is then synthesized by reverse transcriptase

from the target rRNA to form a RNA:DNA duplex by extension from the 3' end of the promoter primer. The enzyme RNase H degrades the RNA of the RNA:DNA duplex. The second primer that binds to the cDNA is extended and a double-stranded DNA molecule is formed. The promoter sequence on the DNA template in the double strand is recognized by RNA polymerase that transcribes it into RNA amplification products (amplicons). Millions of copies of the target are amplified in less than an hour. The amplicons are detected by a single-stranded ester labeled chemiluminescent DNA probe which is complementary to a region of the amplicon. The probe binds to the amplicon to form stable RNA:DNA hybrids that are detected by light emission in a luminometer. Emission is measured as photon signals and reported as Relative Light Units (RLUs).

Assay results are determined by a cutoff based on the total RLUs and the kinetic curve type. A phenomenon known as dual kinetic assay (DKA), where a particular organism displays a characteristic curve, is used when more than one organism such as *C. trachomatis* and *N. gonorrhoeae* are tested simultaneously. For single *M. genitalium* positive detection, the chemiluminescent signal cutoff is ≥50 000 RLUs.

1.7 TREATMENT AND PREVENTION

Urethritis in men caused by *M. genitalium* is often missed while empirical treatment might fail and may lead to chronicity and persistence of the disease.

Due to the growth challenges and subsequent antimicrobial susceptibility testing (AST), the actual minimum inhibitory concentrations (MICs) of the different antimicrobials remain poorly

defined (Taylor-Robinson & Bébéar, 1997; Hamasuna *et al*, 2005). This also poses a problem regarding the formulation of treatment guidelines. Another impediment is the intracellular location of mycoplasmas in human host cells that makes it difficult to clear the infection because the relevant doses of the antibiotics to which *M. genitalium* is susceptible cannot be determined (Jensen, 2006). Treatment must take into consideration the absence of a peptidoglycan-containing cell wall in the *M. genitalium*. The general classes of antimicrobials used for treatment for such a type of infection should therefore exclude those antimicrobial agents that inhibit the synthesis of the peptidoglycan layer of bacterial cell wall such as beta-lactams, cephalosporins, glycopeptides and aminoglycosides (Scholar & Pratt, 2000).

According to Taylor-Robinson & Horner (2001), the AST profile of *M. genitalium* is similar to that of its close relative *M. pneumoniae*. This implies that tetracyclines, erythromycin and azithromycin including fluoroquinolones, that are the most effective for *M. pneumoniae,* should be the relevant therapy for a *M. genitalium* infection. In the CDC treatment guidelines (2006), macrolides, tetracyclines and fluoroquinolones are recommended. In their mechanism of action, macrolides and tetracyclines inhibit bacterial protein synthesis while fluoroquinolones inhibit bacterial genetic replication by interfering with DNA synthesis (Scholar & Pratt, 2000).

As part in the syndromic management, doxycycline has been a drug of choice for treatment of NGU in symptomatic males (Black *et al*, 2008). According to the 2006 CDC treatment guidelines and Takahashi *et al* (2008), azithromycin has become the drug of choice for mycoplasmal NGU over doxycycline. Researchers such as Falk *et al* (2003), Ross & Jensen (2006), Björnelius *et al* (2008) and Wikström & Jensen (2006) have observed higher efficacy of

azithromycin over doxycycline. Ross & Jensen (2006) have suggested the following regimen: a 5-day course of azithromycin i.e. 500 mg on day 1 followed by 250 mg for 4 days, as first-line therapy as single doses are less effective and might generate some resistance to this antimicrobial. Based on the results of the studies mentioned above, the macrolide azithromycin may be the most efficient antimicrobial in the eradication of *M. genitalium*.

To assess compliance to treatment, follow-up after 2-3 weeks should be made on the patient either by telephone or direct communication and a specimen tested for the presence or absence of the organism (Shahmanesh *et al*, 2009). This would help to ensure proper resolution of the disease.

The major set-back in the management of any disease is the development of resistance towards antimicrobial treatment. Björnelius *et al* (2008) have recently reported from their Scandinavian study a more than 50% treatment failure with doxycycline. Bradshaw *et al* (2006) have also reported resistance to single- and multi-dose azithromycin in Australian men infected with *M. genitalium* which they resolved by treatment with a fluoroquinolone moxifloxacin. Falk *et al* (2003) also reported azithromycin to be more effective than tetracycline which failed to clear the infection when they studied a large group of both men and women who were infected by *M. genitalium* and attending an STD clinic in Sweden.

To prevent *M. genitalium* infection and re-infection, sexual partners and their contacts should be assessed and offered empirical treatment. The identification and treatment of high risk men i.e. sexually active males, (men having sex with men (MSM) and men having sex with women

(MSW)), who do not use sheath barriers such condoms, may prevent the spread of infection to their partners thereby reducing the risks of PID and cervicitis (the corresponding disease in women) as indicated by Simms *et al* (2003) and Manhart *et al* (2003), respectively.

1.8 STUDY PROBLEM

There is a significant association between *M. genitalium* and NGU, more specifically in NCNGU of both males and females, as reported by Anagrius *et al*, (2005); Taylor-Robinson & Horner, (2001); and Jensen *et al*, (2004a). The extreme difficulty and/or failure in growing *M. genitalium* on conventional media such as agar, has accounted for the absence of routine cost effective laboratory assays. Clinical diagnosis has been possible only after application of the first PCR-based detection method in 1991 by Jensen and his colleagues (Jensen *et al*, 1991). There is therefore an urgent need for rapid, reproducible molecular assays to detect this fastidious organism. The availability of data by means of NAATs on the prevalence of the disease will aid in the understanding of the pathogenic role of this organism.

Because *M. genitalium* can be present at very low concentrations in some patients (Jensen *et al*, 2004b), factors that have to be taken into consideration when selecting the best NAAT after this comparative study, should be (1) a very low limit of detection (2) generation of high sensitivity and specificity (3) acceptable turn-around-time (from receipt of specimen to issue of results) and (4) cost effectiveness.

1.9 AIM OF THE STUDY

- To compare three NAAT assays namely, a research-only TMA, an in-house adapted real-time PCR and a commercial PCR assay for the detection of *M. genitalium* in urine specimens of South African men with urethritis.

1.10 OBJECTIVES OF THE STUDY

- To obtain first-void urine (FVU) samples from men with signs and/or symptoms of urethritis.
- To detect *M. genitalium* in urine specimens using a research-only TMA analysis.
- To detect *M. genitalium* in urine specimens using real-time PCR assay.
- To detect *M. genitalium* in urine specimens using conventional PCR assay.
- To view *M. genitalium* in urine specimens using electron microscopy.
- To compare the three NAAT methods used in detection of *M. genitalium* on the basis of their sensitivity, specificity, turn-around-time and cost effectiveness.

CHAPTER 2: MATERIALS AND METHODS

2.1 CLINICAL SPECIMENS

2.1.1 Study population

The study population consisted of sexually active males complaining of urethritis, defined as burning on micturition (BOM), frequency, urethral irritation and/or urethral discharge. These patients presented to a private practitioner in Silverton, Pretoria.

2.1.2 Urine specimen collection and transport

First-void urine (FVU) specimens were collected from 300 males presenting with signs and/or symptoms of urethritis from August 2007 to June 2008. The patients did not receive any antimicrobial therapy in the month preceding this visit. All specimens were collected in sterile universal jars and kept at 4^0C until and during transportation to the laboratory. Specimens reached the laboratory within 24 hours of collection.

Upon receipt at the laboratory, 1ml of urine was pipetted into APTIMA® specimen collection tubes containing buffered transport medium (Gen-Probe Inc, USA). These were stored at -70^0C to be used for the transcription-mediated amplification (TMA) assay. Five hundred microliters of the urine was used in the nucleic acid extraction for the conventional polymerase chain reaction (PCR) and the LightCycler (LC) real-time PCR (q-PCR) assays. Two milliliters of the urine was also immediately aliquoted to be used for transmission electron microscopy (TEM) analysis.

2.2 NUCLEIC ACID EXTRACTION

Deoxyribonucleic acid (DNA) was extracted from urine specimens using the Roche AMPLICOR® CT/NG specimen preparation kit, following the manufacturers' instructions (Roche Diagnostics, Germany). Sterile plugged tips were used to add 500µl of CT/NG wash buffer into appropriately labeled screw-cap tubes (Sarstedt). The urine specimens were thoroughly vortexed and 500µl was added to the corresponding tubes containing the wash buffer. The tubes were recapped and the contents mixed by vortexing after which they were incubated for 15 minutes at 37^0C. The tubes were centrifuged at ≥12500 x g for 5 minutes. The supernatant was discarded and 250µl of CT/NG lysis buffer was added using a separate plugged tip for each tube. The tubes were recapped and vortexed. They were incubated at room temperature for 15 minutes. Two hundred and fifty microlitres of CT/NG diluent was added to each tube and mixed by vortexing. This was followed by centrifugation at ≥12500 x g for 10 minutes. The supernatant, containing nucleic acid was stored at -70^0C until further analysis for the conventional PCR and real-time PCR assays.

