CHAPTER 1

EXPERIMENTAL PROPOSAL

1.1 RATIONALE FOR STUDY

Urethritis, an infection that is often sexually acquired, is categorized depending on the presence or absence of *Neisseria gonorrhoeae*, as gonococcal urethritis or non-gonococcal urethritis (NGU). *Chlamydia trachomatis* has been implicated as a major causative agent of NGU. In Chlamydia-negative NGU, however, the aetiology is not completely understood. Pathogens that have been implicated in NGU are *Trichomonas vaginalis* (Pépin *et al,* 2001), *Ureaplasma urealyticum biovar* 2 (Yokoi *et al*, 2007) and *Mycoplasma genitalium* (Jensen, 2006). Although there is evidence that *M. genitalium* may be the cause of male urethritis, the question is still asked whether the organism is merely an invader of damaged tissue caused by another pathogen.

The role of *M. genitalium* in NGU has been difficult to establish, as it is difficult to grow the organism on culture medium or in tissue culture (Jensen *et al,* 1996). Most of the studies on *M. genitalium* were done after the development of molecular diagnostic tests, especially PCR assays specific for the *M. genitalium* MgPa adhesin gene (Jensen *et al*, 1991). Real-time PCR diagnostic, as well as quantitative assays have been developed more recently to detect *M. genitalium* in clinical specimens (Yoshida *et al,* 2002a). In

2006, Gen-Probe Inc (USA) developed a research transcription mediated amplification assay targeting the rRNA of *M. genitalium.*

It is still unclear whether the *M. genitalium* DNA load is higher in specimens from patients with clinical signs of urethritis compared to those without symptoms, i.e is this organism a commensal/passenger or a pathogen? In one study by Jensen *et al* (2004a) in Denmark, significantly higher bacterial loads were seen in specimens from patients with urethral discharge than in those without. However, there were no clear results for patients with urethritis without discharge, and those without symptoms. The detection of *M. genitalium* in higher numbers in urogenital specimens from men with NGU than in asymptomatic men will strengthen the evidence that this organism is a pathogen and not a passenger. Most of the studies on *M. genitalium* were performed in the developed world, and with this study, the role of the organism in urethritis in the developing world was investigated.

Patients with NGU are treated regardless of the detection of *C. trachomatis* or *M. genitalium* with tetracyclines, or macrolides, depending on local recommendations. However, studies have shown that *M. genitalium* may persist after treatment with tetracyclines (Falk *et al*, 2003). In South Africa few studies on *M. genitalium* and its association with NGU, have been performed (Taylor-Robinson *et al*, 2002; Sturm *et al*, 2004; Black *et al*, 2008). In countries like ours, where syndromic management is used for treatment of male urethritis, epidemiological data is important in the effective design of these treatment strategies.

1.2 AIM OF THIS STUDY

This study was done to determine the association of *Mycoplasma genitalium* with urethritis in adult South African men.

1.2.1.1 Research objectives

The following objectives were set:

- To obtain first-void urine (FVU) and endo-urethral swab specimens from men with and without signs and/or symptoms of urethritis.
- To assess presence of urethritis by microscopic examination.
- To establish a NAAT for the detection of *Mycoplasma genitalium*.
- To test all specimens for recognized pathogens associated with urethritis: *Neisseria gonorhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis* and *Mycoplasma genitalium*.
- To determine the bacterial load of *M. genitalium* in positive specimens using q-PCR.
- To investigate the usefulness of the urethral smear in predicting the presence of pathogens.
- To compare and analyze the relationship between clinical signs and symptoms of urethritis, microscopy and bacterial load in *M. genitalium* infection.

1.3 ETHICAL CLEARANCE

Approval to perform this study was obtained from the Medical Research and Ethics Committee (MCREC) of the University of Limpopo (Medunsa Campus): MCREC/P/85/2007:PG.

1.4 THESIS DESIGN

This study is presented in 6 Chapters, with Chapter 1 a general introduction, followed by a review of the literature on *M. genitalium* in Chapter 2. This includes aspects of the organism dealing with taxonomy, characteristics, pathogenesis, epidemiology, diagnosis and treatment.

In Chapter 3 the molecular diagnosis of *M. genitalium* is reviewed. Three NAAT assays, namely a commercial PCR assay, an in-house adapted real-time PCR and a research-only TMA test for the detection of *M. genitalium* in urine specimens from men with and without urethritis are compared and evaluated. The results obtained are discussed.

The role of *M. genitalium* in urethritis in South African men is reviewed in Chapter 4. The occurrence of four common urethral pathogens (including *M. genitalium*) in urine specimens obtained from symptomatic and asymptomatic South African men attending

a family practice in Pretoria, Gauteng Province is investigated. The usefulness of the urethral smear in predicting the presence of pathogens in these patients is examined. The results obtained are analysed and discussed.

In Chapter 5 quantitative PCR assays for the determination of bacterial loads and especially *M. genitalium* is reviewed. The *M. genitalium* loads in urine specimens from men with signs and symptoms of urethritis and from men without any urogenital symptoms are compared and analysed.

In Chapter 6 the results obtained in the previous chapters are briefly discussed to address the initial research question.

A reference list containing all citations used throughout the document is included at the end of the thesis.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Mycoplasma genitalium was first isolated in 1981 by Tully *et al* from two men with non-gonococcal urethritis. Two isolates were grown on SP4, a transport medium that they had developed two years earlier (Tully *et al*, 1979). The strains were designated G-37 and M-30, and shown to be distinct from all other mycoplasma species. These unique isolates were subsequently named as being *Mycoplasma genitalium.* The G-37 isolate has become an American Type Culture Collection (ATCC 33530) strain with its genome being fully sequenced in 1995 (Ueno *et al*, 2008).

Due to its slow cell replication and fastidious growth requirements, culture is not usually used for laboratory diagnosis of *M. genitalium*, hence few epidemiological studies were done in the years following its discovery. However, after the introduction of molecular diagnostic assays, many clinical studies were performed mainly in developed countries. If one reviews the studies that have been performed to date, *M. genitalium* is found in 21% of men with non-gonococcal urethritis (NGU), and in 6% of asymptomatic men (Jensen, 2006). The majority of these studies have shown an association of *M. genitalium* with NGU, whilst in only a few studies no association could be shown (Dolapci *et al*, 2005; Yu *et al*, 2008).

Improvement in laboratory detection methods, particularly with the introduction of the newer nucleic acid amplification tests (NAATs), is playing an important role in elucidating the place of *M. genitalium* among sexually transmitted pathogens, and especially its role in NGU and cervicitis.

2.2 CHARACTERISTICS OF *M. GENITALIUM*

Most of the characteristics of *M. genitalium* are known through the findings from its thoroughly studied, genetically close relative, *Mycoplasma pneumoniae* (Dallo *et al*, 1989, Jensen, 2006). While *M. genitalium* can cause genitourinary tract disease, *M. pneumoniae* is an established pathogen of the respiratory tract and is an important cause of atypical pneumonia (Stein & Baseman, 2005).

2.2.1 Taxonomy

Mycoplasmas are prokaryotes belonging to the family *Mycoplasmataceae* within the order Mycoplasmatales (Prescott *et al*, 2005). The genera *Mycoplasma* and *Ureaplasma* are of the class Mollicutes [*mollis* (soft); *cutis* (skin)] which encompasses bacteria without a cell wall and are popularly termed the naked bacteria (Prescott *et al*, 2005; Jensen, 2006). The terms Mycoplasma and Mollicute are occasionally interchangeably used to refer to the species that belong to this class. The genus

Mycoplasma contains more than 100 species of which 13 are present as human flora (Baseman *et al,* 1984).

Mycoplasmas were initially thought to be viruses, since they could pass through filters that were meant to trap bacteria. However, they became accepted as bacteria when the concept of viruses was much better defined in the 1930s (Freundt *et al*, 1979). In 1995, the International Committee on Systematic Bacteriology Subcommittee (ICSB) on the Taxonomy of Mollicutes defined new mycoplasmas based on their ability to be filtered at very low pore size and absence of a cell wall. The latter being the case even after incubation in medium without antibiotics (Freundt *et al*, 1979; ICSB, 1995). In 2007, these standards were revised to include the deposition of the 16S rRNA gene sequence into a public database, and a phylogenetic analysis of the relationships among the 16S rRNA gene sequences of the novel species and its neighbours (Brown *et al*, 2007).

The phylogenetic tree of evolution shows that mycoplasmas may be descendants of Gram-positive bacteria, presumably of clostridial origin (Stein & Baseman, 2005; Jensen, 2006). This transformation is thought to have occurred through a genome reduction process leading to *M. genitalium* having the smallest genome of all self replicating prokaryotes (Fraser *et al*, 1995). The phylogenetic tree following the level of 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 (Himmelreich *et al*, 1997). The close relationship was confirmed by the similarity in many morphological and antigenic

characteristics including lipid components that the two bacteria share (Hyman *et al*, 1987).

2.2.2 Morphology

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

As *M. genitalium* is too small to be visible under a light microscope, it was first viewed under a transmission electron microscope (TEM) (Tully *et al*, 1983). The electron micrograph of G-37 and M-30 *M. genitalium* strains shows an organism of 0.6 - 0.7 µm in length, 0.3 - 0.4 µm wide near the base and 0.06 - 0.08 µm wide at the terminal tip. The core of the tip has dense parallel tracts called a nap at the neck-like structure that protrudes from the main cell, giving it a pear-like appearance (Tully *et al,* 1983) (Figure 2.1). This differentiated tip structure, is commonly known as the terminal organelle**.**

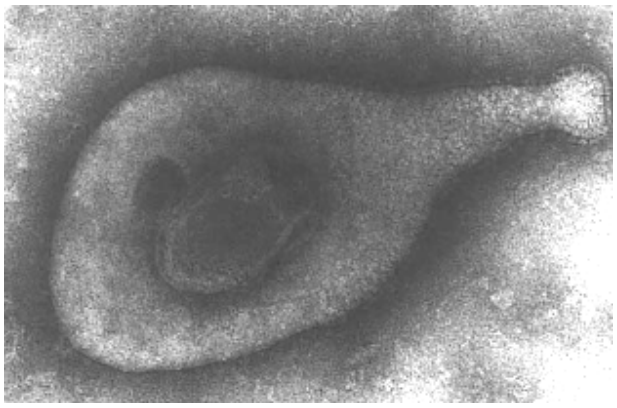


Figure 2.1 TEM of negatively stained intact *Mycoplasma genitalium* cell. (Adopted from Tully *et al*, 1983).

The neck-like region of *M. genitalium* is shorter than that of *M. pneumonia (*Lind *et al*, 1984). The terminal tip organelle is specialized to enable *M. genitalium* to glide along moist/mucous surfaces, as well as to adhere to surfaces such as plastic, red blood cells, Vero monkey kidney cells, and epithelia of eukaryotic host cells (Tully *et al*, 1981; Stein & Baseman, 2005). This attachment organelle is a membrane-bound extension of the cell and is further characterized by an electron-dense core that is part of the mycoplasma cytoskeleton (Krause & Balish, 2001).

M genitalium does not have a peptidoglycan cell wall and therefore lacks cell surface markers. The absence of a cell wall also means that this bacterium has less osmotic stability in the host environment and is therefore prone to changes in its flask-like shape. This lack of a cell wall is a feature that is largely responsible for the two biologic properties of *M. genitalium* namely, no Gram stain reaction and non-susceptibility to

common antimicrobials of the β-lactam class that inhibit bacterial cell wall synthesis (Taylor-Robinson, 1995).

2.2.3 Metabolism

In spite of the small genome possessed by the mycoplasmas, they have the ability to self-reproduce. Whilst other mycoplasmas may utilize arginine (*M. hominis*) or urea (*U. urealyticum*), *M. genitalium* metabolizes glucose, resulting in the production of acid (Taylor-Robinson, 1995; Stein & Baseman, 2005). In keeping with the Mollicutes, the metabolism of *M. genitalium* makes use of substrate (glucose) phosphorylation that is associated with glycolytic kinase enzymes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate kinase or phosphoglycerate kinase for the synthesis of essential nucleotriphosphates (NTPs) for its genome (Pollack *et al*, 2002). *M. genitalium* exclusively makes use of GAPDH, retained in its small genome, during the process of glycolysis, generating energy for the organism (Stein & Baseman, 2005; Svenstrup *et al* 2005).

2.2.4 Genetic make-up

M. genitalium is the smallest existing self-replicating prokaryote with a genome consisting of only 580 kilo basepairs (kb) (Fraser *et al*, 1995). In 1995 *Haemophilus*

influenzae was the first pathogen with a fully sequenced genome (Fleishmann *et al*, 1995). This was followed shortly thereafter with the publication of the complete genomic sequence of *M. genitalium* (Fraser *et al*, 1995). When the genome of *M. genitalium* is compared to the slighter bigger genome of *M. pneumoniae* (816kb), it has been shown that *M. genitalium* contains some subsets of the *M. pneumoniae*'s genomic complement and that the coding genes in the *M. genitalium* genome correspond to certain sequences of *M. pneumoniae*'s genome (Himmelreich *et al*, 1997).

The small genome of *M. genitalium* gives a good indication of the minimal set of genes needed to sustain bacterial life. The minimum set of genes, also called essential genes, in both prokaryotes and eukaryotes, are those described as indispensable for the survival of an organism and are therefore the basis of life for a particular organism. In 2006, Glass *et al* identified 382 of the 482 *M. genitalium* protein-coding genes as essential. A more recent study (Zhang & Lin, 2009) showed that *M. genitalium* needed only 381 essential genes compared to the 642 required by *H. influenzae*. This highlights how the very small *M. genitalium* is capable to survive on its own. When studying the open reading frames (ORFs) of the *M. genitalium* genome, Taylor-Robinson (1995) and Su *et al* (2007) have identified only 480 protein coding regions, while a later study by Ueno *et al* (2008) found 484 coding regions. These identified coding regions include genes for DNA replication, transcription, translation, DNA repair, cellular transport and energy metabolism. It has also been found that *M. genitalium*, unlike other bacteria, uses UGA to code for tryptophan instead of a stop codon, suggesting that expression of its genes is complicated since it would synthesize truncated proteins (Seto *et al*, 2001).

To characterize its genome, *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) as compared to other bacteria (Mombach *et al*, 2006; Bizarro & Schuck, 2007). The G+C content in the DNA of most mycoplasmas 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).

A few genes have been used as target for PCR assays, with the most popular, the *MgPa* DNA gene (coding for the adhesin proteins), the rRNA genes and the housekeeping gene, *gap* (coding for GAPDH) (Jensen *et al*, 1991; Svenstrup *et* a*l*, 2005).

2.3 PATHOGENESIS OF *M. GENITALIUM*

The pathogenesis of *M. pneumoniae* has been studied extensively and due to the close genetic resemblance, certain features in the pathogenesis of *M. pneumoniae* can be applied to *M. genitalium* (Jensen *et al*, 1996). Although *M. pneumoniae* is primarily found in the respiratory tract, and *M. genitalium* in the urogenital tract, both organisms have been shown to cross tissue barriers (Baseman *et al*, 1988; Goulet *et al,* 1995). *M.*

genitalium has been shown to attach to different cell types, including erythrocytes (Morrison-Plummer *et al*, 1987), Vero cells (Jensen *et al*, 1994), fallopian tube cells (Collier *et al*, 1990), respiratory cells (Baseman *et al*, 1996) and spermatozoa (Svenstrup *et al*, 2003). The attachment of M*. genitalium* to Vero cells using its tip structure is shown in Figure 2.2.

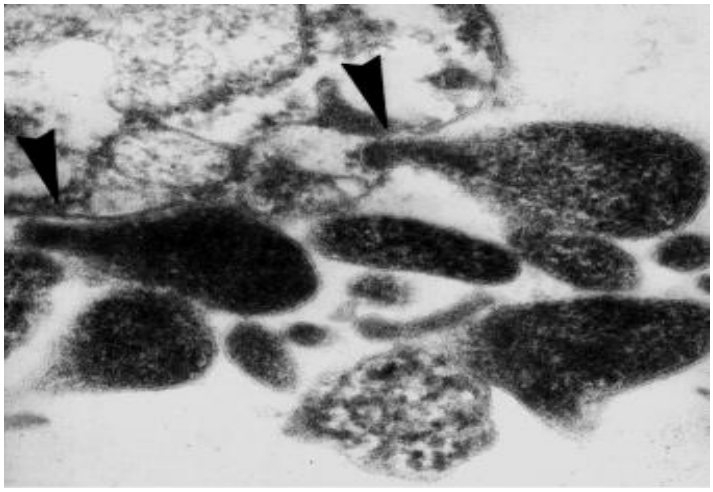


Figure 2.2 An Electron micrograph showing *Mycoplasma genitalium* cells attaching to Vero cells using their tip structures (arrows). (Adopted from Jensen *et al,* 1994).

2.3.1 Virulence of *M. genitalium*

M. genitalium has several virulence factors that are responsible for its pathogenicity. These include the ability to adhere to host epithelial cells using the terminal tip organelle with its adhesins, the release of enzymes (Jensen, 2006; Ueno *et al*, 2008), and the ability to evade the host immune response by antigenic variation (Razin *et al*, 1998).