2.3 MOLECULAR TECHNIQUES FOR THE DETECTION OF *M. GENITALIUM*

2.3.1 Transcription mediated amplification (TMA) assay

The presence of *M. genitalium* was determined in all urine specimens using a research TMA assay, a modification of the APTIMA® Combo2 Assay kit protocol (Gen-Probe Inc., USA) established for *N. gonorrhoeae* and *C. trachomatis*. Primers targeting a 427bp fragment of *M.*

genitalium 16S ribosomal ribonucleic acid (rRNA) gene were used. The *M. genitalium* specific target capture oligomers, primers and probes were designed and synthesized by Gen-Probe, USA.

Gen-Probe general purpose reagents were used in this TMA assay, which is an amplification/DNA probe test, that utilizes target capture for *in vitro* detection of *M. genitalium* rRNA. Briefly, target rRNA is isolated from a specimen by use of capture oligonucleotides contained on magnetic beads. The capture oligo/target complex is removed from solution by powerful magnets and after addition of the relevant primers and enzymes, amplification takes place (Figure 2.1). Amplicons (rRNA) are detected through hybridization with specific chemiluminescent labeled DNA probes. These probes are also labeled with an acridinium ester molecule. A selection reagent, targeting the acridinium ester molecule distinguishes between hybridized and unhybridized probes. The light emitted from the DNA:RNA hybrid is measured in a luminometer and reported as relative light units (RLUs). For a *M. genitalium* positive result, the RLU value should be equal or above 50 000.

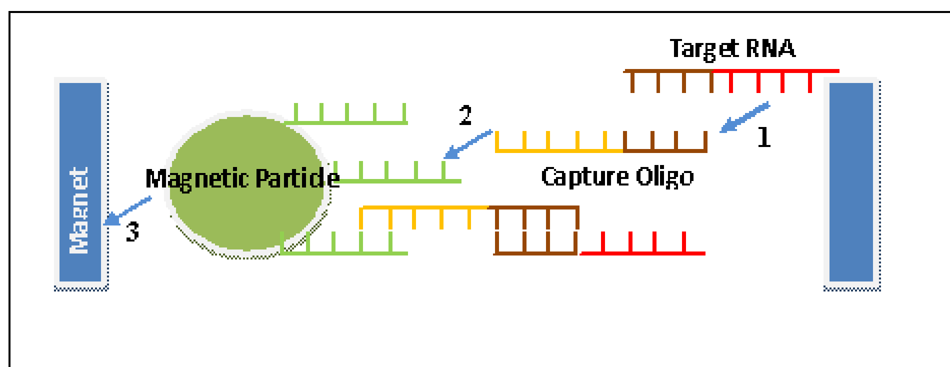


Figure 2.1 Schematic representation of target capture technology.

2.3.1.1 Specimen preparation

The urine specimens stored in the APTIMA® collection tubes were retrieved and allowed to thaw at room temperature. The RNA targets were already released in the APTIMA tubes and preserved in the buffered transport medium solution during the -70^{0}C storage.

The DNA of *M. genitalium* G-37 American Type Culture Collection (ATCC) 33530D strain was used as positive control.

2.3.1.2 Target capture (isolation of rRNA)

The rRNA targets were isolated from the urine solution by the addition of 100µl capture oligomers to 400µl of urine in the appropriate bar-coded tubes of a ten-tube unit (TTU) (Fig. 2.2). Positive (G-37 ATCC 33530D) and negative (water) controls were included in tubes 1 and 2. The TTUs with specimen identification were entered into a database with the appropriate computer software.

Figure 2.2 Bar-coded Ten-Tube Unit (TTU).

The TTUs were covered and placed in a programmed shaking heating block (SB100) where they were vortexed and incubated at 62^0C for 35 minutes. The tubes were allowed to cool to 23^0C for 20 minutes, allowing the target capture oligonucleotides to anneal. The TTUs were placed in a target capture system (TCS) base containing magnets and left for 5 minutes. The supernatant was aspirated from the tubes and 1ml of wash buffer added. After vortexing, the TTUs were replaced in the TCS for 5 minutes, and the supernatant was aspirated again.

2.3.1.3 Amplification

Hundred microlitres of amplification solution, containing 16S rRNA gene specific primers, and 200µl of evaporation preventative oil were added to each tube and incubated at 62^0C for 10 minutes in the SB100. Tubes were allowed to cool from 62^0C to 42^0C for 5 minutes for primer annealing before addition of 25µl of a combination of the enzymes Reverse transcriptase, RNase H and RNA polymerase. The mixture was incubated in the SB100 at 42^0C for 60 minutes to allow amplification.

2.3.1.4 Amplicon Detection

The detection of the rRNA amplicons was performed in a separate room. The SB100 in the post amplification room was preheated to 62^0C and 100µl of acridinium ester-labeled single-stranded DNA chemiluminescent probe specific for *M. genitalium* was added to the mixture in the TTU tubes. These were vortexed and incubated at 62^0C for 20 minutes. Cooling was done at room temperature for 5 minutes and 250µl of Selection Reagent was added, before incubation at 62^0C for 10 minutes. The tubes were cooled to 23^0C for 15 minutes before being placed in the

luminometer (Leader® HC$^+$). They were read using direct acquisition software whereby light emitted by the labeled RNA:DNA hybrids was measured as photon signals.

The signals were reported as relative light units (RLUs). A value above 50 000 was taken as positive for *M. genitalium.*

2.3.2 Conventional polymerase chain reaction (PCR) assay

PCR was performed on the extracted DNA of each specimen by using the *Mycoplasma genitalium* 280/550 IC kit manufactured by Sacace Biotechnologies (Italy). This kit makes use of “hot start” amplification, where the template and primers are kept separate from the *Taq* DNA polymerase by a separating wax; the hot temperature (95^0C) melts this wax, activates T*aq* DNA polymerase enzyme and inactivates the incorporated uracil-N-glycosylase (UNG) before initiation of amplification.

To prevent contamination, all steps were performed in separate clean rooms using sterile filter pipette tips.

2.3.2.1 Master-mix (PCR-mix-1)

The master-mix (PCR-mix-1) was provided in the kit in *Mycoplasma genitalium* 280/550 IC eppendorf amplification tubes. This contained oligonucleotide primers targeting a 281bp region of the 140kDa main adhesion gene (*MgPa*) of *M. genitalium* as described by Jensen *et al* (1991).

2.3.2.2 Amplification

Each PCR-mix-1 tube was labeled in correspondence to the extracted nucleic acid specimen number. Positive (G-37 ATCC 33530D) and negative (water) control tubes were also included. To each PCR-mix-1 tube, 10µl of PCR-mix-2 containing *Taq* DNA polymerase, magnesium chloride ($MgCl_2$) in buffer and DNase free water (provided in the kit) was added, followed by 10µl of the thawed extracted DNA. A volume of 10µl of water was added to the negative control tube and 10µl of the positive control to the positive control tube. The tubes were capped and placed in a thermocycler (GeneAmp PCR System 2700', Applied Biosystems).

Amplification was performed as follows: an initial hold cycle at 95^0C for 2 minutes, followed by 42 cycles of denaturation at 95^0C for 15 seconds, primer annealing at 65^0C for 25 seconds, and extension at 72^0C for 25 seconds. This was followed by a final extension at 72^0C for 1 minute. The amplicons were held at 4^0C in the cycler, awaiting detection.

2.3.2.3 Amplicon detection

Two percent agarose gel (Appendix A) was prepared 1 hour prior to electrophoresis. To the gel, 3µl ethidium bromide (10µg/ml) was added for UV detection.

The amplified products (amplicons) of the specimens and the controls were run on the gel, i.e. using a different sterile tip for each specimen, 15µl of the amplicons was mixed with 5µl of loading dye and loaded on the gel. A size marker (100bp Hyperladder™, Bioline, USA) was included on each gel. Electrophoresis was performed for 1 hour at 70 volts in 1x Tris-Borate-Ethylenediaminetetraacetic acid (EDTA) (TBE) buffer. The gels were viewed and photographed

under UV light to identify the bands. A band of 281bp, that is the amplification product of the *M. genitalium* MgPa gene fragment, was taken as positive for *M. genitalium*.

2.3.3 Real-time PCR (q-PCR) assay

LightCycler real-time PCR (q-PCR) was performed on the nucleic acid that was extracted of each specimen, using the LightCycler FastStart DNA Master^{PLUS} HybProbe kit manufactured by Roche Diagnostics (Germany). The kit uses the LightCycler Carousel-Based System and was suitable for hot-start PCR amplification.

To prevent contamination, all PCR reagents were mixed in a PCR master-mix (cocktail) clean room with all specimens being loaded with sterile filter pipette tips. Non-specific amplification was eliminated by adding 1.5 units (U) of Uracil-N-glycosylase (UNG) (Roche Diagnostics) to the capillary tubes mixture and incubated for 10 min at room temperature.

2.3.3.1 Primers and probes

Primers and probes as described by Svenstrup *et al* (2005) targeting a 190bp fragment of the *M. genitalium gap* gene were used. All primers and probes were synthesized by Metabion International, Germany.

2.3.3.1.1 Primers

Forward primer: mg-gap-605F (5’-GTG CTC GTG CTG CAG CTG T-3’)

Reverse primer: mg-gap-796R (5’-GCT TGA TTT ACT TGT TCA ACA GAT GGA C-3’)

A 20X dilution of a 100µM primer stock solution was prepared to make a 0.5µM working concentration in a final reaction volume of 20µl. A volume of 2µl of the 0.5µM was used in each reaction.

2.3.3.1.2 Probes

Hybridization probes targeting the amplified 190bp *M. genitalium gap* gene were used. They were:

Fluorescein (FL)-labeled probe (donor):

mg-gap-669FL (5'-TGT TGT TCC AGA AGC AAA TGG CAA ACT T-FL-3')

LightCycler (LC) Red640 probe (acceptor):

mg-gap-700LC (5'-LC-Red640-GGG ATG TCA CTC CGT GTT CCA GTG T-phosphate)

A 50X dilution of a 100µM probe stock solution was prepared to make a 0.2µM working concentration in a final reaction volume of 20µl. A volume of 2µl of the 0.2µM was used in each reaction.

2.3.3.2 Amplification mix

From the kit, one vial (labeled 1b) of "Reaction Mix" was allowed to thaw before being briefly centrifuged together with another vial (labeled 1a) of enzyme. From vial 1a, 10µl of enzyme was pipetted into the reaction mix. This was mixed gently by pipetting up and down. The vial was

relabeled "Master Mix". The amplification mix for each specimen was prepared in 15µl, containing master mix, 0.5µM of each primer and 0.2µM of each probe.

Positive (G-37 ATCC 33530D) and negative (water) controls were included. The LightCycler capillary tubes were cooled to 4^0C and 15µl of the amplification mix was added to each. To this, 5µl of nucleic acid template was added and the capillaries were sealed with a stopper. The capillaries were placed into adapters and microcentrifuged at 700 x g for 3 seconds to ensure the mix has entered the capillaries. These were then transferred into the LightCycler 1.5 carousel and amplification was initiated, using the Roche LC program after a self test was completed.

2.3.3.3 Amplification

Amplification was performed as follows: an initial hold cycle at 95^0C for 10 minutes to activate the *Taq* DNA polymerase and deactivate UNG, followed by 45 cycles of denaturation at 95^0C for 15 seconds, primer annealing at 55^0C for 8 seconds, and extension at 72^0C for 8 seconds with a temperature transition rate of 20^0C per second. Simultaneously, fluorescence (640nm) emitted by fluorescence resonance energy transfer (FRET) from mg-gap-669FL donor to mg-gap-700LC Red640 acceptor was measured in channel F2/F1 when the probes annealed to the *gap* target DNA. After the PCR process, a melting program completed the analysis. The amplicons were heated at 95^0C at a temperature transition rate of 20^0C per second without hold. These were cooled to 50^0C for 15 seconds at the same temperature transition rate. The amplicons were then slowly reheated to 95^0C at a temperature transition rate of 0.1^0C per second and finally cooled to 40^0C for 30 seconds at a temperature transition rate of 20^0C per second.

2.3.3.4 Fluorescence data

The fluorescent signal of the FL probe was used as detection of amplification for each specimen. At 260nm when the fluorescent signal reporter of the probe increased to a detectable level (threshold) data were directly transferred from the carousel to the LightCycler software 5.1 (Roche Diagnostics). For each specimen, this was captured and displayed as a sigmoid curve F2/F1, with different colours for every specimen, to form an amplification plot. The PCR products were characterized by a melting curve analysis where a melting temperature ($T_{m)}$ of 67-68^{0}C indicated *M. genitalium*.

2.4 TRANSMISSION ELECTRON MICROSCOPY (TEM)

Fresh, unfrozen specimens were used. Upon receipt, 2ml of each specimen was immediately sent to the TEM unit, where a negative staining technique was performed.

2.4.1 Negative staining technique

Briefly, duplicate urine specimens were mixed with a heavy metal salt, phosphotungstic acid. These were allowed to dry on copper grids of a mesh coated with Formvar, leaving a contrasted electron-dense area surrounding the electron-transparent organism particles. The grids were observed under the electron microscope using the 600 000X objective. For *M. genitalium* positive result, a pear/flask-shaped organism of about 0.6-0.7µm in length, a width of 0.3-0.4µm near the base and 0.06-0.08µm at the tip was to be observed, as described by Tully *et al* (1981).

2.5 TURN-AROUND-TIME AND COST EFFECTIVENESS

The time taken to perform all the assays was estimated as hours spent. The cost to test a specimen by each method was also approximated.

2.6 DATA ANALYSIS

The results of the presence or not of *M. genitalium* as detected by the different assays were entered into a 2x2 table, and the sensitivities and specificities calculated to ascertain the statistical significance of the results. For comparison, an "extended gold standard" was used, where specimens were considered as positive if any two NAATs were positive. This extended gold standard has been used by other researchers where standard culture methods are not practical (Crucitti *et al*, 2003; Gaydos *et al*, 2003).

CHAPTER 3: RESULTS

The study population consisted of 300 sexually active males, attending a private clinic at Silverton, Pretoria. Specimens were collected from August 2007 to June 2008. The mean age of the men was 36.2 years (ranging from 17 to 65 years), and all were complaining of symptoms such as burning on micturition (BOM), frequency, urethral irritation and/or urethral discharge, which was defined as urethritis.

3.1 DETECTION OF *M. GENITALIUM* BY MOLECULAR TECHNIQUES

The TMA assay detected 21% (62/300) *M. genitalium* positives whilst PCR and q-PCR assays detected 14% (43/300) and 16% (48/300) positives, respectively. (Results obtained are shown in Appendix C.)

The three assays together (i.e. a positive result in any of the tests) identified 22% (67/300) *M. genitalium* positives of which 12% (36/300) had BOM and 10% (31/300) had discharge. Thirteen of the 67 (19%) positives were detected by the TMA assay alone, whilst the PCR assay alone detected 3 positives and the q-PCR assay only 2 positives. The number of specimens that were positive in all three assays was 12% (37/300). The distribution of the results from the 3 assays is shown in Figure 3.1.

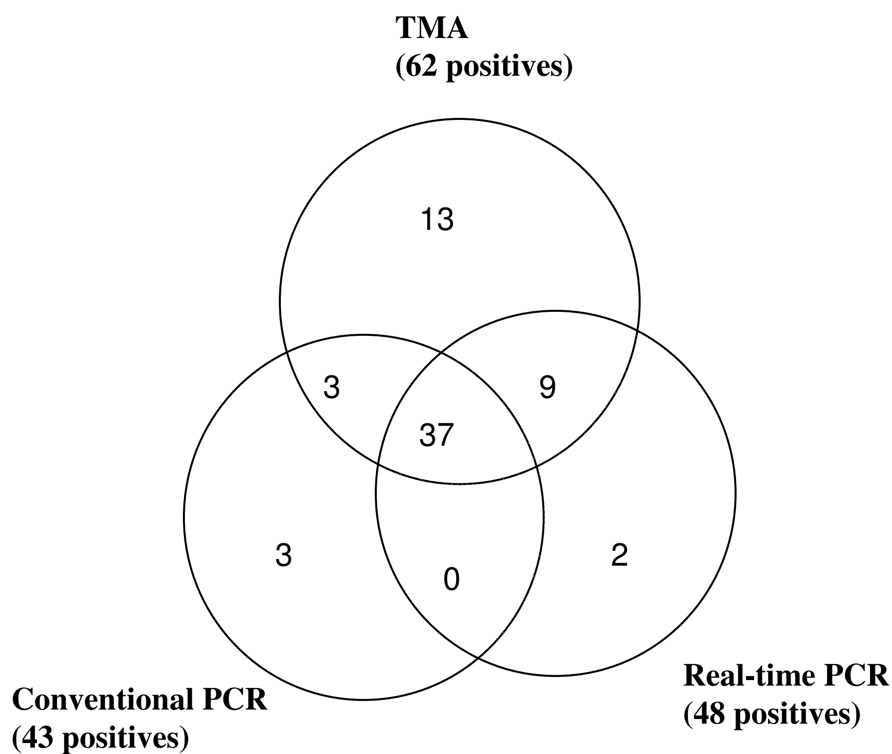


Figure 3.1 Distribution of results obtained for *M. genitalium* using three NAATs.