2.3.1.1 ATTACHMENT AND ENTRY

For all intracellular pathogenic microorganisms, adhesion is a pre-requisite for colonization and infection. Since mycoplasmas lack cell walls and cell wall associated structures such as fimbriae that are normally associated with adhesion, the process of adhesion is mediated by cell membrane bound components that are collectively called adhesins (Burgos *et al*, 2006). *M. genitalium* utilizes the terminal tip organelle that is a complex protein structure, to mediate adhesion. This cell membrane bound protein complex is required for intimate adherence to host target cells and the proteins are collectively called adhesins. Although surface-exposed, these adhesins are linked to the internal cytoskeleton of the tip organelle (Ueno *et al*, 2008).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) of mutant strains that are incapable of haemadsorption has been widely used for the characterization of proteins involved in adhesion (Mernaugh *et al*, 1993). Using this technique, 21 putative protein genes have been identified in the *M. genitalium* genome, but only a few have been characterized as adhesins (Fraser *et al*, 1995; Himmelreich *et al*, 1997).

The major adhesin in the attachment protein complex is the MgPa protein, and together with the P32 (MG318) protein make up the terminal tip organelle (Inamine *et al*, 1989; Reddy *et al*, 1995). The MgPa encodes the P140 (MG191) and P110 (MG192) cytadherence proteins (cytadhesins) at the tip area (Burgos *et al*, 2006; Jensen, 2006).

These proteins are immunogenic both in immunized animals and in humans. Loss of either P140 or P110 results in loss of motility and adherence properties of the entire MgPa attachment organelle (Stein & Baseman, 2005; Burgos *et al*, 2006) thus showing the importance of these proteins in attachment. It was shown that the 140 kilodalton (kDa) P140 (MG191) closely resembles the 170 kDa main adhesion protein (P1) of *M. pneumoniae* whereas the 32 kDa *M. genitalium* protein P32 (MG318) resembles P30 of *M. pneumoniae* (Baseman *et al*, 1988; Stein & Baseman, 2005).

The MG218 and MG317 cytoskeletal proteins were shown to play a role in terminal organelle organization, gliding motility and cytadherence (Pich *et al*, 2008). The MG317 protein contributes to anchoring the electron-dense core of the tip to the cell membrane.

The genes encoding the adherence proteins are located in three different regions of the *M. genitalium* genome. The genes coding for the MgPa adhesins are organized in an operon with three genes (Inamine *et al*, 1988; Inamine *et al*, 1989), consisting of ORF-1 (MG190), ORF-2 (MG191), and ORF-3 (MG192) (Sperker *et al,* 1991). The P32 (MG318) of *M. genitalium* adherence components found on the tip-like terminal structures is located in operons that are a distance away from the MgPa operon. They are expressed together with adherence accessory proteins (M312, M 317 and MG320). The accessory proteins and their analogues in *M. genitalium* are important for clustering of the adhesin at the tip and maintaining the tip of the organism and the shape of the cell, thereby acting like a cytoskeleton (Razin & Jacobs, 1992; Burgos *et al*, 2007). MG218 is grouped in an operon with MG217 and MG219 (Musatovova *et al*, 2003).

M. genitalium penetrates host epithelial cells after attachment in a similar way as *M. penetrans* and *M. fermentans* (Waites *et al*, 2005; Jensen, 2006). The target cell membrane then invaginates in a manner similar to the clathrin-coated pits 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 the infecting organism is delivered 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). This internuclear localization process may take place within 30 minutes after infection.

2.3.1.2 ENZYMES

Besides the role played by the adhesins, Alvarez *et al* (2003) found that during the enzyme-mediated glycolytic pathway, it is the activity of the glycolysis enzyme GAPDH that brings about attachment of *M. genitalium* to human vaginal and cervical mucin in female disease. Thus GAPDH, among other binding proteins, acts as a ligand to receptors mucin and fibronectin, particularly in vaginal and cervical disease.

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) can be released to enhance the pathogenicity of its small genome

(Dhandayuthapani *et al*, 2001; Alvarez *et al*, 2003). MsrA is an antioxidant repair enzyme of the bacterium. It 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).

2.3.1.3 EVASION OF THE HOST IMMUNE RESPONSE

Pathogenesis in mycoplasmas is dependant on an intimate contact with the host cell and therefore, they have to be able to evade the host immune response, adapt to the environment and change phenotypically (Razin *et al,* 1998). The major antigenic determinants of the Mollicutes are their membrane proteins that are expressed on the surface. They are able to generate a high frequency of intragenomic variation in nucleotide sequence or DNA arrangement at selected chromosomal loci, promoting random phenotypic variation as a result of constantly changing host environments (Moxon *et al*, 1994; Arber, 2000). The adaptive potential has been maximized without compromising household functions. Multiple copies of partial gene sequences have been found in most pathogenic bacteria, but as mycoplasmas have very small genomes, the number of mycoplasmal genes involved in diversifying the surface antigens is markedly high (Jensen, 2006).

The basic mechanisms observed in antigenic variation are regulation of the expression of virulent factors by the signal transduction pathways or the natural generation of new phenotypes that are able to survive the host immune response (Razin *et al*, 1998).

When many regulatory genes that could serve as sensors to environmental stimuli or genes that encode transcriptional factors are available, *M. genitalium* can employ antigenic variation caused by molecular switching of events (Rottem, 2003).

In order to escape the host immune attack, proteins P140 and P110 of the MgPa have the ability to undergo antigenic variation thus altering the entire genetic sequence of the MgPa with subsequent generation of variants that are not recognized 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).

Tissue damage seen in *M. pneumoniae* is due to the host cells response and this may reflect what happens in a *M*. genitalium infection. Mycoplasmas have been found to interact with many components of the immune system. This may lead to production of cytokines and macrophage activation. Some cell components may act as super antigens and induce an autoimmune response (Svenstrup *et al,* 2006).

2.3.2 Transmission of *M. genitalium*

M. genitalium is commonly detected in urogenital specimens of sexually active people and their partners attending STI clinics, indicating sexual transmission (Pépin *et al*,

2005; Tosh *et al*, 2007). Like other sexually transmitted pathogens, *M. genitalium* is transmitted between heterosexual partners during unprotected coital activity (Ross & Jensen, 2006; Manhart *et al*, 2007). *M. genitalium* has been shown to adhere to spermatozoa (Svenstrup *et al*, 2003).

Oral sex is not a significant mode of transmission of this organism as it was recently found by Bradshaw *et al* (2009). They did not detect any *M. genitalium* in the pharyngeal swabs of 521 MSM who met at male-only saunas. The organism was present in the urethral and rectal swabs of these men. Further studies are required to determine whether the presence of this organism in the rectum plays a role in the subsequent development of proctitis.

Very little is known on vertical transmission and subsequent colonization of newborn infants by *M. genitalium* (Jensen, 2006). However, Waites *et al* (2005) have reported 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.

2.4 ROLE OF *M. GENITALIUM* IN DISEASE

The genital mycoplasmas represent a group of organisms that have been associated with an array of infections in adults and infants, however, conclusive evidence regarding the pathogenic potential of *Mycoplasma* and *Ureaplasma* spp in many conditions is lacking. This may be due to their fastidious growth requirements, as well as the fact that they are present in healthy persons probably as commensal when in low numbers (Gdoura *et al*, 2007).

2.4.1 *Mycoplasma genitalium* urethritis in men

The urethra is the canal that allows for the flow of urine from the bladder to the outside of the body. When this canal becomes infected, inflammation results in the accumulation of white blood cells in the area and is called urethritis. Urethritis, one of the most common sexually transmitted diseases (STDs) among heterosexual men, is conveniently classified as gonococcal or non-gonococcal urethritis (NGU), depending on the presence or absence of *Neisseria gonorrhoeae.* This probably emanates from the fact that *N. gonorrhoeae* has been identified as the main cause of urethritis. The role of *Chlamydia trachomatis* in NGU is well established (Martin & Bowie, 1999). In Chlamydia-negative NGU however, the aetiology is not completely understood. Pathogens that have been implicated in NGU are *Trichomonas vaginalis* (Pépin *et al,* 2001; Bradshaw *et al*, 2006a) and *Mycoplasma genitalium* (Horner *et al*, 2001). Yokoi *et*

al (2007) have also shown *Ureaplasma urealyticum biovar 2* to be present in symptomatic men; however, the pathogenic role of the ureaplasmas is still unclear.

The role of *M. genitalium* in NGU could not be established before the development of nucleic acid amplification tests (NAATs), as isolating the organism from clinical specimens in culture is extremely difficult. Through the use of various NAATs, this particular mycoplasma has now been found significantly more often in patients with acute NGU than in those without urethritis (Jensen *et al*, 1993; Horner & Taylor-Robinson, 1994; Deguchi *et al*, 1995a; Maeda *et al*, 1998; Totten *et al*, 2001).

In 2001, Taylor-Robinson and Horner reviewed the 19 studies undertaken since the first study that used molecular diagnostic assays (Hooten *et al*, 1988) 23 years previously. These studies were mostly performed in patients attending genitourinary medicine clinics, and in 17 of these, the presence of *M. genitalium* in patients with and without NGU has been determined. The majority of the studies showed that *M. genitalium* was detected significantly more in the urethra of men with NGU (19.8%) than in those without (8.8%). Although there were differences between the studies in terms of design, criteria for diagnosis of urethritis, and laboratory detection methods, the pooled odds ratio showed a value of 2.84 (95% CI 2.24–3.62; $p<0.00001$). In these studies, it was also shown that *M. genitalium* was detected mainly in *C. trachomatis* negative urethritis. The overall prevalence of *M. genitalium* was 23.5% in men with acute non-chlamydial

NGU, and only 5.6% in men without symptoms ($p<0.00001$; OR 5.14, 95% CI 3.38-7.87).

Studies have also shown that the concurrent infection of *M. genitalium* and *N. gonorhhoeae* in urethritis is low compared to simultaneous infection with *C. trachomatis* and *N. gonorhhoeae* (Janier *et al,* 1997). It has been said that *M. genitalium* may be the cause of post-gonococcal urethritis and may persist in the urethra after antimicrobial therapy causing persistent or re-current NGU (Ishihara *et al*, 2004). However, there is not much information available on the association of the organism with chronic NGU.

Although there are many studies showing strong and significant association of *M. genitalium* with NGU, there have been a few studies where no association could be found. When studying the prevalence of *M. genitalium* in men with and without urethritis in Turkey, Dolapci *et al* (2005) found no statistically significant difference between the groups. Yu *et al* (2008) could not demonstrate a significant association either in their study in Hong Kong, although *M. genitalium* was found more (10%) in men with NGU than in asymptomatic men (2%). The findings of these study are not supported by the majority of researchers, and may be attributed to the small number of patients with NGU enrolled (63 and 98 respectively). However, it could be argued that this mycoplasma is only an invader of tissue damaged by other pathogens. The facts that *M. genitalium* is often found in non-chlamydial NGU, has many features in common with *M. pneumoniae* (a known pathogen) and has caused inflammation in the urogenital tracts of inoculated

primates (Taylor-Robinson *et al*, 1985a), have supported the case for *M. genitalium* as pathogen (Taylor-Robinson & Horner, 2001).

2.4.2 *Mycoplasma genitalium* infection in women

The presence of *M. genitalium* in the genital tract of women and its possible association with disease has been demonstrated (Uno *et al*, 1997; Falk *et al*, 2005). Ongoing investigations reported conflicting findings. Casin *et al* (2002) could not show any association of the organism with cervicitis among French women, and Pépin *et al* (2005) showed that in spite of a high prevalence of *M. genitalium* in genital specimens from west African sex workers, only a weak association with cervicitis was seen. In Japanese, American and Swedish studies, the organism was more frequently detected in women with cervicitis than among controls (Uno *et al*, 1997; Manhart *et al*, 2003; Falk *et al*, 2005). In a study among Kenyan women, *M. genitalium* was associated with acute endometritis (Cohen *et al*, 2002), and in Britain, the organism was more frequently detected in women with pelvic inflammatory disease (PID) (Simms *et al*, 2003).

These conflicting reports highlight the need for further investigation into the role of *M. genitalium* in diseases in women. As postulated by McGowin *et al* (2009), although macrophage phagocytosis is an effective method for *M. genitalium* killing, its intracellular localization within vaginal and cervical epithelial cells may provide *M.*

genitalium a survival niche and protection from cellular immune responses thereby facilitating the establishment and maintenance of reproductive tract infection.

2.4.3 *Mycoplasma genitalium* in male and female infertility

Genital mycoplasmas, as inhabitants of the male urethra, contaminate semen during ejaculation, and hence may play a role in male infertility. Gdoura *et al* (2007) reported that in *M. genitalium* positive patients, there was a significant decrease in the sperm concentration. Svenstrup *et al* (2003) have shown that *M. genitalium* adheres to all parts of the human spermatozoa causing sperm agglutination and immotility when many of the organisms were bound. However, when a single *M. genitalium* cell bound to spermatozoa, the neck/midpiece was the preferred attachment site, and no effect on the motility was seen.

In the study by Clausen *et al* (2001) *M. genitalium* could not be correlated with male factor infertility, but there was an association with tubal factor infertility. This has been postulated to be due to inflammatory changes occurring in the fallopian tubes. Grześko *et al* (2009) have shown a significant higher presence of *M. genitalium* in infertile than fertile women.

The studies by McGovin *et al* (2009) and Grześko *et al* (2009) have highlighted the fact that more studies on the role of *M. genitalium* in infertility are needed, as it is still

unclear whether eradication of *M. genitalium* infection will increase the chance of infertile couples to conceive a child.

2.5 EPIDEMIOLOGY OF *M. GENITALIUM* URETHRITIS IN MEN

Before the introduction of molecular techniques, information on the epidemiology of *M. genitalium* was limited due to the absence of routine and effective diagnostic methods (Taylor-Robinson & Horner, 2001). Taylor-Robinson *et al* (1985b), attempted to use serology and culture, and although they failed to isolate *M. genitalium*, they reported presumptive isolation based on colour changes in the mycoplasma broth. They found *M. genitalium* in 7 (32%) of 22 men with NGU, 5 (42%) of 13 men with non-chlamydial NGU and 2 (10%) of 22 without urethritis. However, since the development of the first molecular testing assays, a number of studies investigating the presence of *M. genitalium* in NGU have been carried out.

At first Hooton *et al* (1988) used a DNA probe technique to investigate the prevalence of *M. genitalium* in male genitourinary medicine (GUM) clinic attendees presenting with urethritis. They reported that in the patients diagnosed with NGU, 10% of the men had *M. genitalium* in conjunction with other pathogens, whereas 13% had only *M. genitalium*. This led to the conclusion that *M. genitalium* could cause urethritis independently of *C. trachomatis*.

In the first study that employed PCR techniques by Jensen *et al* (1993), rectal, urethral and throat samples from 99 male STD clinic attendees in Denmark were investigated and *M. genitalium* was detected in 17% of the urethral swabs, but in none of the rectal and throat swabs, indicating that the urogenital tract was the primary site of infection. Furthermore, they found that significantly more men with NGU (27%) were positive for *M. genitalium* than were those without urethritis (9%). In their study on men with urethritis, *M. genitalium* was found more often in *C. trachomatis* negative NGU 12/34 (35%) than in those with chlamydial NGU 1/14 (7%), indicating that the two microorganisms may act as separate causes of urethritis.

Since these early studies, many PCR based investigations have been reported. Jensen (2006) summarized 23 studies carried out unto the year 2003 (Table 2.1). Combining these studies totaling 5455 patients, Jensen (2006) showed an overall *M. genitalium* prevalence of 20.8% in patients with NGU, and 5.9% in asymptomatic subjects. In 16 of the clinical studies, it was possible to calculate the prevalence of *M. genitalium* in men with non-chlamydial NGU. In these studies, the *M. genitalium* prevalence in the NGU group was 19.3% compared to 27.7% of *Chlamydia trachomatis*. Except for studies in Italy (Gambini *et al*, 2000) and the USA (Mena *et al*, 2002), *M. genitalium* prevalence was significantly higher in the non-chlamydial NGU group than in the total NGU group. Among the non-chlamydial NGU patients, 21.9% were *M. genitalium* positive compared to 6.0% of the patients without urethritis.

A large literature review conducted by Deguchi & Maeda (2002) also found *M.*

genitalium to be between 18.4 and 45.5% in all cases of male non-chlamydial NGU patients. Recent studies performed in Norway, Sweden and the USA, have also shown *M. genitalium* rates of between 4.1 – 26.0% in men with symptoms of urethritis (Falk *et al*, 2004; Anagrius *et al*, 2005; Edberg *et al*, 2008; Gaydos *et al*, 2009; Moi *et al*, 2009a). Some of these reports are summarized in Table 2.2. It was shown that *M. genitalium* behaves independently of *Chlamydia trachomatis* and is usually more prevalent in patients with NGU than *C. trachomatis*.

Table 2.1 Clinical studies between 1993 and 2003 of the prevalence of *M. genitalium* as determined by PCR (Adapted from Jensen, 2006)

Investigators	Country	Specimen type	Inclusion criteria for urethritis	%MG+ in NGU	%MG+ in NCNGU	%MG+ in a-sympto matic	Inclusion criteria for controls	Total Number studied
Jensen *et al*, 1993	Denmark	Urethral swab in SP4	Urethritis symptoms + >4 PMNLs/hpf	27.1	35.3	8.5	Asymptomatic men / same setting	99
Horner *et al*, 1993	UK	FVU	Urethritis symptoms + >4 PMNLs/hpf	23.3	27.6	5.7	No symptoms / <5 PMNLs/hpf	164
Blanchard *et al*, 1993	USA	Urethral swab in 2SP	Urethritis symptoms + >4 PMNLs/hpf	14.1	NA	NA	NA	
Deguchi *et al*, 1995a	Japan	Urethral swab in 2SP	Urethritis symptoms + >4 PMNLs/hpf	14.9	18.4	0	No symptoms / <5 PMNLs/hpf	142
Janier *et al*, 1995	France	Urethral swab in 2SP	Urethritis symptoms + >4 PMNLs/hpf	29.0	35.3	8.5	Urethritis symptoms but <5 PMNLs/hpf	273
Lackey *et al* , 1995	USA	NA	>4 PMNLs/hpf	32.3	NA	18.0	<5 PMNLs/hpf	190
Busolo *et al*, 1997	Italy	Urethral swab in SP4	Complaint of discharge and/or discharge	11.5	NA	0	Asymptomatic men / same setting	100
Uno *et al* , 1997	Japan	FVU		NA	NA	1.1	Asymptomatic men	187
Maeda *et al*, 1998	Japan	Urethral swab in TE	Urethritis symptoms + >4 PMNLs/hpf	13.2	26.5	0	Asymptomatic men / same setting	97

Investigators	Country	Specimen type	Inclusion criteria for urethritis	%MG+ in NGU	%MG+ in NCNGU	%MG+ in a-sympto matic	Inclusion criteria for controls	Total Number studied
Bjornelius *et al*, 2000	Sweden	Urethral swab in 2SP	Urethritis symptoms + >4 PMNLs/hpf	30.0	41.4	11.4	No symptoms /<5 PMNLs/hpf	101
Gambini *et al*, 2000	Italy	Urethral swab in PBS	Urethritis symptoms + >4 PMNLs/hpf	29.2	24.5	4.3	No symptoms /<5 PMNLs/hpf	201
Johannisson *et al*, 2000	Sweden	Urethral swab in Amplicor	>4 PMNLs/hpf	14.8	21.6	0.8	<5 PMNLs/hpf	233
Keane *et al*, 2000	UK	FVU	>4 PMNLs/hpf or >9PMNLs/hpf in FVU	33.3	45.0	9.1	<5 PMNLs/hpf	47
Luo *et al*, 2000	China	Urethral swab in saline	Urethritis symptoms	24.1	NA	4.5	Asymptomatic men / same setting	183
Totten *et al*, 2001	USA	FVU	Urethritis symptoms + >4 PMNLs/hpf	22.3	28.2	4.3	No symptoms /<5 PMNLs/hpf	246
Pépin *et al*, 2001	West Africa	Urethral swab in Amplicor	Discharge	16.3	17.7	8.8	Asymptomatic men / same setting	998
Morency *et al*, 2001	Central Africa	Urethral swab in Amplicor	Discharge	41.7	NA	15.0	Asymptomatic men / primary health care	510
Taylor-Robinson *et al*, 2002	South Africa	FVU	Discharge / dysuria	17.7	20.0	8.6	Asymptomatic men / same setting	367
Yoshida *et al*, 2002b	Japan	FVU	>4 PMNLs/hpf	15.1	19.6	2.4	Asymptomatic men / same setting	190

Investigators	Country	Specimen type	Inclusion criteria for urethritis	%MG+ in NGU	%MG+ in NCNGU	%MG+ in a-sympto matic	Inclusion criteria for controls	Total Number studied
Mena *et al*, 2002	USA	Urethral swab (dry) + FVU	Urethritis symptoms + >4 PMNLs/hpf	30.8	28.0	7.6	Asymptomatic men / same setting	285
Eastick *et al*, 2003	UK	FVU	>4 PMNLs/hpf or >9PMNLs/hpf in FVU	16.7	15.2	NA	Asymptomatic men / same setting	54
Dupin *et al*, 2003	France	FVU	Urethritis symptoms + >9 PMNLs/hpf	21.6	28.1	2	Asymptomatic men / same setting	193
Totals				**20.8**	**21.7**	**5.9**		

MG: *Mycoplasma genitalium*

NGU: Non gonococcal urethritis

NCNGU: Non chlamydial non gonococcal urethritis

FVU: First void urine

SP: Sucrose phosphate

PMNLs/hpf: Polymorphonucleur leukocytes per high power field

Table 2.2 Clinical studies conducted after 2003 of the prevalence of *M. genitalium* in men with urethritis

Investigators	Country	Specimen type	%MG+ in NGU	%MG+ in a-sympto matic	Inclusion criteria for controls	Total Number studied
Falk *et al,* 2004	Sweden	FVU	7.0	NA	NA	519
Dolapci *et al*, 2005	Turkey	FVU	6.34	NA	NA	63
Anagrius *et al*, 2005	Sweden	Urethral swab	13.6	1.2	No symptoms /<5 PMNLs/hpf	501
Leung *et al*, 2006	UK	FVU	5.3%	NA	NA	680
Yokoi *et al*, 2007	Japan	FVU	4.1	NA	NA	390
Black *et al,* 2008	South Africa	FVU	14.4	NA	NA	438
Yu *et al*, 2008	Hong Kong	Urethral swab	10.0	2	Asymptomatic men	507
Edberg *et al*, 2008	Sweden	Urethral and rectal swab	7.7	0.7	NA	381
Moi *et al* , 2008a	Norway	FVU	8.6	2.1	No symptoms	8468
Chalker *et al*, 2009	UK	Urethral swab in SP4	11.5	0	No symptoms /<5 PMNLs/hpf	280
Manhas *et al* , 2009	India	FVU	6.0	NA	NA	100
Taylor-Robinson *et al*, 2009	Russia	Urethral swab in TE	26.0	10	No signs or symptoms	172
Gaydos *et al*, 2009	USA	FVU	22.4	7.3	<5 PMNLs/hpf	292

MG: *Mycoplasma genitalium*

NGU: Non gonococcal urethritis

FVU: First void urine

SP: Sucrose phosphate

PMNLs/hpf: Polymorphonucleur leukocytes per high power field

2.5.1 Prevalence of *M. genitalium* in Africa

Not many studies investigating *M. genitalium* infection have been carried out in Africa. However, studies done in West Africa (Pépin *et al*, 2001), Central Africa (Morency *et al*, 2001), and South Africa (Taylor-Robinson *et al*, 2002; Sturm et al, 2004; Black *et al*, 2008) all showed similar results. *M. genitalium* was found in 14.4 – 28.2% of men with symptoms of urethritis.

When comparing the African studies with those done in other parts of the world, Jensen (2006) found the odds ratios for being *M. genitalium* positive both in patients with NGU and non-chlamydial NGU lower. The difference could be ascribed to the definition of urethritis used in these African studies, where microscopy was not always performed, and the presence of symptoms was used to define urethritis. However, the higher prevalence of *M. genitalium* in men without symptoms may also reflect the importance of *M. genitalium* in populations with high burdens of sexually transmitted infections.

The role of *M. genitalium* in South African men with urethritis will be discussed in more detail in Chapter 4.

2.6 LABORATORY DIAGNOSIS OF *M. GENITALIUM*

In any infectious disease, it is essential to identify the infecting microorganism. This can be done by using either conventional methods such as microscopy, culture and antigen / antibody detection, or the detection of its nucleic acids, by a variety of methods. The type of specimen (eg. urine, endourethral swabs, endocervical swabs) taken also plays a role in the identification of the pathogen.

2.6.1 Culture

Sucrose phosphate based culture media (SP4) played a major role in the discovery of *M. genitalium*. This medium designed to isolate mycoplasmas and spiroplasmas was developed by Tully *et al* in 1979 at the National Institute of Health (NIH), Maryland, USA, and consisted of mycoplasma broth base, tryptone, peptone, glucose, deionized water, supplemented with sterile tissue culture supplement containing glutamine, 25% yeast extract, 2% TC Yeastolate, fetal bovine serum, penicillin and 0.1% phenol red, with the final pH adjusted to 7.5. Two isolates were found from 13 samples collected from men with NGU attending a genitourinary medicine clinic in London in 1980 (Tully *et al* 1981). Evidence of mycoplasmal growth was an increase in turbidity as well as the acidic pH change from red to yellow of the phenol red indicator, due to glucose fermentation by the organism (Hussain *et al,* 1999). The layer of cells adhering to

the container surface was scraped off to inoculate a solid agar medium (0.6% agarose or 0.8% Noble agar in broth base) before anaerobic incubation at 37^0C. Signs of growth in the medium occurred very slowly; with a colour change only occurring after 50 days. This approach for culture yielded two strains and these were designated G-37 and M-30. The G-37 became the type strain *M. genitalium* (Tully *et al*, 1983).

Even though strains G-37 and M-30 were re-isolated from the original specimens after they had been frozen, further attempts to isolate the organism by culture of specimens from the urogenital tract, have proved difficult. A study carried out by Taylor-Robinson *et al* (1985b) on specimens from men with NGU that had been thought to have the organism, on the bases of presumptive isolation by observation of colour-change in the mycoplasma broth, failed to yield growth of the organism. A colour change in the mycoplasma broth medium could also be caused by other bacteria or by human cells that may grow in the rich SP4 medium. Samra *et al* (1988) also used SP4 and a modified medium that contained horse serum to try to isolate *M. genitalium* from a variety of clinical specimens, and observed a colour change in 15% of the specimens. However, the organism could not be subcultured. The success of SP4 medium has also been reported from China where 8 strains of *M. genitalium* were reportedly isolated from urogenital tract specimens from patients attending a STD clinic (Luo *et al*, 1999). During a six month period, Baseman *et al* (2004) were able to establish axenic primary cultures of *M. genitalium* from vaginal and cervical

specimens using the SP4 medium. Aliquots of cervical and vaginal specimens were processed for culture by first diluting samples in SP-4 medium, followed by filtration and incubation incubated at 37℃ in an atmosphere of 10% (vol/vol) CO_2 until colour changes were observed. Positive broth cultures were regularly passaged and clones incubated on SP4 agar.

Considering the property of *M. genitalium* to adhere to moist surfaces and with the knowledge of propagation of fastidious strains of *M. hominis* in cell culture, attempts were made to grow *M. genitalium* in cell cultures (Hopps & Del Giudice, 1984) In Denmark, Jensen *et al* (1996) could recover 5 new strains by co-culture of *M. genitalium* with Vero monkey kidney cells. This approach proved to be efficient but extremely time consuming. For most strains, more than six months were required from the time the cell culture was inoculated to the time the strains were ready for cloning (Jensen *et al*, 1996). Furthermore, some strains did not grow on solid medium after filtration and therefore they were difficult to purify. The Ultroser HY serum substitute was shown to be superior to foetal calf serum when isolation was attempted in Vero cells (Jensen *et al*, 1996). However, cell assisted growth of *M. genitalium* has also been reported with standard cell culture medium, containing foetal calf serum though no new strains have been recovered (Totten *et al,* 2001).

More recently, Hamasuna *et al* (2007) in Japan, 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 PCR. Isolation of the organism in pure culture was very time consuming, and took over a year.

Laboratory culture methods to isolate *M. genitalium* from different urogenital specimens stay challenging and therefore there are only a few research strains available (Edberg *et al*, 2008). The difficulty in culture and subsequently difficulty to do antimicrobial susceptibility testing means that limited information on the minimum inhibitory concentrations (MICs) for relevant therapeutic drugs is available.

2.6.2 Microscopy and staining

The detection of *M. genitalium* using an ordinary light microscope is not possible as this organism is almost the size of a virus (0.22 - 0.45 µm) (Jensen, 2006; Hamasuna *et al*, 2007). Furthermore, conventional Gram or other staining techniques cannot be used due to the absence of the peptidoglycan cell wall in the membrane structure of *M. genitalium.* The organism can only be clearly observed under the transmission electron microscope (TEM) or scanning electron microscope (SEM) (Tully *et al*, 1983; Prescott *et al*, 2005).

2.6.3 Serology

Due to the antigenic similarities between *M. genitalium* and *M. pneumoniae,* there is a considerable rate of cross-reaction between the two species. This has significantly hampered the use of specific serology for the diagnosis and epidemiological studies of *M. genitalium*. A number of serological techniques have been employed to identify *M. genitalium,* but in most studies the diagnostic performance has not been validated (Jensen *et al*, 2006).

A variety of assays, for instance the complement fixation test (CFT), metabolism inhibition test, indirect haemagglutination test, and disc growth inhibition have shown cross-reaction between *M. genitalium* and *M. pneumoniae* (Jensen *et al,* 2006). This cross-reactivity was reported by Lind *et al* (1984) using micro-immunofluorescence, crossed immuno-electrophoresis and heamadsorption tests. A study by Furr and Taylor-Robinson (1984) showed a four-fold or greater rise in antibodies to *M. genitalium* in men with NGU using a micro-immunofluorescence assay but the correlation to presumptive isolation was poor. In chimpanzee model studies, both Taylor-Robinson *et al* (1985a) and Tully *et al* (1986) showed that antibodies were slow to develop after urogenital inoculation.

Wang *et al* (1997) studied the seroprevalence of antibodies to *M. genitalium* in different populations using Lipid-Associated Membrane proteins (LAMPs) in an enzyme immunoassay (EIA) test that was partially validated. LAMPs are

mycoplasma surface proteins that are Triton X- extracted and purified to be used as antigens in a LAMP-EIA for detection of antibodies to several different species of mycoplasmas (Perez *et al*, 1998). The Wang *et al* (1997) assay was validated using serum and urine specimens from 104 patients from whom 40 (38%) were LAMP-EIA positive. From the 40 positive patients, 38% had positive PCR results. The LAMP-EIA assay appears to be one of the most promising of the serological assays for the diagnosis of male patients with NGU.

Jurstrand *et al* (2007) conducted a study to evaluate the association between *M genitalium* antibodies and pelvic inflammatory disease (PID) and ectopic pregnancy using the LAMP-EIA method (Wang *et al*, 1997). The study was done on sera obtained from patients with clinical PID and ectopic pregnancy as well as sera from asymptomatic women in Sweden. The LAMP-EIA showed that 17% (33/193) of the PID patients were *M genitalium* positive as compared to 18% (15/82) of the ectopic pregnant patients and 15% (36/246) of the healthy women. No significant association could be demonstrated between *M genitalium* antibodies and PID or ectopic pregnancy. The LAMP antigen was specific for different genotypes of *M. genitalium* since no cross-reactivity was observed with other mycoplasmas.

2.6.4 Molecular techniques

The application of molecular technology created a major impact for the detection of *M. genitalium* in clinical specimens. Studies using DNA probes (Hooton *et al* in 1988) were followed by the development of a conventional PCR assay in 1991 by Jensen *et al*. A real-time PCR assay was developed by Yoshida *et al* in 2002a. Gen-Probe® has developed a research transcription mediated amplification assay for the detection of *M. genitalium* that was first evaluated in a clinical study by Hardick *et al* in 2006a. Some of these assays will be discussed in more detail in Chapter 3.

2.6.4.1 DNA PROBES

Although Göbel *et al* introduced the first molecular assay using radioactive labeled oligonucleotide probes targeting the 16S rRNA for the detection of *M. genitalium* in 1987; the detection limit of the 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 $6x10^4$ genome copies.

2.6.4.2 POLYMERASE CHAIN REACTION (PCR) BASED METHODS

The first PCR assay to detect *M. genitalium* in clinical specimens was developed by Jensen *et al* in 1991 using the MgPa DNA sequence that was thought to be conserved, since the attachment protein is vital in the pathogenesis of mycoplasmas. It was later found that the region of MgPa flanked by the MgPa 1/MgPa 3 primers appeared variable. An *EcoR1* restriction site was present in G-37 strains, as well as in the M-30 and other respiratory tract isolates, but not in any of the Danish strains studied (Jensen *et al* 1991). This observation evoked research into the genetic variability of the *Mycoplasma genitalium's* genome and the realization that there was a need for another PCR assay. Different authors have independently amplified different fragments of the adhesion/attachment organelle MgPa to improve the sensitivity and specificity of PCR based assays and thereby to enable *M. genitalium* to be detected reliably in different clinical urogenital specimens (Taylor-Robinson & Horner, 2001). A hemi-nested PCR assay developed by Palmer *et al* (1991) has been proved to work, though the assay was prone to contamination due to carryover. Assays targeting variable regions may also produce false negatives when a mutation appears in one of the primer binding sites. A slight modification by designing a new outer forward

primer (MgPa1) was compared by Deguchi *et al* (1995b) to the original Jensen *et al* (1991) PCR and was found to be in agreement. This primer pair (MgPa1/MgPa3) has since been widely used and forms the base of the microwell-plate-based PCR assay developed by Dutro *et al* (2003). It was also used in a multiplex PCR assay developed by Lee *et al* (2007) for the simultaneous detection of *C. trachomatis, N. gonorrhoeae, M. genitalium* and *U. urealyticum.*

Molecular approaches that followed targeted different genes such as the 16S rRNA (Yoshida *et al,* 2002a; Jensen *et al* in 2003) and the *gap* gene which encodes the glyceraldehydes-3-phosphate dehydrogenate enzyme of the glycolysis pathway (Svenstrup *et al* in 2005).