3.1.1 Summary of TMA assay results

The results for the TMA assay, where a RLU ≥50 000 was taken as a positive result for *M. genitalium*, are shown in Table 3.1.

Table 3.1 TMA test results for *M. genitalium* assay (Gen-Probe).

SPECIMEN	POSITIVE FOR *M. GENITALIUM*	NEGATIVE FOR *M. GENITALIUM*
Patients with urethritis (N=300)	21% (62/300)	79% (238/300)

3.1.2 Summary of PCR assay results

M. genitalium was detected by electrophoretic analysis of the PCR amplification products in 14% (43) of the 300 specimens (Fig. 3.2) and the results are shown in Table 3.2.

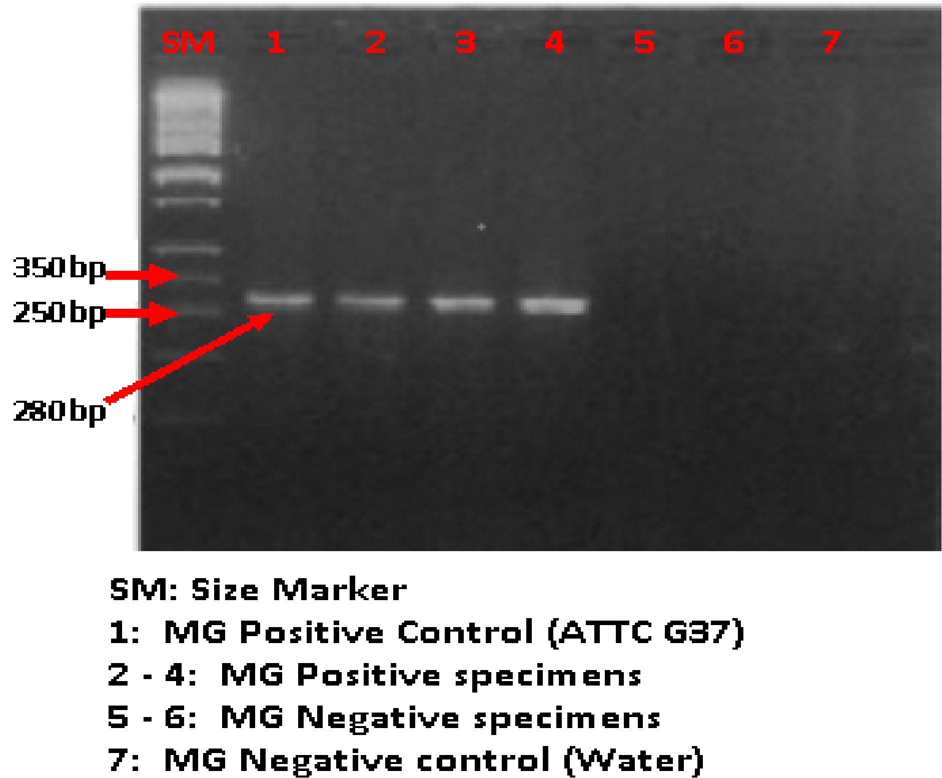


Figure 3.2 Electrophoretic analysis of the PCR products obtained from urine specimens.

Table 3.2 PCR test results for *M. genitalium* 280/550 IC (Sacace Biotechnologies).

SPECIMEN	POSITIVE FOR *M. GENITALIUM*	NEGATIVE FOR *M. GENITALIUM*
Patients with urethritis (N=300)	14% (43/300)	86% (257/300)

3.1.3 Summary of q-PCR assay results

The q-PCR products were characterized by melting curve analysis and a melting temperature (T_m) of 67-68°C indicated *M. genitalium* as depicted in Fig. 3.3. The q-PCR detected 16% (48/300) positives. The results are summarized in Table 3.3.

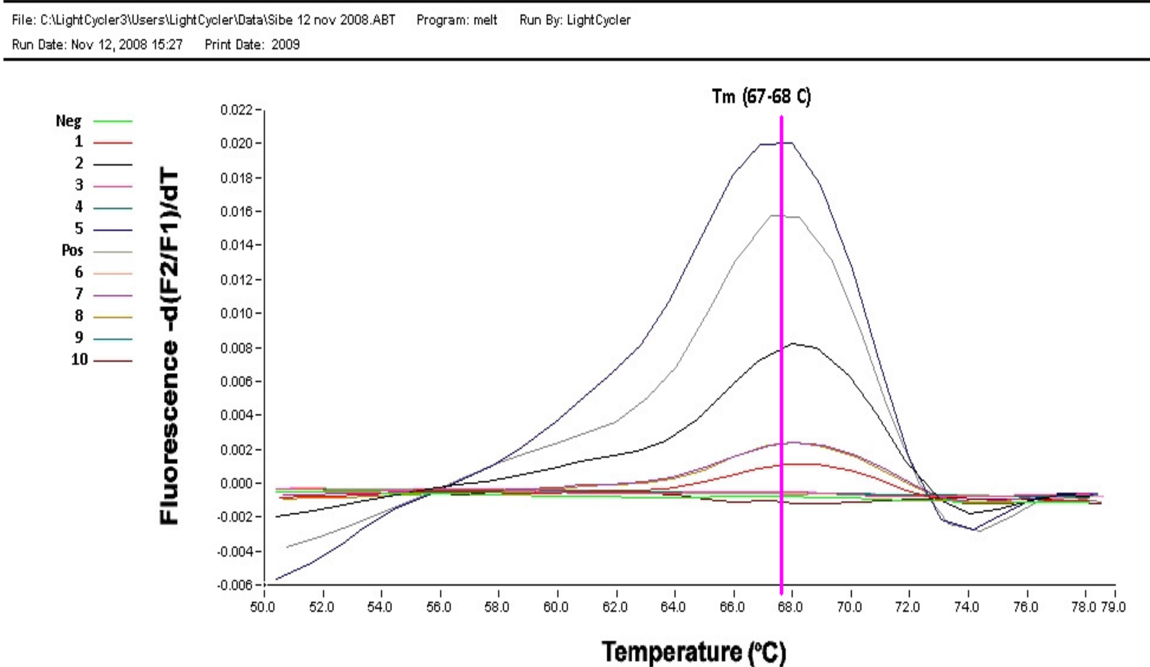


Figure 3.3 q-PCR melting curve analysis of *M. genitalium*.

Table 3.3 q-PCR test results for *M. genitalium* LightCycler FastStart DNA Master^{PLUS} HybProbe (Roche Diagnostics).

SPECIMEN	POSITIVE FOR *M. GENITALIUM*	NEGATIVE FOR *M. GENITALIUM*
Patients with urethritis (N=300)	16% (48/300)	84% (252/300)

3.2 COMPARISON OF THE THREE ASSAYS IN THE DETECTION *OF M. GENITALIUM*

3.2.1 Comparison of TMA, q-PCR and PCR assays

The TMA assay detected more *M genitalium* positives, namely 21% (62/300) when compared to the 16% for q-PCR (48/300) and14% for the PCR assays (43/300) (Fig 3.4).