Some of the PCR assays were combined with probe hybridization of the amplified products in microwell titre plates. Yoshida *et al* (2002a) developed a method for detecting the presence of *M. genitalium, M. hominis, U. parvum* and *U. urealyticum* from clinical samples by microwell plate hybridization of amplified 16S rRNA genes with species specific probes. The microwell plate based PCR of Dutro *et al* (2003) made used of the MgPa1/3 primer pair for amplification. This was also followed by hybridization with a specific probe and colorimetric detection in microwell plate format. An internal inhibition control to determine the limit of the assay detection was used in this assay.

2.6.4.3 REAL-TIME POLYMERASE CHAIN REACTION (q-PCR)

The real-time PCR (q-PCR) assay is one of the most recent developments and modifications of the traditional (conventional) PCR for the diagnosis of human pathogens. It has several advantages over the earlier DNA probe or PCR based assays, most notably the shortened time required for performance (Bustin, 2005). This assay is a combination of conventional PCR amplification and simultaneous automated detection of amplified PCR products (amplicons) using fluorescent probes in the same reaction tube (Espy *et al*, 2006). This eliminates the labour intensive post amplification detection of amplicons by agarose gels or probe hybridization with reduced risk of carryover contamination. Both the amplification and detection can be completed within an hour which is much faster than DNA probes or conventional PCR assays that may take two or more hours.

There are a number of different fluorescent probe chemistries available for the q-PCR assay. Among these are (1) hydrolysis probes including Taqman probes, molecular beacons and scorpions (2) hybridization probes, incorporating dual fluorescence resonance energy transfer (FRET) and (3) intercalating dyes like SYBR green that bind to double stranded DNA and (4) fluorescent labeled primers such as Scorpion™, Sunrise™ or Lux™ (Gunson *et al*, 2006). All of these chemical reactions rely on the transfer of light energy from the donor to the adjacent acceptor dye molecule, a process known as FRET (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. 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 first q-PCR assay for the detection of *M. genitalium* was established and published in Japan by Yoshida *et al* (2002a) who used the TaqMan probe reaction targeting the 16S rRNA gene in urine specimens of men with non-chlamydial NGU and found the assay to be highly sensitive (98.8%). Jensen *et al* (2004a) developed and validated a quantitative Taqman probe PCR detecting a fragment of the *MgPa* adhesin gene. This assay also contained an internal processing control to detect PCR inhibition. With this assay they could detect less than 5 genome copies without cross-reaction with other mycoplasmas. Svenstrup *et al* (2005) developed a quantitative real-time hybridization probe assay on the Roche LightCycler targeting the *gap* housekeeping gene. There was a good linear correlation between the LightCycler assay and the Taqman assays. Chen *et al,* (2005) evaluated five potential *M. genitalium* target genes for real-time PCR assays. These were the adhesin gene (MG191), DNA polymerase (MG262), dihydrolipoamide dehydrogenase (MG271), *gap* (MG301) and lactate dehydrogenase (MG460). All the genes proved to be suitable, but the adhesin gene primers could successfully be incorporated in multiplex assays. Some of the primers used by different researchers in PCR and q-PCR are shown in Table 2.3.

Table 2.3 Primers used in the detection of *M. genitalium* in PCR assays.

Target gene	Name	Sequence	Reference
MgPa	MgPa1 MgPa3	AGT TGA TGA AAC CTT AAC CCC TTG G CCG TTG AGG GGT TTT CCA TTT TTG C	Jensen *et al*, 1991
MgPa	Mg1 (out fwd) Mg3 (in fwd) Mg2 (out rev)	TGT CTA TGA CCA GTA TGT AC GTA ATT AGT TAC TCA GTA GA CCG TTG AGG GGT TTT CCA TTT TTG C	Palmer *et al*, 1991
MgPa	Mg1a (out fwd)	GTG TAA CTT ACC AGT GGC TTT GAT C	Deguchi *et al*, 1995b
MgPa	MgPaW1 MgPaWR1	AAG TGG AGC GAT CAT TAC TAA C CCG TTG TTA TCA TAC CTT CTG A	Mena *et al*, 2002
MgPa	MgPa1-mod MgPa3-mod	TGA AAC CTT AAC CCC TTG G AGG GGT TTT CCA TTT TTG C	Dutro *et al*, 2003
16S rRNA	Mge1 Mge2	GAA TGA CTC TAG CAG GCA ATG GCT G ATT TGC TCA CTT TTA CAA GTT GGC T	Sasaki *et al*, 1992
16S rRNA	Mg16S-45F Mg16S-447R	TAC ATG CAA GTC GAT CGG AAG TAG C AAA CTC CAG CCA TTG CCT GCT AG	Jensen *et al,* 2003
16S rRNA	My-ins MGSO-2	GTA ATA CAT AGG TCG CAA GCG TTA TC CAC CAC CTG TCA CTC GGT TAA CCT C	Yoshida *et al*, 2002a
P115	P115-74 P115-173	CCC ATC GTC AAG GTA CAA TGA TGA GCA TTT TTC AAG TTC AAC TGC AAA GG	Dupin *et al,* 2003
gap	Mg-gao-605f Mg-gap-794r	GTG CTC GTG CTG CAG CTG T GCT TGA TTT ACTTGT TCA ACA GAT GGA C	Svenstrup *et al*, 2005

Recently in Sweden, Edberg *et al* (2008*)* compared q-PCR (targeting both 16S rRNA and adhesin proteins) to a conventional PCR assay targeting 16S rRNA. They found real-time *MgPa* gene PCR to be more sensitive than conventional PCR, and to have a considerably increased sensitivity compared with real-time 16S rRNA gene PCR for the detection of *M. genitalium.*

2.6.4.4 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 of the first generation NAATs for CT and NG by introducing technologies such as target capture to remove 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*,* which is a modification of the second generation APTIMA® Combo 2 CT/NG assay protocol (Hardick *et al,*

2006a). The TMA assay combines the technologies of target capture, transcription mediated amplification and detection. This assay was compared to a multitarget (*MgPa* and 16S rRNA) real-time PCR assay and was more sensitive (98.1% vs 91.8%) and slightly less specific (98.1% vs 99.5%) (Hardick *et al*, 2006a).

2.6.4.5 TYPING METHODS

In order to understand transmission and pathogenesis of *M. genitalium* it has become important to type the different strains. After the first PCR assay (Jensen *et al*, 1991), several molecular methods have been reported that could be useful for strain typing. These include short tandem repeat (STR) analysis of putative lipoprotein genes (MG309) (Ma & Martin, 2004), single nucleotide polymorhisms (SNPs) in the rRNA genes (Ma *et al*, 2008), restriction fragment length polymorphisms (RFLP) of the MG192 (*mgpC*) gene (Musatovova *et al*, 2006) and SNPs in the MG191 (*mgpB*) conserved gene (Hjorth *et al*, 2006). In 2008, Ma *et al* compared some of these typing methods and found that a combination of the MG309-STRs and MG191-SNPs was efficient for general epidemiological studies.

2.7 TREATMENT

There is increasing recent evidence that indicates that *M. genitalium* has an aetiological role in the causation of non-chlamydial NGU and cervicitis. *M. genitalium* infection is often missed, if not ignored, due to the lack of rapid appropriate diagnostic methods including culture. *M. genitalium* lacks a cell wall and is therefore inherently resistant to beta-lactam antibiotics and any other cell wall inhibitors. In general, the mycoplasmas are susceptible to antibiotics that inhibit protein synthesis, and the majority of these antibiotics have a bacteriostatic effect (Björnelius *et al,* 2008). The Centers for Disease Control and Prevention, USA (CDC) recommends the use of macrolides, tetracyclines and fluoroquinolones for the treatment of *M. genitalium* infections. In their mechanism of action, macrolides and tetracyclines inhibit bacterial protein synthesis whilst fluoroquinolones inhibit bacterial genetic replication by interfering with DNA synthesis (Scholar & Pratt, 2000).

The exact minimum inhibitory concentrations (MICs) cannot be determined because it is not easy to culture the organism in artificial media (Taylor-Robinson & Bébéar, 1997; Hamasuna *et al*, 2005). This also poses a problem for 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). However, with the recent advances in growth of *M. genitalium* on Vero cells, combined with the development of quantitative PCR assays, we may be able to obtain better information which will assist in the formulation of treatment guidelines.

In 1998 Hannan performed an *in vitro* susceptibility study on 8 previously isolated and cell culture adapted *M. genitalium* strains from Denmark. A commercially available, liquid medium (Mycoplasma Experience Ltd) containing serum, fresh yeast extract and glucose was used in the cultivation and MIC testing of the organism. MIC tests were performed in 96-well, microdilution sensititre plates by mixing the compound dilutions with an equal volume of *M. genitalium* (containing$1x10^3$ – $1x10^5$ colony forming units/ml). All the *M. genitalium* isolates were susceptible to azithromycin but not to doxycycline and ciprofloxacin.

Falk *et al* (2003) compared the treatment efficacy of tetracyclines and azithromycin in *M. genitalium* infected patients. Women and men, attending a STD clinic in Sweden, who were positive for *M. genitalium*, were included. Patients with urethritis (>4 PMNLs/hpf) and/or cervicitis (PMNLs > epithelial cells) were treated with doxycycline or lymecycline. The patients not receiving initial treatment, but with a positive *M. genitalium* test (PCR), were treated with azithromycin. All patients were re-tested 4 to 5 weeks after treatment commenced. It was found that tetracyclines, although being effective against *C. trachomatis*, could not eradicate *M. genitalium* in men with symptomatic

urethritis. Azithromycin was more active than the tetracyclines and had superior mucosal cell penetration. In 2006, the Centers for Disease Control and Prevention, USA (CDC) treatment guidelines for STDs recommended 1g azithromycin orally in a single dose, or 100mg doxycycline orally twice daily for 7 days. There were concerns about azithromycin treatment failure and an extended 5 day regimen, consisting of 500mg on the first day, followed by 250g for 4 days was recommended by Björnelius *et al* (2003). Bradshaw *et al*, (2006b) reported treatment failure with the single dose azithromycin among Australian men with *M. genitalium* positive NGU and the infection was only cleared after treatment with moxifloxacin for an extended period of time (400mg/day for 10 days).

The major set-back in the management of any infectious disease is the development of antimicrobial resistance. Björnelius *et al* (2008) have reported in their Scandinavian study a greater than 50% treatment failure with a 9 days doxycycline treatment. After the failure of single dose azithromycin in the Australian study, Jensen *et al* (2008) investigated the reason for occasional treatment failure with this drug. They found that macrolide resistance correlated with azithromycin treatment failure. This resistance was shown to be due to mutations occurring in region V of the 23S rRNA gene. This is also well described in other Mollicutes (Morozumi *et al*, 2005; Wolff *et al*, 2008). These findings raised further concern about the use of single-dose azithromycin treatment for NGU of unknown etiology.

Pereyre *et al (*2004) compared the activities of some of the newer quinolones (garenoxacin, gatifloxacin, gemifloxacin with those of four fluoroquinolones, moxifloxacin, levofloxacin, ciprofloxacin, and ofloxacin against different human *Mycoplasma* and *Ureaplasma* species. The susceptibility testing was performed by an agar dilution method. Garenoxacin, a des-fluoro(6)-quinalone exhibited the highest activity. Yasuda *et al*, 2005) investigated the bacteriological efficacy of some fluoroquinolones in the treatment of *Mycoplasma genitalium* by testing positive patients daily (with PCR) after treatment. The bacteriological efficacy of gatifloxacin was superior to that of levofloxacin and tosufloxacin.

In order to prevent *M. genitalium* infection and re-infection, sexual partners and their contacts should be assessed comprehensively and offered empirical treatment. The identification and treatment of males with high risk of mycoplasmal infection may prevent spread to their female partners thereby reducing the risks of cervicitis and complications such as PID (Simms *et al*, 2003; Uno *et al,* 1997).

It was shown that *M. genitalium* should be taken in to account for diagnosis and when designing treatment of adult male urethritis, empirical treatment that is often used for urethritis might fail and lead to chronicity and persistence of the disease in a patient.

CHAPTER 3

EVALUATION OF NUCLEIC ACID AMPLIFICATION TESTS (NAATs) FOR THE DETECTION OF *MYCOPLASMA GENITALIUM* IN URINE SPECIMENS

3.1 INTRODUCTION

Culture techniques for *M. genitalium* have achieved some success, but it remains difficult, as it requires elaborate media, is time-consuming, and has low yields (Jensen *et al*, 1996; Baseman *et al*, 2004.) Therefore, laboratory diagnosis depends on the use of nucleic acid amplification tests (NAATS) targeting different genes. Polymerase chain reaction (PCR) assays have been developed for the detection of *M. genitalium* from different clinical samples such as urine and swabs from the cervix and urethra (Jensen, 2006). In 1991, Jensen *et al* developed a conventional PCR assay targeting the adhesion (*MgPa*) gene and in 2002 the first real-time (q-PCR) assay targeting the 16S rRNA was published by Yoshida *et al* (a). More recently, in 2006, the diagnostic commercial company Gen-Probe (USA) developed a transcription mediated amplification (TMA) assay for research use (Hardick *et al*, 2006a). As more researchers are showing the association of *M. genitalium* with male urethritis, highly sensitive assays for the detection of this pathogen are required to treat most of the infected persons to prevent transmission to sexual partners.

3.1.1 Specimen type

Various specimen types, including first void urine (FVU), endourethral swabs, cervical swabs and vaginal swabs can be used to detect *M. genitalium* infection in males and females. In view of the fact that different NAATs target different genome sequences, make use of different specimen preparation techniques and may have different amplicon detection methods, they may have different sensitivities and specificities in relation to the specimen types used for the detection of *M. genitalium.*

Cervical and urethral swabs have been the traditional specimens to detect genital pathogens such as *N. gonorrhoeae* and *C. trachomatis*, but recent studies have shown that self-collected vaginal swabs and urine specimens (e.g. first void urine (FVU)) are as sensitive (Gaydos & Quinn, 2005; Van de Wijgert *et al*, 2006). The advantages of self-collected swabs and FVU are that they are easy to obtain, non-invasive and are usually preferred by patients. Wroblewski *et al*, (2006) compared different specimen types for detection of *M. genitalium* in women using PCR and TMA assays and concluded that vaginal swab specimens were the most sensitive specimen type for the detection of the pathogen. This was confirmed by Moi *et al* (2009b) who 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 Oslo, Norway. Jensen *et al* (2004b) compared FVU and urogenital swabs for the detection of *M. genitalium* and *C.*

trachomatis in men and women by PCR, and also found cervical swabs to be preferred in women, but in men, FVU was the better specimen type. However, the cytotoxic effects of urine may hamper results if the specimen is 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 of results.

M. genitalium requires proper transport methods as it has no cell wall and is sensitive to drying and heat. A cold chain should be maintained and specimens have to be delivered to the laboratory within 24 hours of collection. The urine collection tubes (Gen-Probe Inc, USA) for TMA testing have the advantage of keeping urine samples stable for up to 28 days at room temperature (Gen-Probe Inc, 2001). A temperature of -70^0C is recommended for longer storage of specimens, (Murray *et al*, 2003). Carlsen *et al* (2009) have shown that the *M. genitalium* DNA load as well as the detection rate decreased after storage of clinical specimens. It is therefore recommended that the DNA is extracted from the specimens before storage.

3.1.2 Nucleic acid extraction methods

Researchers developing NAATs for the detection of *M. genitalium* have used different specimen preparation techniques. The method used by Jensen *et al* in 2004a has also been used is other studies (Edberg *et al*, 2008; Svenstrup *et al*, 2005). In this method, swabs (transported in SP4 medium) and concentrated

urine pellets were mixed with Chelex 100 slurry in Tris-EDTA buffer to release the DNA.

Dutro *et al* (2003) have compared three different commercial extraction methods in their microwell-plate-based PCR assay for *M. genitalium* detection from urine. The first was a DNA purification method marketed by Bio-Rad Laboratories, USA, called Instagene Matrix®. With this method urine samples are prepared on the day of collection by freezing in buffer, followed by thawing, cell lysis and deproteination to release DNA. The second method used was the Roche AMPLICOR® CT/NG specimen preparation kit. In this method, the urine is washed by adding buffer and then heated. The harvested bacteria are lysed and centrifuged to remove proteins. The third method was a MasterPure® DNA purification kit by Epicenter (USA). In this method cells are lysed, proteins removed by proteinase K and centrifugation and DNA collected in supernatant. The Roche and Epicenter extraction methods gave similar results, and were shown to be suitable for the extraction of *M. genitalium* from urine specimens. These two methods were also successfully used to detect *M. genitalium* by Wroblewski *et al* (2006) comparing PCR and TMA assays for various genital specimen types. Some researchers (Deguchi *et al*, 2002; Yoshida *et al,* 2002a) made use of proteinase K digestion followed by classic phenol-chloroform purification and ethanol precipitation of the DNA.