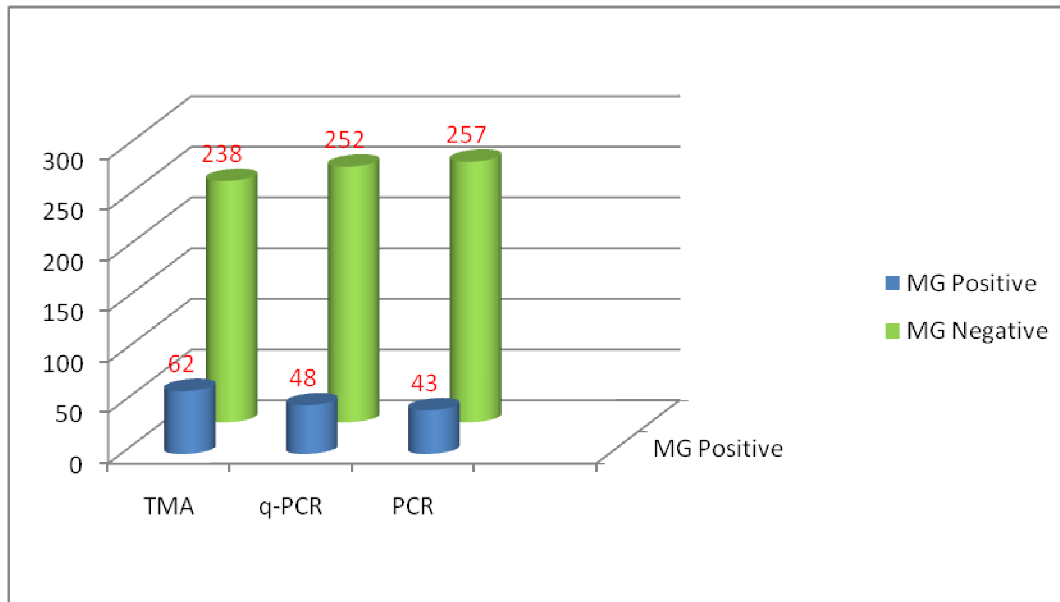


Figure 3.4 Graphic representation of TMA, q-PCR and PCR assay results in the study group (N=300).

3.2.2 Detection by TMA and q-PCR assays

Both TMA and q-PCR assays detected 46 positives (15%) (Table 3.4). TMA assay detected an additional 16 positives (5%) that were not detected by q-PCR while q-PCR detected 2 positives (1%) that were not picked up by the TMA. The sensitivity and specificity of the TMA assay were

96% and 94%, respectively, compared to 74% sensitivity and 99% specificity (Tables 3.4 and 3.5) of the q-PCR assay.

Table 3.4 Comparison of TMA and q-PCR assays in all specimens.

		q-PCR +	q-PCR -	**Total**
TMA	+	46	16	62
	-	2	236	238
	Total	48	252	300

Sens	96%
Spec	94%
PPV	74%
NPV	99%

Sens (sensitivity); Spec (specificity); PPV (positive predictive value); NPV (negative predictive value).

Table 3.5 Comparison of q-PCR and TMA assays in all specimens.

		TMA +	TMA -	**Total**
q-PCR	+	46	2	48
	-	16	236	252
	Total	62	238	300

Sens	74%
Spec	99%
PPV	96%
NPV	94%

Sens (sensitivity); Spec (specificity); PPV (positive predictive value); NPV (negative predictive value).

3.2.3 Detection by TMA and PCR assays

TMA and PCR assays together detected 40 positives (13%) (Table 3.6). There were 22 positives (7%) detected by TMA assay and not by PCR whereas PCR detected 3 positives (1%) that were

not picked up by the TMA. The sensitivity and specificity of the TMA assay were 88% and 91%, respectively, compared to 61% sensitivity and 98% specificity (Tables 3.6 and 3.7) of the PCR assay.

Table 3.6 Comparison of TMA and PCR assays in all specimens.

		PCR		
		+	-	**Total**
TMA	+	40	22	62
	-	3	235	238
	Total	43	257	300

Sens	88%
Spec	91%
PPV	61%
NPV	98%

Sens (sensitivity); Spec (specificity); PPV (positive predictive value); NPV (negative predictive value).

Table 3.7 Comparison of PCR and TMA assays in all specimens.

		TMA		
		+	-	**Total**
PCR	+	40	3	43
	-	22	235	257
	Total	62	238	300

Sens	61%
Spec	98%
PPV	88%
NPV	91%

Sens (sensitivity); Spec (specificity); PPV (positive predictive value); NPV (negative predictive value).

3.2.4 Detection by q-PCR and PCR assays

Both q-PCR and PCR assays detected 37 positives (12%) (Table 3.8). q-PCR detected an additional 11 positives (4%) that were not detected by PCR whereas PCR detected 6 positives (2%) that were not picked up by the q-PCR. The sensitivity and specificity of the q-PCR assay were 81% and 95%, respectively, compared to 73% sensitivity and 97% specificity (Tables 3.8 and 3.9) of the PCR assay.

Table 3.8 Comparison of q-PCR and PCR assays in all specimens.

		PCR +	PCR -	**Total**
q-PCR	+	37	11	48
	-	6	246	252
	Total	43	257	300

Sens	81%
Spec	95%
PPV	73%
NPV	97%

Sens (sensitivity); Spec (specificity); PPV (positive predictive value); NPV (negative predictive value).

Table 3.9 Comparison of PCR and q-PCR assays in all specimens.

		q-PCR +	q-PCR -	**Total**
PCR	+	37	6	43
	-	11	246	257
	Total	48	252	300

Sens	73%
Spec	97%
PPV	81%
NPV	95%

Sens (sensitivity); Spec (specificity); PPV (positive predictive value); NPV (negative predictive value).

3.3 EXTENDED GOLD STANDARD

The different assays were compared to and evaluated using an extended gold standard, where due to the lack of a recognized gold standard, a specimen was deemed positive when any two NAATs were positive for the organism. According to this definition, there were 49 specimens positive for *M. genitalium*.

3.3.1 TMA assay

The TMA detected 62 positives, of which 49 were also detected by the extended gold standard, and 13 by the TMA only. There were no positive results obtained by the extended gold standard that the TMA did not detect. The sensitivity and specificity of the TMA assay were 100% and 95%, respectively (Table 3.10)

Table 3.10 Comparison of TMA assay and extended gold standard.

		Extended gold standard		
		+	-	**Total**
TMA	+	49	13	62
	-	0	238	238
	Total	49	251	300

Sens	100%
Spec	95%
PPV	80%
NPV	100%

Sens (sensitivity); Spec (specificity); PPV (positive predictive value); NPV (negative predictive value).

3.3.2 q-PCR assay

The q-PCR assay detected 48 positives of which 46 were detected by the extended gold standard. Two specimens tested positive for *M. genitalium* by q-PCR only, and 3 specimens detected by the extended gold standard were not detected by q-PCR. The sensitivity and specificity of the q-PCR assay were 92% and 95%, respectively (Table 3.11).

Table 3.11 Comparison of q-PCR assay and extended gold standard.

Extended gold standard

		+	-	**Total**
	+	46	2	48
q-PCR	-	3	249	252
	Total	49	251	300

Sens	94%
Spec	99%
PPV	96%
NPV	99%

Sens (sensitivity); Spec (specificity); PPV (positive predictive value); NPV (negative predictive value).

3.3.3 Conventional PCR

PCR and the extended gold standard assays detected 40 positives. There were 3 positives (1%) detected by PCR assay and not by the extended gold standard whilst the extended gold standard detected 9 positives (3%) that were not picked up by the PCR. The sensitivity and specificity of the PCR assay were 78% and 97%, respectively (Table 3.12).

Table 3.12 Comparison of PCR assay and extended gold standard.

		Extended gold standard		
		+	-	**Total**
PCR	+	40	3	43
	-	9	248	257
	Total	49	251	300

Sens	82%
Spec	99%
PPV	93%
NPV	97%

Sens (sensitivity); Spec (specificity); PPV (positive predictive value); NPV (negative predictive value).

3.3.4 Summary of assay performance

The sensitivities, specificities, negative and positive predictive values of the three NAATs compared to the extended gold standard are compared in Table 3.13. The sensitivity of the TMA assay was higher (100%) than the q-PCR (94%), and the PCR assays (82%). The PCR assays were highly specific (99%), while the TMA was less specific (95%).

Table 3.13 Comparison of NAATs' performance in detection of *M. genitalium*.

Test	**Sensitivity (%)**	**Specificity (%)**	**Negative predictive value (%)**	**Positive predictive value (%)**
PCR	82	99	97	93
q-PCR	94	99	99	96
TMA	100	95	100	80

3.4 RESULTS OF TRANSMISSION ELECTRON MICROSCOPE (TEM)

The electron micrograph of a positive specimen taken under the transmission electron microscope (TEM) revealed a structure that is very small, flask-shaped with a protruding tip and a dense center (Fig. 3.5).

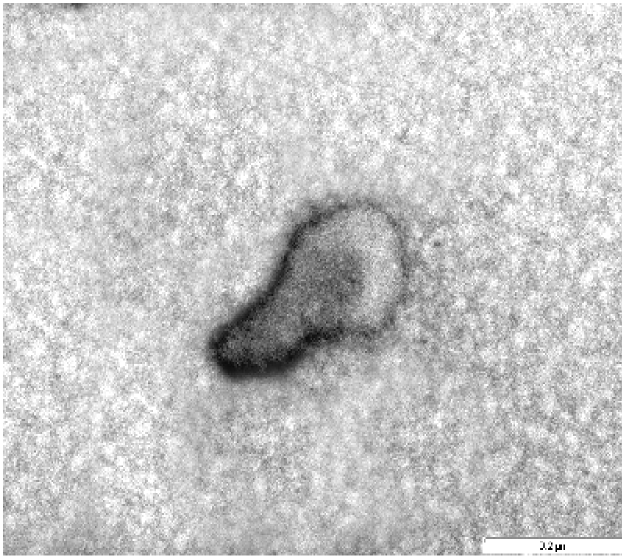


Figure 3.5 TEM of *M genitalium* (600 000X). Picture taken by Dr Chantélle Baker, EM unit University of Limpopo, Medunsa Campus.

3.5 TURN-AROUND-TIME AND COST EFFECTIVENESS

Table 3.14 is a summary of the approximate turn-around-time of each assay. The PCR assay took 2 hours, the q-PCR 1 hour and the TMA 4 hours to complete.

Table 3.14 Turn-around-time of the assays.

ASSAY	SPECIMEN PREPARATION	REAGENT PREPARATION	AMPLIFICATION	DETECTION	TOTAL
PCR	55min	22min	60min	5min	2h 22min
q-PCR	55min	5min	8min	0min	1h 8min
TMA	0min	5min	240min	0min	4h 5min

min (minutes); h (hours).

Table 3.15 is a summary of the total cost per assay. Prices were calculated at the time when the assays were performed. The testing of controls was taken into consideration when the total cost per specimen was calculated. The total cost per specimen was R104 for PCR, R120 for q-PCR and R184 for TMA.

Table 3.15 Cost per specimen for the assays.

ASSAY PRICE PER SPECIMEN	COLLECT	SPECIMEN PREP	PRIM/ PROB	AMP	DETECT	PLASTIC WARE	TOTAL
PCR	2	15.15	0	69.8	9.59	8	104.54
q-PCR	2	7.51	76.25	34.2	0	0	119.96
TMA	23.15	0	28.6	132.8	0	0	184.55

COLLECT (collection); PREP (preparation); PRIM (primers); PROB (probes); AMP (amplification); DETECT (detection). Currency in RSA rands.

CHAPTER 4: DISCUSSION

The purpose of this study was to establish a convenient cost effective method to detect *M. genitalium* in clinical specimens. Although many published clinical studies have used different assays to detect the pathogen, most of these were done using conventional PCR. In this study, we have compared a commercial PCR assay with a real-time PCR assay and a transcription mediated amplification assay for the detection of *M. genitalium* in first void urine specimens collected from men with signs and symptoms of urethritis.

The results indicated that *M. genitalium* was detected by the TMA, q-PCR and PCR assays in 62/300 (21%), 48/300 (16%) and 43/300 (14%), of the patients respectively. A total number of 67 (22%) positives of the 300 first void urine (FVU) specimens from men with urethritis symptoms were identified by the three assays together. The agreement among the three assays was 37/67 (55%) positives. Of the 67 positives, 36 had burning on micturition and 31 had symptoms of a discharge. These results show a strong association of *M. genitalium* with symptomatic urethritis, as was found in previous studies (Leung *et al*, 2006; Anagrius *et al*, 2005).

The comparison between the assays was based on the "extended gold standard" concept, where a specimen is deemed positive when any two NAATs are positive. This method is convenient in cases like these, where standard techniques for the detection of the organism are not available or are impractical. The assumption that a positive result obtained by only one test, is a false positive, may however be biased. One way to eliminate this is to verify the amplification

products by DNA sequencing. This was not done in this study, as there was insufficient specimen left for further testing.

4.1 TMA ASSAY

The TMA assay was the most sensitive (100%) of the NAATs used (q-PCR (94%) and PCR (82%)). This may be due to the fact that the target (RNA) in TMA is more abundant in organisms than DNA which was the target for the other two PCR assays. In addition to this, the use of the APTIMA® urine transport tubes may also have contributed to the high sensitivity, as the transport media in the tubes is designed to stabilize RNA during shipment and storage. The TMA assays make use of magnetic beads and a target capture system before amplification to exclude inhibition, eliminating the incorporation of individual amplification controls (Chong *et al*, 2003). The specificity of the TMA was lower than that of the other assays (95% vs 99%), but this was due to the additional 13 specimens where *M. genitalium* was detected. All these specimens were from men with BOM only, which may mean that the more sensitive TMA assay was able to detect the low copies of the organism, as was shown by other researchers (Hardick *et al,* 2006; Wroblewski *et al*, 2006). As these discrepant results may also have been caused by contamination, the assay was repeated from the original specimens, but the results did not change. There were no positives detected by both other assays that were not detected by the TMA assay.

4.2 q-PCR ASSAY

Real-time PCR has revolutionized the way clinical microbiology laboratories diagnose many human microbial infections. This testing method combines PCR chemistry with fluorescent

probe detection of amplified product in the same reaction vessel, minimizing the risk of releasing amplified nucleic acids into the environment. In this study, the q-PCR performed well compared to the other two assays. Although its sensitivity was lower than that of the TMA assay (94% vs 100%), the specificity of this test was higher (99% vs 95%). The sensitivity was high compared to conventional PCR. The sensitivity of our q-PCR was higher compared to other studies such as the study by Jurstrand *et al* (2005) where they compared q-PCR and PCR assays in the detection of *M. genitalium* in FVU of men. The sensitivity of their q-PCR assay sensitivity was 72% with no significant difference compared to their PCR assay (68%). The lower sensitivities in the two PCR assays compared to the TMA test may have been due to lower concentrations of the starting DNA material, which when improved by either using an alternative method of DNA extraction, changing the type of the specimen or increasing the volume of the specimen may produce better results.

In our study we used the q-PCR assay targeting the housekeeping gene *gap,* encoding glyceraldehyde-3-phosphate-dehydrogenase of *M. genitalium,* as described by Svenstrup *et al* (2005). The *gap* gene was chosen by the authors, as sequencing of the gene revealed no variation between the prototype strains and 4 clinical isolates. The q-PCR test did not detect 3 positive specimens detected by the other two NAATs. This may be due to low copy numbers in the specimens as they were all from men with BOM only.

M. genitalium was detected by this test only in two specimens. This may have been due to contamination during specimen preparation but these two were negative by the conventional PCR that used the same DNA extracts. As the conventional PCR has a lower sensitivity, the

DNA extraction from the original specimens should have been repeated to rule out contamination. Unfortunately, there was not enough urine left to do this, or to perform sequence analysis of the amplicons. One of these specimens was from a man with urethral discharge and microscopy confirmed urethritis, and as it was shown by Svenstrup *et al* (2005) that the *gap* target is unique to *M. genitalium* and the primers do not cross-react with other mycoplasmas or urogenital pathogens, this may be a true positive, missed by the other assays.

The q-PCR may be useful to quantitatively detect the bacterial load of *M. genitalium* and results compared with another method such as TMA in monitoring treatment of patients attending STI clinics. Previous findings by Hardick *et al* (2006) have shown the TMA assay to be more sensitive (98%) than q-PCR (92%) when they tested the presence of *M. genitalium* in male urine samples. They used the criteria that we also used in this study, of any two targets being positively amplified, as their extended gold standard.

The q-PCR assay is continuing to show global recognition and utilization in the detection of urogenital pathogens. Recently, Bradshaw *et al* (2009) have used the q-PCR assay to determine the prevalence of sexually transmitted infections (STIs) in (among other specimen types) FVU of men who have sex with men (MSM) in Australia. *M. genitalium* could be detected among common pathogens such as *Neisseria gonorrhoeae* (*N. gonorrhoeae*) and *Chlamydia trachomatis* (*C. trachomatis*).

4.3 CONVENTIONAL PCR

In our study, we used a conventional PCR assay, commercially available from Sacace (Italy) to detect *M. genitalium* from urine specimens. The assay did not perform as well as the newer NAATs. The specificity was good (99%), but the test was not very sensitive (82%), as it missed 9 positives, detected by the other NAATs. This was to be expected, as the test makes use of agarose gel electrophoresis to detect amplicons. Further more, it was the last assay to be evaluated, and the prolonged storage of the extracted DNA may have contributed towards the poor PCR assay sensitivity compared to other studies. For example, Mena *et al* (2002) found a sensitivity of 87% when they used the PCR method to detect this organism in urine specimens.