As shown by Dutro *et al* (2003), inhibitors in urine specimens may play a role in generating false negative results, hence the inclusion of internal amplification controls. The technique of target capturing as included in the TMA assays from Gen-Probe may eliminate amplification inhibition. In this method, the rRNA targets are released in the transport medium and attach to specific capture oligomers. 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) that are coated with magnetic particles. These magnetic particles are taken out of solution by powerful magnets, washed and the inhibitor-free rRNA targets are released for amplification (Figure 3.1).

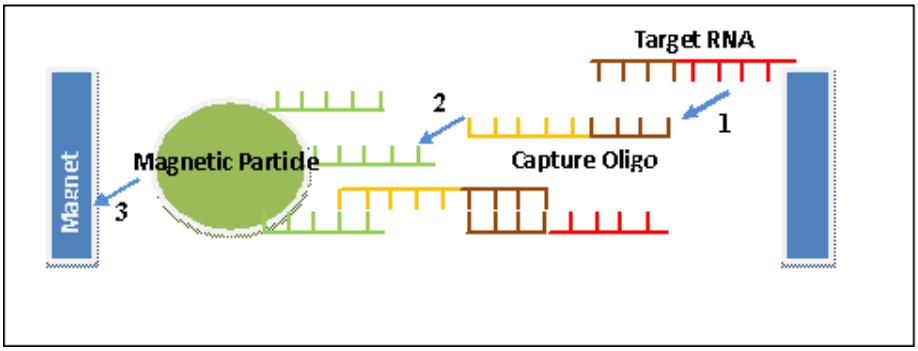


Figure 3.1 Schematic representation of target capture technology.

Modern robotic methods, e.g. the MagNa Pure® LC method (Roche, USA) and DTS 400 instrumentation system (Gen-Probe Inc, USA) have also been used to extract nucleic acids from clinical specimens for *M. genitalium* testing (Hardick *et al*, 2006a; Huppert *et al*, 2008; Moi *et al*, 2009b).

3.1.3 Nucleic acid amplification assays.

As explained in Chapter 2 (Section 2.6.4), the application of molecular technology had a major impact towards the detection of *M. genitalium* in clinical specimens. Conventional and real-time PCR assays, as well as a TMA test have been developed for the detection of *M. genitalium* in clinical specimens. Different researchers have independently amplified different fragments of the organism's genome; this was elaborated upon in Chapter 2 (Section 2.6.4.2 and Table 2.3).

3.1.3.1 OVERVIEW OF DIFFERENT POLYMERASE CHAIN REACTION (PCR) ASSAYS

The first PCR assay to detect *M. genitalium* in clinical specimens was developed by Jensen *et al* in 1991 using the adhesion/attachment organelle MgPa DNA sequence that was thought to be conserved. Since then, different researchers have independently amplified different fragments of the *MgPa* gene to improve the sensitivity and specificity of PCR based assays and thereby to enable *M. genitalium* to be detected reliably from different clinical urogenital specimens (Taylor-Robinson & Horner, 2001). All the PCR assays make use of the same technology: sequential cycles of double strand denaturing, primer annealing and template extension. This process is driven by a DNA polymerase enzyme, usually *Taq* DNA polymerase, in a thermal cycler, an instrument that can reach

the three different temperatures in a very short time. The amplicon detection takes place after amplification by either agarose gel electrophoresis, or specific probe hybridization.

The selection of primers for the PCR assay can be critical as was demonstrated by the experience of several researchers using primers targeting the *MgPa* gene (Jensen *et al*, 1991, Palmer *et al*, 1991; Peterson *et al*, 1995). This adhesin gene thought to be conserved, turned out to be variable, and highlighted the need to find alternative primer targets. This led to the development of new PCR assays, including a modification of the original MgPa primers (Deguchi *et al*, 1995b; Totten *et al*, 2001) and the use of primers targeting a 16S rRNA gene (Jensen *et al*, 2003; Eastick *et al*, 2003).

As with all PCR assays, these tests are prone to contamination, require separate amplicon detection, and cannot be used in quantification analysis. There was a need for faster, one tube assays in which the target copy number could be determined. The real-time polymerase chain reaction (q-PCR) that was subsequently developed makes use of fluorescent reporter molecules to monitor the production of amplification products during each cycle of the PCR reaction (Bustin, 2005). This combines the DNA amplification and detection steps into one homogeneous assay and eliminates the need for gel electrophoresis to detect amplification products. Its simplicity, specificity, and sensitivity, together with its potential for high throughput, quantification, and the ongoing introduction of new

chemical reactions, more reliable instrumentation and improved protocols, has made q-PCR the highly suitable for the detection of DNA (Bustin, 2005).

The concepts underlying fluorescence-based q-PCR are relatively simple and four different principles are commonly used for detection (Klein, 2002). All four technologies are based on the measurement of fluorescence during the PCR reaction. The simplest and cheapest principle is based on the intercalation of double-stranded DNA-binding dyes (eg SYBR Green). Already established PCR assays can easily be adapted to incorporate this technology, and there is no need for any additional fluorescence-labeled oligonucleotides. The drawback of this technology is that specific and nonspecific PCR products can both be detected (Bustin *et al*, 2005). Therefore, these assays require optimization of the PCR conditions and differentiation between specific and nonspecific PCR products using melting-curve analysis (Klein, 2002). The three other principles are based on the introduction of an additional fluorescence-labelled oligonucleotide primers (such as Scorpion™, Sunrise™ or Lux™) or probes (eg. Taqman probes) (Gunson *et al*, 2006). Sufficient amounts of fluorescence are only released either after cleavage of the hydrolysis probe (Heid *et al*, 1996) or during hybridization of a molecular beacon (Tyagi & Kramer, 1996) or during the hybridization of two probes to the amplicon (Wittwer *et al,* 1997). Introduction of these probes in the assays increases the specificity of the quantified PCR product and allows the development of multiplex reactions.

Rather than looking at the amount of DNA target accumulated after a fixed number of cycles, real-time assays determine the point in time during cycling when amplification of a PCR product is first detected. This is determined by identifying the cycle number at which there is a distinguishable increase in fluorescence above the background (Bustin, 2005). This cycle number is referred to as the threshold cycle (Ct). The Ct is determined at the exponential phase of the PCR reaction and is inversely proportional to the copy number of the target. Therefore the higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed, and the lower the Ct (Bustin, 2005).

The first q-PCR assay for the detection of *M. genitalium* was established and published by Yoshida *et al* (2002a) who used the TaqMan probe chemistry targeting the *M. genitalium* 16S rRNA gene in urine specimens of Japanese men with non-chlamydial NGU and found the assay to be highly sensitive (98.8%). Jensen *et al* (2004a) developed and validated a quantitative Taqman probe PCR detecting a fragment of the *MgPa* adhesin gene. With this assay they could detect less than 5 genome copies without cross-reaction with other mycoplasmas. Svenstrup *et al* (2005) developed a quantitative real-time hybridization probe assay on the Roche LightCycler targeting the *gap* housekeeping gene. There was a good linear correlation between the LightCycler assay and the Taqman assays. In 2005, Chen *et al* evaluated five potential *M. genitalium* target genes for real-time PCR assays. These were the adhesin gene

(MG191), DNA polymerase (MG262), dihydrolipoamide dehydrogenase (MG271), *gap* (MG301) and lactate dehydrogenase (MG460). All the genes proved to be suitable, but the adhesin gene primers could successfully be incorporated in multiplex assays. Recently in Sweden, Edberg *et al* (2008*)* compared two q-PCR assays (targeting the 16srRNA and adhesin proteins respectively) to a conventional PCR assay targeting 16SrRNA. They found real-time *MgPa* gene PCR to be more sensitive than conventional PCR, and to have a considerably increased sensitivity compared with real-time 16S rRNA gene PCR for the detection of *M. genitalium.* Real-time PCR has also been used in antibiotic susceptibility testing of *M. genitalium* where growth of the organism in Vero cells was monitored by a quantitative PCR assay (Hamasuna *et al*, 2005).

3.1.3.2 TRANSCRIPTION MEDIATED AMPLIFICATION

Gen-Probe Inc (USA) has developed a research TMA assay targeting 16S rRNA of *M. genitalium*. The rRNA is isolated from specimens using the target capture method where the rRNA is extracted and purified with the help of capture probes attached to magnetic particles. 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 RNaseH) 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. Copy DNA (cDNA) 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 RNaseH degrades the RNA of the RNA:DNA duplex. The second primer that binds to the cDNA creates a double-stranded DNA molecule. The promoter sequence in the DNA template in the double strand is recognized again by RNA polymerase that transcribes it into RNA amplification products (amplicons). Billions of copies of the target are amplified in less than an hour (Gen-Probe, 2009).

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. However, this assay is for research purposes only and the kit is therefore not commercially available.

3.1.4 Study problem and aims

The extreme difficulty and/or failure in growing *M. genitalium* on conventional media such as agar, has accounted for the absence of routine laboratory assays. There is a need for rapid, reproducible molecular assays to detect this fastidious organism. As *M. genitalium* can be present at very low concentrations in some patients (Jensen *et al*, 2004a), factors that have to be taken into consideration when selecting the best NAAT should be (1) a very low limit of detection (2) generation of high sensitivity and specificity (3) cost effectiveness and (4) acceptable turn around time (from receipt of specimen to issue of results).

This study was undertaken to compare three NAAT assays, namely a commercial PCR assay, an in-house adapted real-time PCR and a research-only TMA test for the detection of *M. genitalium* targeting different genes in urine specimens of men with and without urethritis.

3.2 MATERIALS AND METHODS

3.2.1 Clinical specimens

3.2.1.1 SAMPLE SIZE

The prevalence of *M. genitalium* in South Africa varies between 8% and 17% according to different authors, and therefore a prevalence of 16% was used to calculate the sample size. A sample size of 370 cases and controls (296:74) has been calculated with 90% confidence interval, and 80% power to detect an OR of $\geq$ 2 using EpiInfo.

3.2.1.2 STUDY POPULATION

The study population consisted of sexually active males complaining of urethritis, defined as burning on micturition (BOM), frequency of micturition, urethral irritation and/or urethral discharge. These were first time attendees who did not receive any antibiotic therapy in the preceding month. Specimens were also obtained from asymptomatic males in the same age group as controls, who also did not receive any antibiotic therapy in the preceding month. All these patients attended a family practice in Silverton, Pretoria and gave verbal consent for participation. The study was carried out between August 2007 and June 2008.

3.2.1.3 SPECIMEN COLLECTION AND TRANSPORT

After general examination by the physician, endourethral swab specimens were collected from each patient and smeared onto microscope slides. This was used for microscopic examination following Gram staining (Chapter 4). Thereafter, first-void urine (FVU) specimens were collected for NAATs into sterile universal jars and kept at 4^0C until 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 (Gen-Probe Inc, USA) containing buffered transport medium and stored at -70^0C for the transcription-mediated amplification (TMA) assay. The remainder 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.

3.2.2 Nucleic acid extraction

Deoxyribonucleic acid (DNA) was extracted from urine specimens using the Roche AMPLICOR® CT/NG specimen preparation kit, according to the manufacturers' instructions (Roche Diagnostics, Germany). The urine specimens were thoroughly vortexed and sterile plugged tips were used to add 500 µl to

appropriately marked tubes (Sarstedt) containing the wash buffer. The tubes were recapped and the contents thoroughly 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 in a microcentrifuge (Mikro 200R, Hettich). The supernatant was discarded and 250 µl 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.

3.2.3 Nucleic acid amplification assays for detection of *M. genitalium*

Three different NAATs were used to detect *M. genitalium* in urine specimens. The DNA of *M. genitalium* G-37 American Type Culture Collection (ATCC) strain 33530D was included as positive control and distilled water as negative control for all the analyses.

3.2.3.1 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 (http://www.sacace.com/products.php). 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 wax is melted at high temperature (95^0C), *Taq* DNA polymerase is activated, and the incorporated uracil-N-glycosylase (UNG) is inactivated before initiation of amplification. To prevent contamination, all steps were performed in separate clean rooms using sterile filter pipette tips.

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

Each PCR-mix-1 tube was labeled in correspondence to the extracted nucleic acid specimen number and controls. To each PCR-mix-1 tube, 10 µl 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 10 µl of the thawed extracted nucleic acid or control. 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.

Two percent agarose gel in 0.5 x Tris-borate-EDTA (TBE) buffer containing 3 µl ethidium bromide (10 µg/ml) was prepared an hour prior to electrophoresis. A size marker (100 bp Hyperladder™, Bioline, USA) was included on each gel. Electrophoresis was carried out for 1 hour at 80 volts after which the bands were visualized using a transilluminator. A band of 281bp, that is the amplification product of the *M. genitalium* MgPa gene fragment, was taken as positive for *M. genitalium*.

3.2.3.2 REAL-TIME PCR (Q-PCR) ASSAY

An in-house q-PCR method was used by adapting the LightCycler method of Svenstrup *et al* (2005). Primers targeted a 190 bp fragment of the *M. genitalium gap* gene and had the following sequences:

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')

The probes designed to hybridize to the amplicons 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)

Primers and probes were diluted in a 100 µM stock solution. All probes and primers were synthesized by Metabion international AG, Germany.

To prevent contamination, the PCR reagents were mixed in a PCR master-mix room prior to amplification. Non-specific amplification was eliminated by the inclusion of 1.5 units of Uracil-N-glycosylase (UNG) (Roche Diagnostics) in the master mix.

PCR master mixes were prepared to contain 0.5 µM of each primer, 0.2 µM of each probe, and 2.0 µl of LightCycler® FastStart DNA Master^{PLUS} Hybridization Probe (Roche Diagnostics) (containing hotstart *Taq* DNA polymerase, reaction buffer, $MgCl_2$, and dNTP mix (with dUTP in stead of dTTP)). The volume was

adjusted with molecular grade water for each reaction to contain 15 µl of master mix.

After addition of 5 µl of specimen or control to the appropriate capillaries, they were capped, and centrifuged at <200 x g for 10 seconds in a microcentrifuge (Mikro 200R, Hettich) to ensure that the mixture descended into the capillaries. These were then loaded into the LightCycler 5.1 (Roche Diagnostics). 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 the mg-gap-669FL donor to the mg-gap-700LC Red640 acceptor was measured in channel F2/F1 when the probes annealed to the *gap* target DNA. After the amplification, melting point analysis was performed. 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. Fluorescent signal data was directly transferred to the LightCycler software 5.1 (Roche Diagnostics). For each specimen, the crossing point (Ct) was determined from the amplification plot. The PCR products were

characterized by the melting curve analysis where a melting temperature ($T_{m)}$ of 67-68^{0}C indicated *M. genitalium* (Svenstrup *et al,* 2005).

3.2.3.3 TRANSCRIPTION MEDIATED AMPLIFICATION (TMA) ASSAY

The presence of *M. genitalium* was determined in the 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*. Gen-Probe general purpose reagents were used in this TMA assay, using primers and a probe targeting a 427bp fragment of the 16S rRNA gene of *M. genitalium*. The primers and probe were synthesized by Gen-Probe Inc (USA). The method as recommended by the manufacturers was used (Gen-Probe, 2006).

The stored urine specimens in the APTIMA® specimen 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 rRNA targets were isolated from the urine solution by the addition of 100 µl capture oligomers to 400 µl of urine in the appropriate bar-coded tubes of a ten-tube unit (TTU) (Figure 3.2).

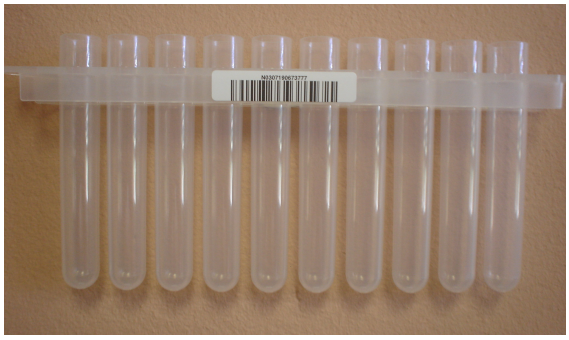


Figure 3.2 Bar-coded Ten-Tube Unit (TTU).

The TTUs with specimen identification were entered in the appropriate application software on the computer. 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 TTU tubes were placed in a target capture system (TCS) 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. The extracted, washed rRNA was then used in amplification.

Hundred microliters of amplification solution, containing 16S rRNA gene specific primers, and 200 µl 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 before addition of 25 µl of a combination of the three enzymes; reverse transcriptase, RNase H and RNA polymerase. The mixture was incubated in the SB100 at 42^0C for 60 minutes to allow amplification.

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 100 µl 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 250 µl 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 the APTIMA 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* (Hardick *et al,* 2006a).

3.2.4 Data analysis

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). The results were entered into a 2x2 table, and the chi square test used to determine the performance of each assay. The specificities and sensitivities were calculated and compared (EpiOnline).