PCR has been shown to be a highly specific assay, as was also shown in this study (99%). As a basic method to detect *M. genitalium* infection, PCR assay had been the most used and reliable technique after five different previously isolated *M. genitalium* strains, including the type strain G-37 could all be detected by this method whereas DNAs from mycoplasmas other than *M. genitalium* yielded negative results (Jensen *et al*, 1991; Jensen, 2006).

In our study, PCR detected 3 positives that were not detected by the other two NAATs. These may be true positive results, however they are more likely due to contamination. In conventional PCR assays, there are many “hands-on” steps where contaminants can be introduced. Although precautions have been taken to avoid this, including separate areas, unidirectional flow, meticulous laboratory techniques and the use of uracil-N-glycosylase, contamination may still occur. Another possible explanation for these positive results may lie in the primer design. The kit manufacturers state that there was no cross reaction with other pathogens in a group control,

but it may be that cross reaction with other mycoplasmas. The question remains whether we are dealing with true *M. genitalium* positive specimens, missed by the other two NAATs. With improvement in sample preparation and increased amount of the DNA starting material, the PCR assay could perform better.

On cost and turn-around-time, the q-PCR assay was the most rapid among all three assays and less costly than the TMA assay, therefore would be given the first choice. There was about 2 hours' difference in turn-around-time between PCR and TMA assays. The TMA, although the most expensive of the three assays, was the most sensitive and would be chosen as the second best assay.

Our transmission electron microscopy (TEM) results showed a very small organism, flask-shaped, with a protruding end and a dense center. This micrograph is comparable to the *M. genitalium* structure observed by other authors such as Tully *et al* (1983) and Taylor-Robinson *et al* (1995). The use of electron microscopy is difficult, time consuming and requires fresh specimens. It is therefore not recommended for diagnostic purposes.

4.4 CONCLUSIONS

M. genitalium is associated with urethritis, and as a sexually transmitted infection (STI), should be included in treatment and prevention strategies. In this era of opportunistic infections due to the human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) pandemic, it is important that *M. genitalium* is routinely tested for. The absence of a "gold standard" method for the diagnosis of *M. genitalium* as a STD, poses a threat in our HIV/AIDS-

stricken country. HIV/AIDS patients, due to immunodeficiency, lack the ability to clear other infections hence the infection would be compounded. Thus far the diagnosis in clinical samples is research-based and has highlighted the need for routine sensitive, specific, cost effective and rapid assays for *M. genitalium* testing in clinical specimens.

Owing to the published information by Jensen (2006) on the genetic mutation of the usual target *MgPa* gene, the rate of mutation in our area may need to be detected for proper management of *M. genitalium* infection in our STD clinics. Further tests such as *M. genitalium* DNA sequencing may be used to verify a true positive *M. genitalium* genome in the case of inconclusive results

In our study, both the TMA and real-time PCR assays performed well and can be used in clinical studies. These assays would assist in obtaining clear-cut local epidemiological information on the prevalence of *M. genitalium* disease for comparison with local and global studies. This is important as highly sensitive assays for the detection of *M. genitalium* are required if most of the infected persons are to be treated to prevent transmission to sexual partners.

CHAPTER 5: REFERENCES

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APPENDICES

APPENDIX A

A.1 *10X *TBE buffer preparation*

Reagent	*Mass*
Tris base	105.0g
Boric acid	55.0g
*EDTA	7.45g

A volume of 800ml distilled water (dH2O) was added to the reagents above. This was made up to 1000ml using an acid or alkaline to obtain a pH of 8.0.
To make up 1X TBE, 900ml dH2O was added to 100ml of 10X TBE prepared above (1000ml total volume) and this was sterilized by autoclave.

*TBE (Tris/Boric acid/Ethylenediaminetetraacetic acid); EDTA (Ethylenediaminetetraacetic acid)

A.2 *2% Agarose gel preparation*

A 0.6g agarose powder was weighed out and mixed with 1X TBE buffer prepared above. The mixture was heated until all the agarose was dissolved. The gel (with 3ul ethidium bromide added and mixed) was allowed to cool to 60^0C poured out into a gel tray. This was ready for an electrophoresis run.

APPENDIX B

B.1 *TMA APTIMA reagents*

(2-8^0C storage)

1. Target capture B containing noninfectious nucleic acid in a buffered solution with <5% detergent.
2. Enzyme containing reverse transcriptase and RNA polymerase dried in N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic acid (HEPES) buffered solution with <10% bulking agent as a preservative.
3. Amplification containing nucleic acids dried in buffered solution with <5% bulking agent.
4. Probe containing noninfectious chemiluminescent DNA probes (<500ng/vial) dried in succinate buffered solution with <5% detergent.
5. Noninfectious DNA of *M. genitalium* G-37 American Type Culture Collection (ATCC) 33530D strain in a buffered solution with <55% detergent as positive control.
6. Water as negative control.

(2-30^{0}C storage)

7. Amplification reconstitution solution containing aqueous solution with preservatives.
8. Enzyme reconstitution solution containing HEPES buffered solution with surfactant and glycerol.
9. Probe reconstitution solution containing succinate buffered solution with <5% detergent.
10. Selection reagent containing 600mM borate buffered solution with surfactant.

(15-30^{0}C storage)

11. Target capture containing buffered salt solution in solid phase (<0.5 mg/ml) and capture oligomers.
12. Wash solution containing 10mM HEPES buffered solution with <2% detergent.
13. Buffer for deactivation fluid containing 800mM bicarbonate buffered solution.
14. Oil containing silicone.

APPENDIX C

C.1 *Results sheet*

Specimen Number	BOM and /Dis	TMA	PCR	q-PCR
1	N	N	N	N
2	N	N	N	N
3	N	N	N	N
4	Y	N	N	N
5	N	N	N	N
6	N	N	N	N
7	Y	N	N	N
8	N	N	N	N
9	N	N	N	N
10	N	N	N	N
11	N	N	N	N
12	N	N	N	N
13	N	N	N	N
14	Y	N	N	N
15	N	P	N	N
16	N	N	N	N
17	N	N	N	N
18	Y	P	N	P
19	Y	P	P	N
20	N	N	N	N
21	N	N	N	N
22	N	N	N	N
23	N	N	N	N
24	Y	N	N	N
25	N	N	N	N
26	N	N	N	N
27	Y	N	N	N
28	N	N	N	N
29	N	N	N	N
30	N	N	N	N
31	N	N	N	N
32	N	N	N	N
33	N	N	N	N
34	N	N	N	N
35	N	N	N	N
36	N	N	N	N
37	N	N	P	N
38	N	P	P	N
39	N	N	N	N
40	N	N	N	N
41	N	N	N	N
42	N	N	N	N
43	N	N	N	N
44	N	N	N	N
45	Y	N	N	N
46	N	N	N	N
47	N	N	N	N
48	Y	P	P	N
49	N	N	N	N
50	N	N	N	N
51	N	N	N	N
52	N	N	N	N
53	N	N	P	N
54	Y	N	N	N
55	N	N	N	N
56	N	N	N	N
57	Y	N	N	N
58	Y	P	N	N
59	Y	N	N	N
60	Y	N	N	N
61	N	N	N	N
62	N	N	N	N
63	N	N	N	N
64	Y	N	N	N
65	N	N	N	N
66	N	N	N	N
67	Y	N	N	N
68	N	N	N	N
69	N	N	N	N
70	N	N	N	N
71	N	N	N	N
72	N	P	N	N
73	N	P	N	N
74	N	N	N	N
75	N	P	N	N
76	N	N	N	N
77	N	N	N	N
78	N	P	N	N
79	N	N	N	N
80	Y	N	N	N
81	N	N	N	N
82	N	N	N	N
83	Y	P	N	N
84	Y	N	N	N
85	N	N	N	N
86	Y	P	N	N
87	Y	N	N	N
88	Y	N	N	N
89	Y	N	N	N
90	N	N	N	N
91	N	N	N	N
92	Y	N	N	P
93	N	N	N	N
94	Y	P	P	N
95	N	N	N	N
96	N	N	N	N
97	N	N	N	N
98	N	N	N	N
99	N	P	N	N