3.3 RESULTS

3.3.1 Study population

Three hundred men with signs and/or symptoms of urethritis, and 75 asymptomatic men were recruited for the study. Of the symptomatic men, 94 (31%) were patients with visible urethral discharge, and the remaining 206 (69%) patients complained of BOM without visible discharge on examination. Patients' ages ranged from 19-64 years, with the mean of 37.0 years. The mean age of the asymptomatic men was 36.5 years (range 17 – 65 years).

3.3.2 Detection of *M. genitalium* by molecular techniques

From the total number of specimens (n=375) *M. genitalium* was detected in 60 of the urine specimens using TMA (16.0%), whilst PCR and q-PCR assays detected

M. genitalium in 48 (12.8%) and 55 (14.7%) respectively. Of the 60 positive specimens detected by TMA, 52 (87%) were from symptomatic patients, and 8 (13%) were from patients without symptoms. Fourty three (90%) of the *M. genitalium* positive results obtained by PCR were detected in symptomatic men, while 5 (10%) were from asymptomatic men. Among the 55 positive results found by q-PCR 48 (87%) were from urethritis patients and 7 (13%) were from men without urethritis symptoms. The distribution of the results obtained by the 3 assays from patients with signs and symptoms of urethritis is shown in Figure 3.3 (A) and from asymptomatic patients in Figure 3.3. (B).

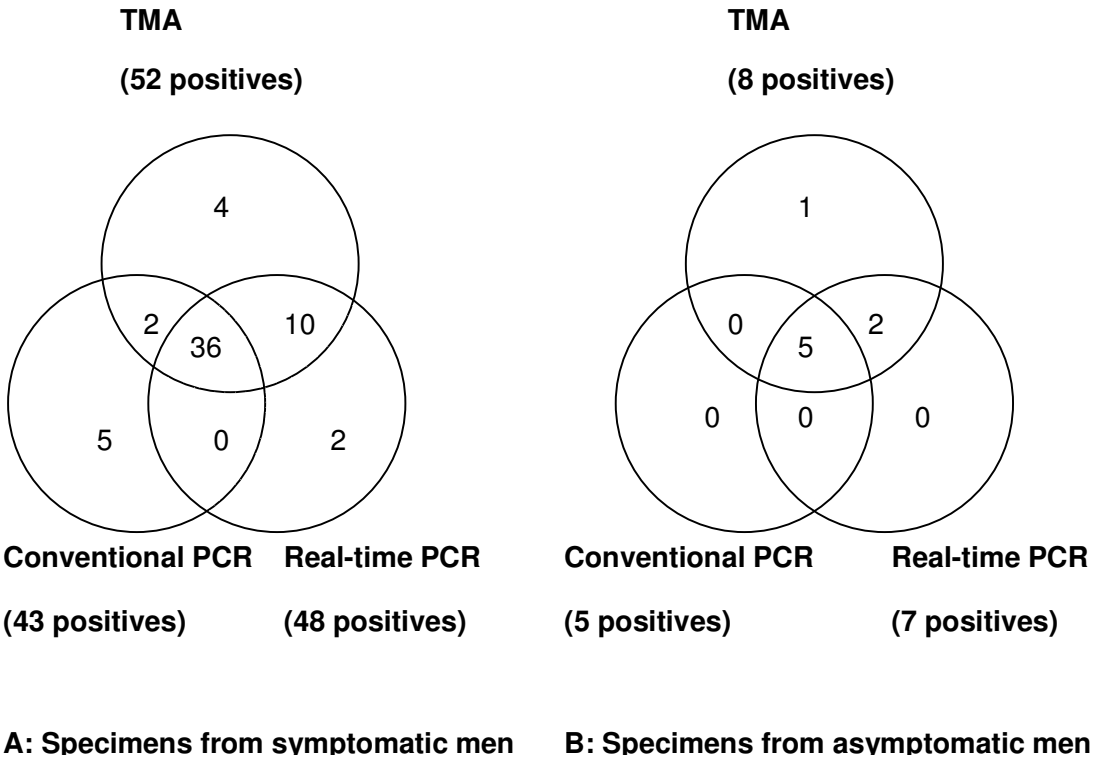


Figure 3.3 Comparison of *M. genitalium* detection in symptomatic and asymptomatic men using three NAATs.

Among the symptomatic group, all three assays detected *M. genitalium* in 36 specimens, the TMA + q-PCR detected an additional 10, and 2 more were detected by TMA + PCR. In the asymptomatic group *M. genitalium* was detected by all 3 NAATs in 5 specimens while TMA + q-PCR detected 2 more, and only TMA detected 1 additional positive. No pathogens were detected by both PCR and q-PCR, and not by TMA.

Using the extended gold standard, i.e a positive result for any 2 NAATs. *M. genitalium* was detected in 48 (16%) symptomatic and 7 (9%) asymptomatic men. Of the 48 specimens from symptomatic men, 21 (44%) were from men with urethral discharge, and 27 (56%) were from men with BOM only.

3.3.2.1 CONVENTIONAL PCR

PCR products, amplified using the 280/550 IC (Sacace Biotechnologies) kit, were analysed by agarose gel electrophoresis (Figure 3.3). *M. genitalium* was detected in 48 (12.8%) of the 375 specimens.

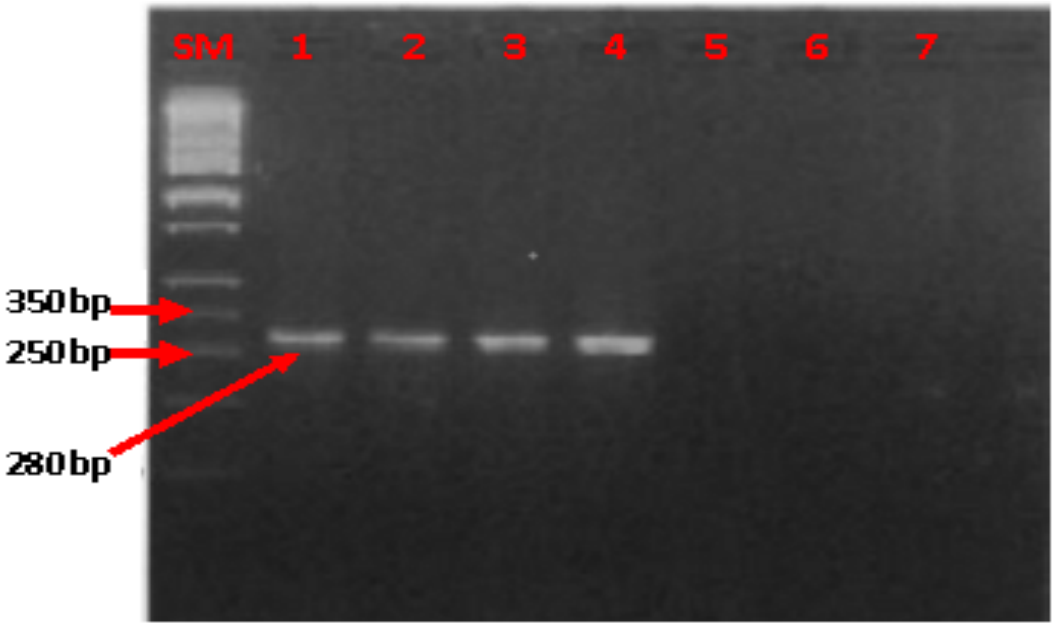


Figure 3.4 Electrophoretic analysis of the PCR products obtained from urine specimens.

SM: Size marker; 1: MG Positive Control (ATTC G37); 2-4: MG Positive specimens; 5-6: MG Negative specimens; 7: MG Negative Control (water)

When PCR was evaluated against the extended gold standard (any 2 positive NAATs), sensitivity and specificity of the PCR assay was 78% and 98% respectively. The positive and negative predicted values were 90% and 96%.

3.3.2.2 REAL-TIME PCR (q-PCR)

The q-PCR products were characterized by melting curve analysis and a melting temperature (T_m) of 67-68°C was indicative of the *M. genitalium* product (Fig. 3.4). The q-PCR detected the pathogen in 55/375 (14.7%) specimens. Against the extended gold standard, the sensitivity and specificity of the q-PCR assay was 96% and 99% respectively. The positive and negative predicted values were 96% and 99%.

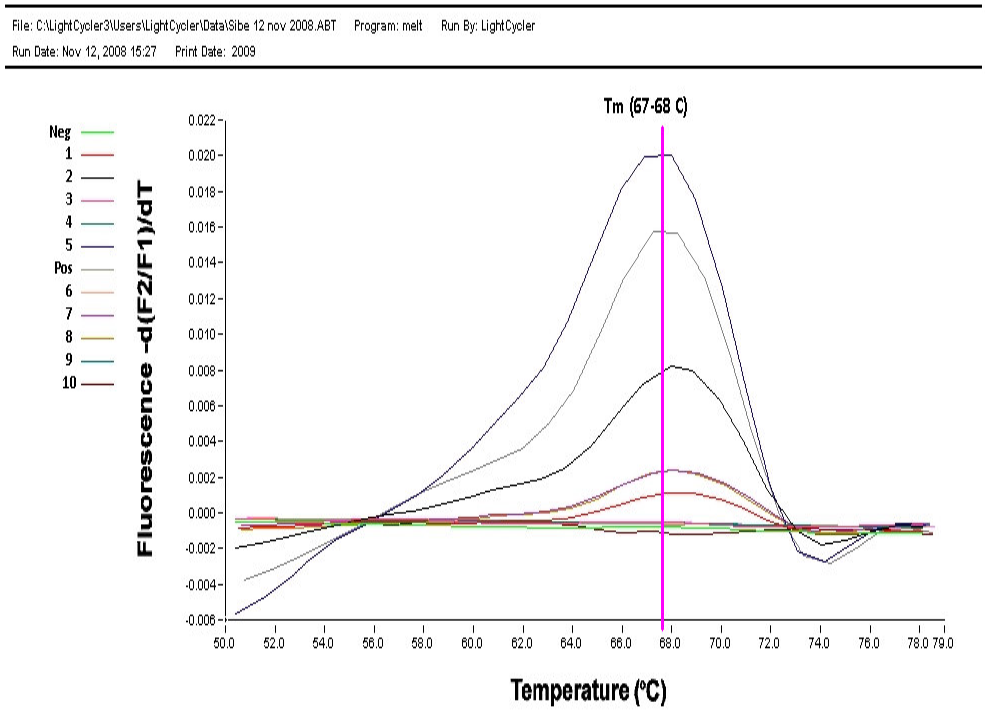


Figure 3.5 q-PCR melting curve analysis of *M. genitalium*

3.3.2.3 TRANSCRIPTION MEDIATED AMPLIFICATION

A result of ≥50 000 RLU's as detected by the luminometer was interpreted as a positive result for *M. genitalium*. TMA detected *M. genitalium* in 60 of the 375 men (16.0%). The sensitivity and specificity of the assay were 100% and 98% when compared to the extended gold standard. The positive and negative predicted values were 92% and 100% respectively.

3.3.2.4 SUMMARY OF ASSAY PERFORMANCE

The sensitivities, specificities, negative and positive predictive values of the three NAATs are compared in Table 3.1.

Table 3.1 Comparison of NAATs' performance in detection of *M. genitalium.*

Test	Sensitivity (%)	Specificity (%)	Negative predictive value (%)	Positive predictive values (%)
c-PCR	78	98	96	90
q-PCR	96	99	99	96
TMA	100	98	100	92

3.4 DISCUSSION

The purpose of this study was to evaluate three novel methods for the detection of *M. genitalium* in clinical specimens. There are many published clinical studies that have used different assays to detect the pathogen, bit the majority of these were done using conventional PCR (Jensen, 2006). In this study, we have compared a commercial PCR assay with a real-time PCR assay and a transcription mediated amplification (TMA) assay. First void urine specimens collected from men with and without signs and symptoms of urethritis were used to compare the abilities of each assay to detect *M. genitalium*.

The analysis to evaluate efficiency of assays was based on using 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 inefficient specimen left for further testing.

All three assays were highly specific (98-99%) in the detection of *M. genitalium.* However, where q-PCR and TMA demonstrated high sensitivities (96% and

100%), the sensitivity of the conventional PCR assay was low (78%). The TMA performed slightly better than the q-PCR assay, and both performed considerably better than the commercial (conventional) PCR assay.

3.4.1 Conventional PCR

In this study, a conventional PCR assay, commercially available from Sacace (Italy) was used do detect *M. genitalium* in urine specimens. The assay did not perform as well as the newer NAATs. The specificity was good (98%), but the test was not very sensitive (78%), as it missed 12 that were positive by the other NAATs. This finding was not unusual, as the test makes use of agarose gel electrophoresis to detect amplicons. This detection method is not as sensitive as methods that incorporate southern blot analyses or probe hybridization techniques.

Low bacterial loads in the specimens may contribute to the poor performance of an assay, as was shown by Jensen *et al*, 2004b. In their study, comparing urine and urogenital swab specimens for the detection of *M. genitalium* by PCR, it was shown that 28% of swab specimens and 14% of urine specimens contained less than 10 genome equivalents of *M. genitalium* DNA. According to the manufacturers of the assay used in the current study (Sacace Biotechnologies, Italy), the kit allows for the detection of *M. genitalium* DNA in 100% of the tests

with a sensitivity of not less than 1000 copies/ml. Of the 12 specimens that were negative, 2 were from asymptomatic men, and the remaining 10 from men with BOM only, without microscopic evidence of urethritis. This suggests that the bacterial loads were probably too low for the kit's detection limits.

The PCR test that we used, targets the *MgPa* gene, which have been used in numerous PCR assays (Table 2.3) as the adhesin gene was thought to be rather conserved owing to the fact that the attachment protein is vital in the pathogenesis of mycoplasmas. It was later found that the region of *MgPa* flanked by the MgPa 1/MgPa 3 primers first used, appeared variable, with differences between the prototype strains (G-37 and M-30) and clinical strains (Jensen *et al* 1991). This problem was subsequently addressed by various authors using different primer sequences, with varying results (Jensen, 2006). In 2003, Jensen *et al* compared a hemi-nested PCR assay using a modification of the original MgPa-1 primer, with a PCR assay targeting the 16S rRNA of *M. genitalium* in urethral swab specimens. The 16S rRNA primers detected only one more positive than the MgPa primers (97.5% agreement). As with most commercial assays, the exact primer sequences used in the kit are not known, and it may be that the target falls within a variable region of the gene. If there is a mutation in the primer binding area, a false negative result will be generated. Besides low copy numbers, this may also account for the 12 specimens detected by the other two NAATs that were missed by the conventional PCR test.

In this study, PCR detected 5 positives that were not detected by the other two NAATs. All five were from patients with BOM only. 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. As we used the same extracted DNA for the q-PCR assay, we assumed that the contamination did not occur during specimen preparation, and only repeated the PCR assays. However, there was no change in the results. Another possible explanation for these positive results may again 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 and especially *M. pneumoniae*, its close relation, and *M. hominis*, a known colonizer of the urogenital tract (Taylor-Robinson & Furr, 1998) may have occurred. However, the question remains whether we are dealing with true *M. genitalium* positive specimens, missed by the other two NAATs.

3.4.2 Real-time PCR

Real-time PCR has revolutionized the way clinical microbiology laboratories diagnose many human microbial infections. This testing method combines PCR with fluorescent probe detection of amplified product in the same reaction vessel,

minimizing the risk of releasing amplified nucleic acids into the environment. In general, both PCR and amplified product detection are completed in an hour or less, which is considerably faster than conventional PCR detection methods.

In this study, the q-PCR performed well compared to the other two assays, and even compared to the TMA test. Although its sensitivity was lower than that of the TMA assay (96% vs 100%), the specificity of this test was higher (99% vs 98%). In this study a q-PCR assay targeting the housekeeping gene *gap,* encoding glyceraldehyde-3-phosphate-dehydrogenase of *M. genitalium,* as described by Svenstrup *et al* (2005) was used. The *gap* gene was chosen by the authors, as sequencing of the gene revealed no variation between the prototype strains and 4 clinical isolates. Furthermore, there is only a single copy of the gene in the genome (ideal for quantification assays), and it is different from other species, including its close relation, *M. pneumoniae* with only 72.3% homology (Fraser *et al,* 1995).

In this study, the q-PCR test did not detect 2 positive specimens that were found by the other two NAATs. Both were from patients with BOM only. In the study by Svenstrup *et al* (2005) 246 urethral swab specimens were tested with the LightCycler *gap* gene assay as well as with a Taqman q-PCR assay targeting the adhesion gene. Of these 246 specimens, 82 were randomly selected among those positive for *M. genitalium* and 164 from those found negative by conventional PCR. The *gap* assay missed 4 of the positives and the Taqman

only 1. However, the copy numbers in those specimens were low (<3 copies/µl). Furthermore, the LightCycler assay is performed in a smaller volume (20 µl) compared to the Taqman assay (50 µl) allowing for more template to be tested. The use of a larger test volume may be beneficial, but the cost of the test increases as more reagents are needed, and there may not be sufficient material for re-testing.

Chen *et al* (2009) evaluated 5 possible *M. genitalium* targets in Taqman real-time PCR assays, of which the *gap* gene was one. Compared to the other target genes (adhesin, DNA polymerase, dihydrolipoamide dehydrogenase and lactate dehydrogenase), the *gap* gene assay yielded a slightly lower fluorescence signal, but all the assays had similar analytical sensitivities and specificities showing no cross reaction with other mycoplasmas or urogenital pathogens.

In this study, *M. genitalium* was detected by the q-PCR 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 both Svenstrup *et al* (2005) and Chen *et al* (2009) 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.

3.4.3 Transcription Mediated Amplification

PCR assays are all based on the amplification of DNA using a DNA polymerase, while TMA amplifies RNA in a DNA background using three different enzymes, reverse transcriptase, RNaseH and RNA polymerase. TMA assays have been shown to be highly sensitive and specific for the detection of sexually transmitted pathogens (Levett *et al,* 2008: Chernesky *et al*, 2009). The TMA assay developed by Gen-Probe Inc (USA), targets rRNA, a molecule present in multiple copies per cell, thereby increasing the sensitivity of detection relative to the sensitivities of PCR assays that target single-copy genes (Wroblewski *et al*, 2006).

In this study, TMA performed well compared to the other two assays. There were no positives detected by the other assays both, which were not detected by the TMA assay. Although its specificity was lower than that of the q-PCR assay (98% vs 99%), TMA was highly sensitive compared to the other assays (100% vs 96% and 78%). This may be expected, as the target concentration (RNA) is so much higher than that of DNA, the target for the PCR assays (Klein, 2002). 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.

Hardick *et al* (2006a) compared the performance of the Gen-Probe TMA research assay to that of a multitarget real-time PCR for *M. genitalium* detection in urine samples from male patients. The TMA assay also showed higher sensitivity than the q-PCR assay (98.1% vs 91.8%), and slightly lower specificity (98.1% vs 99.5%). Wroblewski *et al* (2006) compared the TMA assay with a conventional PCR assay for the detection of *M. genitalium* in various genital specimen types. The TMA and PCR results were concordant for 98.2% of vaginal swab specimens, 97.6% of cervical swab specimens and for 96% of the urine specimens from men, with the TMA tests detecting more positives than the PCR test.

In this study, the TMA assay detected 5 positives that were not detected by the other two NAATs. Of these, 4 specimens were from men with BOM only, and one from an asymptomatic man, 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,* 2006a; Wroblewski *et al*, 2006). The discrepant results may also have been caused by contamination, and therefore the assay was repeated from the original specimens. The results were unchanged. Unfortunately, as the specimens may have been contaminated during

transferring of the urine to the transport tubes, repeating the test in this instance will not resolve the matter.

None of the assays used in this study included an internal amplification control for inhibition, allowing for false negative results. The PCR kit from Sacace allows for the use of an internal control, but as it needs to be included during the specimen preparation stage, using a Sacace specimen preparation kit, it was not used in this study. The specimens for PCR were all prepared using the same kit (Roche AMPLICOR® CT/NG Specimen preparation kit). 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). However, a study by Jang *et al* (2005) has shown that inhibition may still occur, albeit in a small proportion (≤1%) of patients. Their study was performed on women, and it has been reported that urine inhibition may play a bigger role in specimens from females than from males, especially for the testing of *N. gonorrhoeae.*

In our study, both the TMA and real-time PCR assays performed well and can be used in clinical 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 4

AETIOLOGICAL AGENTS OF URETHRITIS IN SYMPTOMATIC SOUTH AFRICAN MEN

4.1 INTRODUCTION

Urethritis is one of the commonest sexually transmitted conditions in heterosexual men, and is conveniently classified as gonococcal or non-gonococcal urethritis (NGU), depending on the presence or absence of *Neisseria gonorrhoeae*. It has been reported that adult male urethritis comprises 60% of STI presentations in the Gauteng province of South Africa (Lewis *et al*, 2007).

The aetiological role of *Chlamydia trachomatis* in NGU is well established (Martin & Bowie, 1999). However, in Chlamydia-negative NGU, the aetiology is not completely understood. Pathogens that have been implicated in Chlamydia negative NGU are *Trichomonas vaginalis* (Pépin *et al,* 2001; Bradshaw *et al*, 2006a) and *Mycoplasma genitalium* (Horner *et al*, 2001). The eoidemiology of *M. genitalium* in NGU world-wide has been discussed in Chapter 2 (Sections 2.4 and 2.5).

Besides *M. genitalium* and *T. vaginalis*, Yokoi *et al* (2007) have also demonstrated the presence of *Ureaplasma urealyticum biovar 2* in symptomatic men; however, the pathogenic role of the ureaplasmas is still unclear. Viruses such as adenoviruses and herpes simplex virus-1 have also been implicated in the aetiology of NGU (Bradshaw *et al*, 2006a; Martin 2008).

At healthcare facilities in South Africa, syndromic management for STDs is widely practiced. Guidelines for the syndromic management of STDs for controlling sexually transmitted infections in South Africa were first formulated in 1996 by the National Department of Health (DOH, 1996). Formulating these guidelines requires knowledge of the aetiologies of the common clinical syndromes and antimicrobial susceptibility profiles of the prevalent pathogens (Ballard *et al*, 2002).

4.1.1 Aetiology of urethritis in South African men

Few studies on *M. genitalium* and its role in NGU, using molecular diagnostic techniques have been performed 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 clinician 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, and ligase chain reaction (LCR) assays (Abbott Laboratories, USA) were used to test for *C. trachomatis* and *N. gonorrhoeae*. *N. gonorrhoeae* was detected in 47.3% of symptomatic and 8.6% of asymptomatic men. *M. genitalium* was detected in 16.7% and *C. trachomatis* in 16.7% of the 96 men with NGU, and both organisms were detected in 8.9% men without clinical symptoms. *M genitalium* and *C. trachomatis* were detected simultaneously in less than 1% of men with or without symptoms of urethritis. These researchers did not test for *T. vaginalis*.

Ballard *et al*, (2002) studied the coexistence of urethritis with genital ulcer disease in South African men, and recruited 186 mine workers with genital ulcers in Carletonville. Chlamydial and gonococcal infections were diagnosed by LCR, and *M. genitalium* by PCR in Copenhagen. Although none of the men complained of urethritis symptoms, 99 (53%) of the men were diagnosed with urethritis using microscopy (>5 PMNLs/hpf). In these men with urethritis, 45.4% were infected with *N. gonorrhoeae* and among those with NGU, 18.0% 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 urethritis with microscopy) and 100 asymptomatic men in their study. Urethral swab specimens were collected and tested for *N. gonorrhoeae* and *C. trachomatis* using a strand displacement assay

(BD ProbeTec™ET). *M. genitalium* and *T. vaginalis* were detected by in-house PCR tests. *N. gonorrhoeae* was detected in 52% of the patients and not in any of the controls. *Chlamydia trachomatis*, *M. genitalium* and *T. vaginalis* were detected in 16%, 5% and 6% of patients and in 8%, 3% and 12% of controls respectively.

In a more recent study from Johannesburg by Black *et al* (2008), 430 men with male urethritis syndrome (MUS) attending a STI clinic were recruited. The diagnosis was confirmed with microscopy, and urine specimens were collected for laboratory testing. Chlamydial and gonococcal infections were diagnosed by PCR (Roche Amplicor®) and *M. genitalium* and *T. vaginalis* by in-house real-time PCR assays. *N. gonorrhoeae* was the commonest organism (62.3%) followed by *C. trachomatis* (19.3%), *M. genitalium* (14.4%) and *T. vaginalis* (4.9%).

4.1.2 Diagnosis of urethritis

The diagnosis of urethritis traditionally requires microscopic examination of Gram stained endourethral smears where the visualisation of five or more polymorphonuclear leukocytes (PMNLs) per high power field (hpf) is considered as positive (Desai & Robson, 1982). However, urethral pathogens are often detected in symptomatic men with less than 5 PMNLs/hpf, especially when sensitive NAATs are used (Janier *et al*, 1995; Haddow *et al*, 2004).

Epidemiological studies of NGU frequently restrict enrollment to men with signs and symptoms of urethritis confirmed with microscopy.

In the study by Bradshaw *et al* (2006a), the aetiology of NGU was studied by enrolling men with urethritis symptoms (329), regardless of microscopy, and 307 asymptomatic control subjects. It was found that laboratory evidence of urethritis was associated with the presence of urethral discharge. *M. genitalium* (11%) and *C. trachomatis* (22%) were most frequently detected in this group and if microscopy was used as sole criterion for urethritis, a significant proportion of *C. trachomatis* (32/73 (32%)) and *M. genitalium* (14/38 (37%)) would not have been diagnosed.

The use of the Gram-stained urethral smear for the diagnosis and management of urethritis has been questioned (Landis *et al*, 1988; Janier *et al*, 1995) It was shown that in studies of NGU in which enrollment is not limited to microscopic diagnosis, more pathogens are detected.

4.1.3 Aims of the study

This study was done to determine the occurrence of urethral pathogens in urine specimens obtained from symptomatic and asymptomatic South African men attending a family practice in Pretoria, Gauteng Province.

4.2 MATERIALS AND METHODS

4.2.1 Patients and Specimens

Men presenting to a family practitioner in Pretoria, South Africa, with urethral discharge and/or complains of burning on micturition (BOM) were recruited who did not receive antimicrobial therapy in the preceding month, and as control group men without symptoms that did not receive any antimicrobial treatment in the preceding month were included (see Chapter 3 (Section 3.2.1)). This study was carried out between August 2007 and June 2008 and the recruited patients gave verbal consent for participation. After a general examination, an endourethral swab specimen was taken from each patient for microscopic examination following Gram staining. Thereafter a first catch urine sample was collected for transcription mediated amplification (TMA) testing into a urine collection tube (Gen-Probe Inc, USA). The specimens were delivered to the laboratory within 24 hours.

4.2.2 Microscopy

Microscopic examination of Gram stained smears was performed by two laboratory microscopists independently, and discrepant results were resolved by a third microscopist. Urethritis was diagnosed when five or more

polymorphonuclear leukocytes (PMNLs) were present per high power field (hpf; x1000 magnification) (Desai & Robson, 1982).

4.2.3 Molecular detection of pathogens

Urine samples were tested for *N. gonorrhoeae* and *C. trachomatis* using the APTIMA® Combo2 assay (Gen-Probe Inc, USA) following the manufacturer's instructions. *T. vaginalis and M. genitalium* were tested by the transcription-mediated amplification-based analyte-specific-reagent (ASR) testing (Gen-Probe Inc, USA) as described previously (Chapter 3, Section 3.2.4.3). TMA values >30000 relative light units (RLUs) were considered positive for *T. vaginalis*, and > 50000 RLUs positive for *M. genitalium.* Purified nucleic acids from a laboratory *T. vaginalis* isolate, and *M. genitalium* genome (ATCC G-37, 33530D) were used as positive controls in the research TMA assays (Gen-Probe Inc, USA).

4.2.4 Data analysis

The specimens were grouped based on clinical and microscopic evidence of urethritis. The results of the presence or not of pathogens in cases and controls were entered into a 2x2 table and the Chi square test was used to calculate the statistical significance of any association found.

4.3 RESULTS

4.3.1 Study population

Three hundred men with signs and/or symptoms of urethritis, and 75 asymptomatic men were enrolled. Of the symptomatic men, 94 were patients with visible urethral discharge, and the remaining 206 patients complained of BOM without visible discharge on examination. The ages of the men with urethritis ranged from 19-64, with the mean of 37.0 years. The mean age of the asymptomatic men was 36.5 years (range 17 – 65 years).

4.3.2 Microscopy

Microscopic evidence of urethritis was seen in a total of 54 patients; 35 (37.7%) from patients with discharge (n=94) and 19 (9.7%) from those with BOM only (n=206). Discrepant results were resolved in 6 cases by the 3rd microscopist. None of the asymptomatic men displayed microscopic evidence of urethritis.

4.3.3 Urethral organisms detected in urine specimens

Among the 300 symptomatic men, one or more pathogens were detected in 129 (43%) men. *M. genitalium* was the most frequently detected pathogen, 52

(17.3%) followed by *N. gonorrhoeae,* 50 (16.7%), *C. trachomatis,* 37 (12.3%) and *T. vaginalis,* 24 (8.0%). In the asymptomatic group (75) one or more pathogen were detected in 14 (18.6%) men, with *N. gonorrhoeae* found in 2 (2.6%), *C. trachomatis in* 4 (5.1%), *T. vaginalis* in 1 (1.3%) and *M. genitalium* in 7 (9.0%). The results obtained in this study were compared to that found in previous South African studies, and is shown in Figure 4.1.

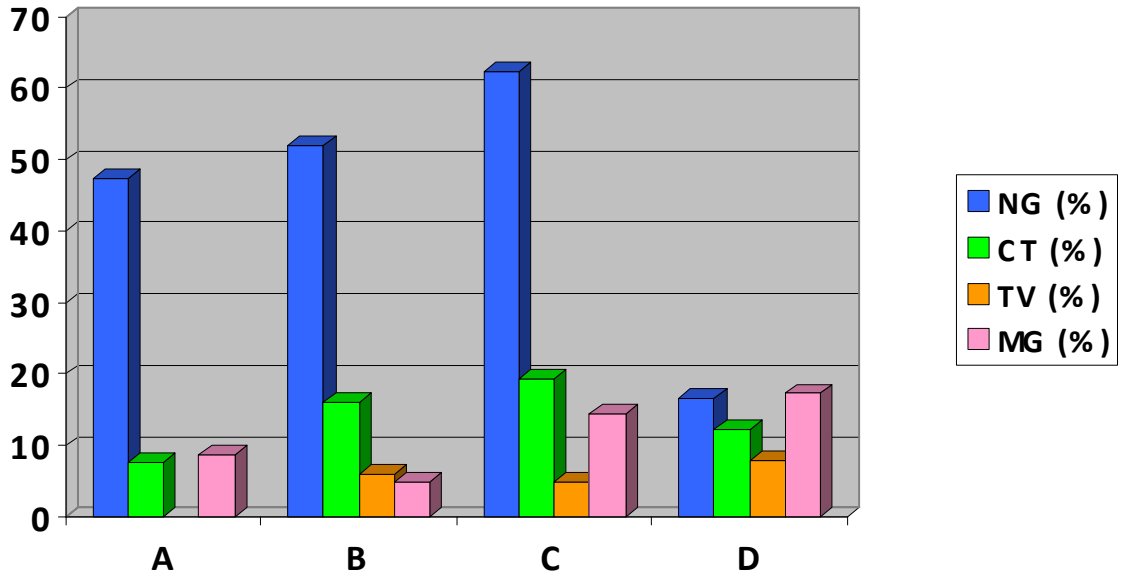


Figure 4.1 Aetiology of urethritis in South African studies.

Where:

A Taylor-Robinson *et al*, 2002; 182 men with signs and symptoms of urethritis, Johannesburg, Gauteng

B: Sturm *et al* 2004; 341 men with male urethritis syndrome, Durban, KwaZulu-Natal

C: Black *et al*, 2008; 430 men with male urethritis syndrome, Johannesburg, Gauteng

D: This study; 300 men with signs and symptoms of urethritis, Pretoria, Gauteng

All the pathogens were significantly less detected in the asymptomatic group (*N. gonorrhoeae* p<0.001; *C. trachomatis* p = 0.04; *T. vaginalis* p = 0.03; *M. genitalium* p = 0.04). A comparison of pathogens detected in the two groups is shown in Figure 4.2.

Figure 4.2 Urethral pathogens detected in men with and without symptoms of urethritis.

4.3.3.1 RESULTS OF PATHOGENS DETECTED BASED ON CLINICAL SYMPTOMS

The breakdown of the pathogens based on clinical symptoms is shown in Figure 4.3. *N. gonorrhoeae* was detected in 42 (44.6%) of the 94 patients with discharge, followed by *M. genitalium* 26 (27.7%), *Chlamydia trachomatis* 18 (19.1%) and *T. vaginalis* 6 (6.4%). Of these, 19.1% occurred as mixed infections. Among the 206 patients without discharge, *M. genitalium* was detected most

frequently 26 (12.7%), followed by *Chlamydia trachomatis* 19 (9.2%), *T. vaginalis* 18 (6.3%) and *N. gonorrhoeae* 8 (3.8%) Mixed infections were seen in only 2.9%. There were significant differences for the detection of *N. gonorrhoeae*, *C. trachomatis* and *M. genitalium* but no significant differences for *T. vaginalis* detection between these two groups.