Specimen Number	BOM and /Dis	TMA	PCR	q-PCR	Specimen Number	BOM and /Dis	TMA	PCR	q-PCR	Specimen Number	BOM and /Dis	TMA	PCR	q-PCR
100	N	N	N	N	**133**	N	**P**	**P**	N	**166**	N	N	**P**	N
101	N	N	N	N	**134**	N	N	N	N	**167**	N	N	N	N
102	N	N	N	N	**135**	N	N	N	N	**168**	N	N	N	N
103	**Y**	**P**	**P**	**P**	**136**	N	N	N	N	**169**	**Y**	N	N	N
104	**Y**	N	N	N	**137**	N	N	N	N	**170**	N	N	N	N
105	N	N	N	N	**138**	N	N	N	N	**171**	N	N	N	N
106	N	N	N	N	**139**	N	N	N	N	**172**	N	**P**	**P**	N
107	**Y**	**P**	N	N	**140**	N	N	N	N	**173**	N	N	N	N
108	**Y**	N	N	N	**141**	N	N	N	N	**174**	**Y**	N	N	N
109	N	N	N	N	**142**	**Y**	N	N	N	**175**	**Y**	**P**	**P**	N
110	N	N	N	N	**143**	N	**P**	**P**	**P**	**176**	N	N	N	N
111	N	**P**	N	N	**144**	N	N	N	N	**177**	N	N	N	N
112	N	**P**	N	**P**	**145**	N	N	N	N	**178**	N	N	N	N
113	N	N	N	N	**146**	N	N	N	N	**179**	**Y**	**P**	**P**	N
114	N	N	N	N	**147**	N	N	N	N	**180**	N	**P**	**P**	**P**
115	N	N	N	N	**148**	N	N	N	N	**181**	**Y**	N	N	N
116	**Y**	N	N	N	**149**	**Y**	N	N	N	**182**	N	N	**P**	N
117	N	N	N	N	**150**	N	**P**	**P**	N	**183**	N	N	N	N
118	N	N	N	N	**151**	N	**P**	N	N	**184**	N	N	N	N
119	N	N	N	N	**152**	N	N	N	N	**185**	**Y**	N	N	N
120	N	N	N	N	**153**	N	N	N	N	**186**	N	N	N	N
121	N	**P**	**P**	N	**154**	N	N	N	N	**187**	N	N	N	N
122	**Y**	N	N	N	**155**	N	N	N	N	**188**	N	N	N	N
123	**Y**	N	N	N	**156**	N	**P**	N	N	**189**	**Y**	N	N	N
124	N	N	N	N	**157**	N	N	N	N	**190**	**Y**	N	N	N
125	N	N	N	N	**158**	N	**P**	N	N	**191**	**Y**	N	N	N
126	**Y**	N	N	N	**159**	N	N	N	N	**192**	N	**P**	**P**	N
127	**Y**	N	N	N	**160**	N	N	N	N	**193**	N	N	N	N
128	N	**P**	**P**	N	**161**	N	N	N	N	**194**	**Y**	N	N	N
129	N	N	N	N	**162**	N	N	N	N	**195**	N	N	N	N
130	N	**P**	**P**	N	**163**	N	N	N	N	**196**	N	N	N	N
131	**Y**	N	N	N	**164**	N	N	N	N	**197**	N	N	N	N
132	N	N	N	N	**165**	N	N	N	N	**198**	N	N	N	N

Specimen Number	BOM and /Dis	TMA	PCR	q-PCR
199	N	P	N	P
200	Y	N	N	N
201	N	N	N	N
202	N	N	N	N
203	N	N	N	N
204	Y	N	N	N
205	Y	N	N	N
206	Y	N	P	N
207	N	N	N	N
208	N	N	N	N
209	N	N	N	N
210	N	N	N	N
211	Y	N	N	N
212	N	P	P	N
213	N	N	N	N
214	N	N	N	N
215	N	N	N	N
216	Y	N	N	N
217	N	N	N	N
218	N	N	N	N
219	N	N	N	N
220	Y	N	N	N
221	N	N	N	P
222	N	N	N	P
223	N	N	N	P
224	N	N	N	N
225	Y	N	N	N
226	N	P	P	P
227	Y	N	N	N
228	Y	N	N	N
229	Y	N	N	N
230	N	N	N	P
231	N	N	N	N

Specimen Number	BOM and /Dis	TMA	PCR	q-PCR
232	Y	N	N	N
233	N	N	N	N
234	N	P	N	P
235	N	N	N	N
236	N	N	N	N
237	Y	N	N	N
238	N	N	N	N
239	Y	N	N	N
240	N	N	N	N
241	N	N	N	N
242	N	N	P	P
243	N	N	N	N
244	Y	P	N	P
245	N	N	N	N
246	N	N	N	N
247	N	P	N	N
248	N	P	N	N
249	N	N	N	N
250	N	N	N	N
251	Y	P	P	N
252	Y	N	N	P
253	Y	P	P	P
254	N	N	N	N
255	Y	P	N	N
256	N	P	N	N
257	N	P	N	N
258	N	N	N	N
259	Y	N	N	N
260	Y	P	P	N
261	Y	N	N	N
262	Y	N	N	N
263	Y	P	N	N
264	N	N	N	N

Specimen Number	BOM and /Dis	TMA	PCR	q-PCR
265	N	P	P	N
266	N	N	N	N
267	N	N	N	N
268	N	P	N	P
269	N	P	N	N
270	N	N	N	N
271	N	N	N	N
272	N	P	N	N
273	N	N	N	N
274	N	P	N	N
275	N	N	N	N
276	N	P	N	N
277	N	P	N	N
278	N	N	N	N
279	N	N	N	N
280	N	N	N	N
281	N	P	N	N
282	N	P	N	N
283	N	N	N	N
284	N	N	N	N
285	N	N	N	N
286	N	N	N	P
287	N	N	N	N
288	N	P	N	N
289	N	N	N	N
290	N	P	N	N
291	N	P	N	N
292	N	P	N	N
293	N	N	N	P
294	N	N	N	N
295	N	P	N	P
296	N	N	N	N
297	N	P	N	N

Specimen Number	BOM and /Dis	TMA	PCR	q-PCR
298	N	N	N	P
299	N	N	N	N
300	Y	N	N	N

BOM (burning on micturition); Dis (discharge); TMA (transcription mediated amplification); PCR (polymerase chain reaction, conventional); q-PCR (real-time PCR); Y (yes); N (no/negative); P (positive).

APPENDIX D

D.1 *M. genitalium positive results*

Spec no.	Age	Dis	BOM	TMA	PCR	q-PCR
15	47		1	1		
18	35	1		1		1
19	27	1		1	1	1
37	43		1		1	
38	40		1	1	1	1
48	25	1		1	1	1
58	32	1		1	1	1
72	71		1	1	1	1
73	28		1	1		1
75	47		1	1	1	1
78	47		1	1		
83	38	1		1		1
86	26	1		1	1	1
88	33	1		1		1
92	40	1				1
94	40	1		1	1	1
99	56		1	1		
103	35	1		1	1	1
107	37	1		1		
111	24		1	1	1	1
112	25		1	1		1
121	23		1	1	1	1
128	34		1	1	1	1
130	34		1	1	1	1
133	50		1	1	1	1

Spec no.	Age	Dis	BOM	TMA	PCR	q-PCR
143	45		1	1	1	1
150	40		1	1	1	1
151	21		1	1		
156	32		1	1	1	1
158	29		1	1	1	1
166	41		1		1	
172	58		1	1	1	1
175	26	1		1	1	1
179	27	1		1	1	1
180	27		1	1	1	1
192	36		1	1	1	1
199	22		1	1		1
206	35	1			1	
212	27		1	1	1	
226	30		1	1	1	1
234	50		1	1		1
242	57		1			1
244	29	1		1	1	1
247	38		1	1		
248	35		1	1	1	1
251	28	1		1	1	1
253	30	1		1	1	1
255	21	1		1		
256	38		1	1	1	1
259	32	1		1		
260	33	1		1	1	1
263	30	1		1		
265	42		1	1	1	1

Spec no.	Age	Dis	BOM	TMA	PCR	q-PCR
268	65		1	1	1	
269	28	1		1		
272	40	1		1	1	1
274	35	1		1		
276	64		1	1		1
279	27	1		1	1	1
281	28		1	1	1	1
282	23	1		1		
288	32	1		1	1	1
290	28	1		1		1
291	29	1		1	1	
292	29	1		1	1	1
295	45		1	1	1	1
297	22	1		1		
	36.23 Mean age	31	36	62	43	48
				92.537	64.18	71.64

(yellow)	Agreement all 3 tests
(orange)	Agreement TMA + PCR
(magenta)	Agreement TMA + q-PCR
(blue)	TMA only
(light green)	PCR only
(green)	q-PCR only

Total number of positives by all three tests (N) =67

Agreement among the three tests =37

Agreement between TMA and PCR =3

Agreement between TMA and q-PCR =9

Positive by TMA only =13; Positive by PCR only =3; Positive by q-PCR only =2

Spec no. (specimen number); Dis (discharge); BOM (burning on micturition); TMA (transcription mediated amplification); PCR (polymerase chain reaction, conventional); q-PCR (real-time PCR).