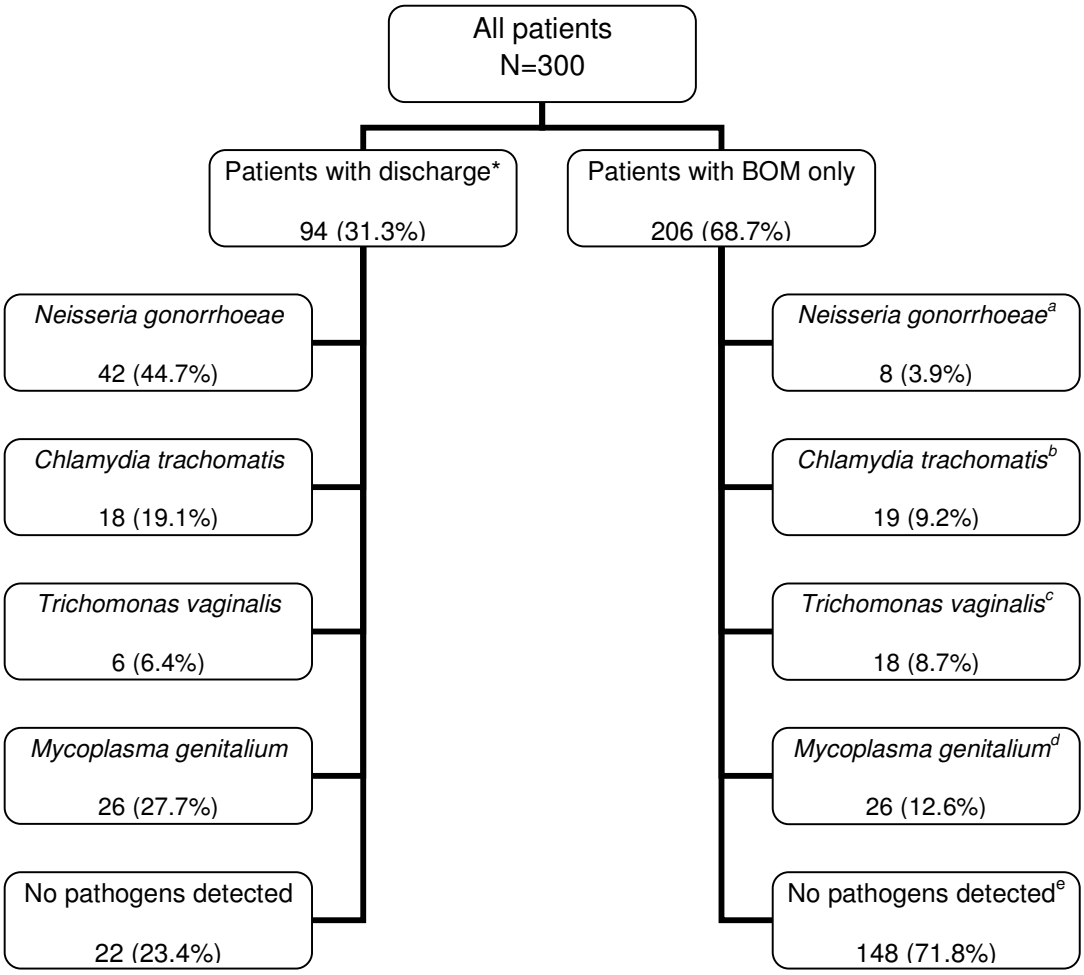


Figure 4.3 Pathogens detected according to symptoms (n=300).

*with or without BOM; [a]p<0.001; [b]p = 0.02; [c]p = 0.6; [d]p = 0.002; [e]p<0.001

4.3.3.2 RESULTS OF PATHOGENS DETECTED BASED MICROSCOPY

Figure 4.4 shows the pathogens in relation to microscopic evidence of urethritis. Only *N. gonorrhoeae* and *C. trachomatis* were significantly associated with microscopic evidence of urethritis.

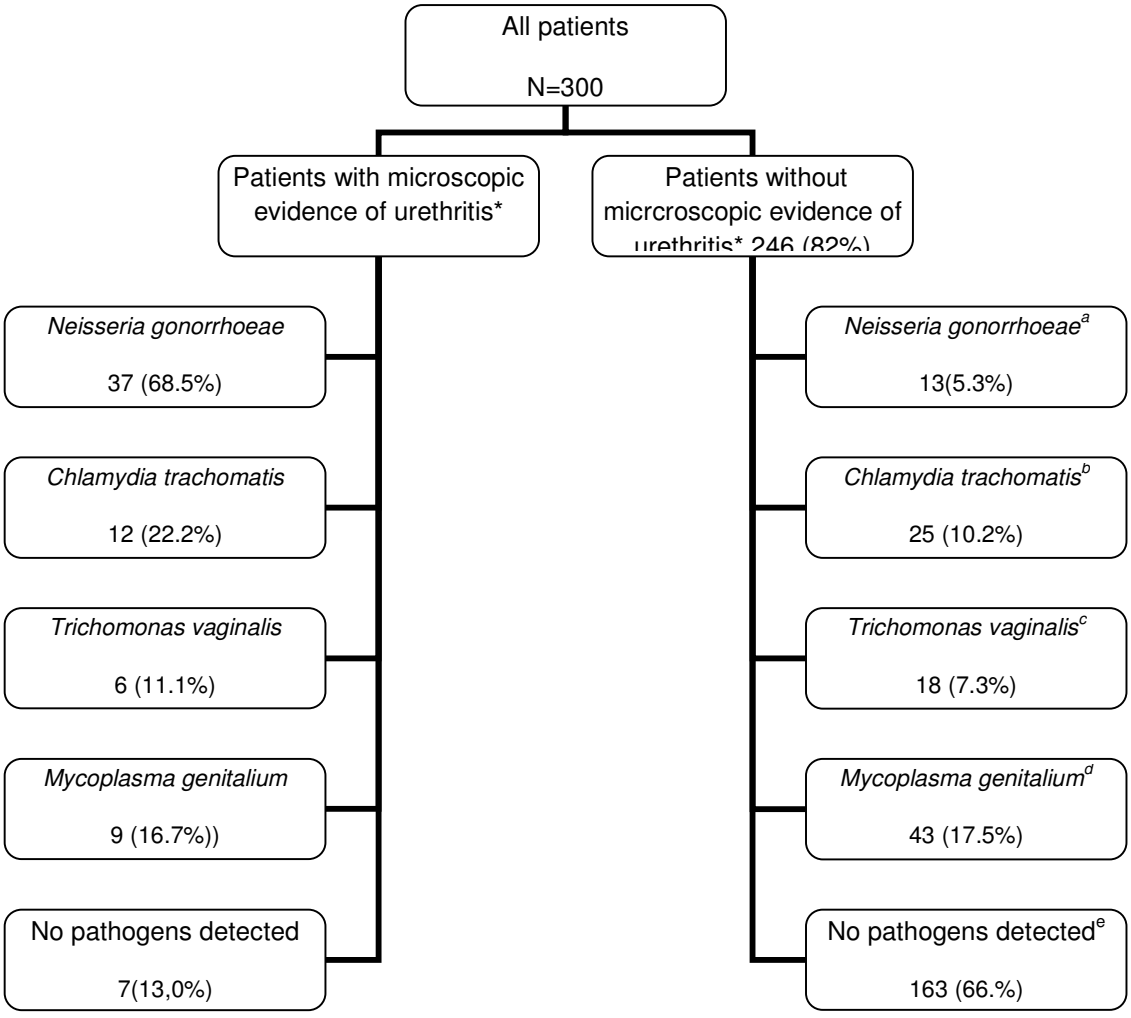


Figure 4.4 Pathogens in relation to microscopic evidence of urethritis (n=300).

*>4 PMNLs/hpf; [a]p<0.001; [b]p = 0.02; [c]p = 0.4; [d]p = 1.00; [e]p<0.001

4.3.3.3 MIXED INFECTIONS

Mixed infections were seen in 9.3% (28/300) of all the specimens from symptomatic men and in 1 asymptomatic man. It was significantly more seen in those with discharge (19/94; 20.2%) than in those with BOM only (9/206; 4.4%). The most common mixed infection was with *N. gonorrhoeae* and *M. genitalium* (11/300; 3.7%) followed by *N. gonorrhoeae* and *C. trachomatis* (6/300; 2.0%). The asymptomatic man was infected with *C. trachomatis* and *T. vaginalis.*

4.4 DISCUSSION

This study evaluated urethral pathogens according to a clinical diagnosis of urethritis based on clinical signs and symptoms and laboratory based diagnosis on microscopic evidence of the presence of inflammatory cells.

In keeping with the findings of many studies from developed and developing countries there is a high burden of urethritis pathogens amongst men presenting with urethritis be it with complaints of urethral discharge with or without BOM. *M. genitalium* was the commonest pathogen detected (17.3%) in all patients studied and was found in a higher percentage of men with NGU than *C. trachomatis*. In men with visible discharge, *Neisseria gonorrhoeae* was the most frequently (44.6%) detected pathogen. This is in keeping with the findings of most studies.

All the pathogens were significantly less frequently detected in the asymptomatic group; a finding that strengthens the case for *M. genitalium's* role in causing urethritis in men. Despite the use of the sensitive TMA assays employed in this study, no pathogen could be diagnosed in 57% of all patients studied. However, it must be emphasized that we did not test for other possible less frequent causes of urethritis e.g. genital herpes, adenoviruses and ureaplasmas.

Only a few studies on the aetiology of male urethritis which included *M. genitalium* have been carried out in South Africa (Taylor-Robinson *et al*, 2002; Sturm *et al*, 2004; Black *et al*, 2008). In all these studies, *N. gonorrhoeae* and *C. trachomatis* were the most commonly isolated organisms. In this study *N. gonorrhoeae* was detected in only 16.7% of all patients which is much lower than that in the above mentioned South African studies (47.2% to 62.3%). However, in patients with urethral discharge *N. gonorrhoeae* was diagnosed in 44.6% of patients in our study, a prevalence rate similar to that reported from our other South African studies. In previous studies conducted in the Pretoria region and reported from our center, *N. gonorrhoeae* was found in 39.0% and 44.7% of men with urethritis (De Jongh *et al*, 2007; 2009).

C. trachomatis was detected in 12.3% of all patients and this falls within the same range as reported by the other South African studies (7.7% to 19.3%). In the 1996 STD review of South African studies by Pham-Kanter *et al*, prevalences of 12 -13% were reported. These studies used various diagnostic methods such

antigen detection and culture for diagnosing chlamydial infection whilst our study used the TMA assay.

T. vaginalis was seen more frequently in this study at 8.0% compared to 4.9% to 6.0% in the other studies. This was also higher than in a study in Pretoria (De Jongh *et al*, 2009), where *T. vaginalis* was found in 5.5% of men with signs and symptoms of urethritis. *M. genitalium,* the most commonly detected pathogen in all patients in this study, was also more prevalent (17.3%) than that reported in the other South African studies (5.0% to 14.4%).

Multiple infections in males with urethritis have been reported previously in South Africa (Taylor-Robinson *et al*, 2002; Black *et al*, 2008; De Jongh *et al,* 2009). In this study, multiple infections were seen in 9.3% of patients, with a combination of *N. gonorrhoeae* and *M. genitalium* the most common.

N. gonorrhoeae, *C. trachomatis* and *M. genitalium* were detected significantly more in men with urethral discharge, whilst *N. gonorrhoeae* and *C. trachomatis* were significantly more associated with urethritis based on microscopic evidence of urethritis. There was no significant difference for the detection of *M. genitalium* or *T. vaginalis* whether there was microscopic evidence or not. This is contrary to findings reported for European studies where *M. genitalium* has been shown to be associated with microscopic evidence of urethritis (Ross & Jensen, 2006).

However, this is similar to the results obtained by Bradshaw *et al* (2006a) in Australia. In developing countries including South Africa healthcare practitioners do not have the time, nor the skills to define urethritis based on microscopy, and mainly use symptoms for diagnosis and therapy. As shown in this study, if microscopy was used as the only criterion for diagnosis, many *T. vaginalis* and *M. genitalium* infections would have been missed. The value of the Gram-stained urethral smear for the management of men with urethritis is limited (Landis *et al,* 1988, Bradshaw *et al,* 2006a) and this study concurs with that finding.

Patient urine collected in the Gen-Probe tubes proved to be an easy and convenient specimen to analyse. The urine is stable for 28 days at room temperature, and can be used to test for all four pathogens. The TMA assays make use of magnetic beads and a target capture system before amplification to exclude inhibition, eliminating the cumbersome incorporation of individual amplification controls (Chong *et al*, 3003). However, a study by Jang *et al* (2005) showed that inhibition may still occur, albeit in a small proportion (≤1%) patients compared to the 12% occurring with the Cobas AMPLICOR® assay. The latter study was performed on women, and it has been reported that urine inhibition plays a bigger role in specimens from females than in males, especially for the testing of *N. gonorrhoeae (*Mahoney *et al,* 1998*;* Martin *et al*, 2000).

The Gen-Probe APTIMA® assays for the detection of *N. gonorrhoeae* and *C. trachomatis* in urine specimens have been evaluated and compared to other

commercial assays and showed high sensitivities and specificities. For *C. trachomatis* sensitivities and specificities ranged from 92.1 to 100% and 98.9 to 100% respectively, and for *N. gonorrhoeae* 98.5 to 100% and 91.3 to 100% (Moncada *et al*, 2004; Lowe *et al*, 2006; Levett *et al*, 2008). Sensitivity and specificity of the TMA assay for detection of *T. vaginalis* have been reported as 98.6% and 99.1% with the TMA assay identifying more positives than a real-time PCR assay, indicating that the TMA assay is more sensitive (Hardick *et al*, 2006b). TMA was also shown to be a reliable test for detecting *M. genitalium* in urine specimens as was discussed in Chapter 3 of this thesis.

This study highlighted the necessity of epidemiological studies of this nature for the proper understanding of the aetiology of adult male urethritis. Currently the syndromic management for adult male urethritis in this country consists of a single dose cefixime (400mg, oral) and a 7 day course of doxycycline (100mg twice daily, oral). Although doxycycline is the antibiotic of choice for the treatment of *C. trachomatis*, it has become apparent that *M. genitalium* often persists in the urethra of infected men after treatment (Falk *et al*, 2003). Azithromycin as a single dose has become the antimicrobial of choice for the treatment of male urethritis in some countries (Bjornelius *et al*, 2008). However, Jensen *et al* (2008) have reported the emergence of resistance of *M. genitalium* to azithromycin, and advocate a longer duration treatment with azithromycin for up to 7 days.

In clinical settings such as ours where microscopy is not used at all for the management of adult male urethritis, this study has demonstrated that the treatment of patients with BOM should be re-visited. In patients with visible urethral discharge the current therapeutic regimen appears appropriate. However, for patients presenting with BOM only in the absence of urethral discharge the administration of a long course azithromycin plus metronidazole may be appropriate as this combination will cover the commoner pathogens such as *M. genitalium, C. trachomatis* and *T. vaginalis.* Such a regimen will be cost effective and prevent the development of antimicrobial resistance by removing selective pressure.

CHAPTER 5

QUANTITATIVE DETECTION OF *MYCOPLASMA GENITALIUM* IN SOUTH AFRICAN MEN WITH AND WITHOUT SYMPTOMS OF URETHRITIS

5.1 INTRODUCTION

Due to the difficulties in isolating *M. genitalium* from clinical samples, only a few studies investigating the role of the organism in human disease had been undertaken before the development of PCR-based assays (Taylor-Robinson *et al*, 1985a; Samra *et al*, 1988). Since the development of nucleic acid amplification tests (NAATs), a significant association of *M. genitalium* and non-gonococcal urethritis (NGU) has been demonstrated (Jensen *et al,* 1993; Maeda *et al,* 2001; Totten *et al*, 2001).

With the introduction of quantitative real-time PCR (q-PCR) assays, the *M. genitalium* load in the urogenital tract of symptomatic men can be determined (Yoshida *et al,* 2002a). The assessment of bacterial loads in specimens from symptomatic and asymptomatic men can provide useful information for understanding the pathogenic role of *M. genitalium* in the urogenital tract. Quantitative detection of the organism in patients following antimicrobial

treatment can be useful to demonstrate response to therapy and to determine the susceptibility of the organism especially where culture is difficult.

5.1.1 Quantification using real-time PCR technology

The introduction of real-time PCR technology has significantly improved and simplified the quantification of nucleic acids and has been widely used by scientists for molecular diagnostics. The wide use of real-time PCR methods has highlighted some of the critical points and limitations of these molecular assays (Klein, 2002).

As described in Chapter 3 (Section 3.1.3.1), four different principles are commonly applied for real-time PCR detection. All four methods are based on the measurement of fluorescence during the PCR reaction (Bustin, 2005). As the amount of emitted fluorescence is proportional to the amount of PCR product, the PCR reaction can be monitored. This linear correlation between PCR product and fluorescence intensity can be used to calculate the amount of template present at the beginning of the reaction (Klein, 2002).

The least suitable method for quantification studies is the use of double-stranded DNA-binding dyes to measure fluorescence, as these studies require careful optimization to eliminate non-specific PCR product detection (Bustin *et al*, 2005).

The three other principles, based on the introduction of an additional fluorescence-labelled oligonucleotide are useful in quantification studies (Bustin, 2005). Sufficient amounts of fluorescence are only released either after cleavage of the hydrolysis probes (Heid *et al,* 1996) or during hybridization of oligonucleotide probes to the amplicon (Wittwer *et al,* 1997).

Real-time PCR assays are characterized by a wide dynamic range of quantification, a high technical sensitivity (< 5 copies) and a high precision (< 2% standard deviation) (Bustin, 2005). Jordan (2000) reported the development of many assays for the detection and quantification of various infectious agents (Jordan, 2000). Critical and strict evaluation of newly developed diagnostic assays is a prerequisite to obtain reliable data. These diagnostic assays detect and quantify nucleic acids from live and dead pathogens, whereas classical microbiological tests detect only live and in many cases replicating pathogens (Klein, 2002).

Quantification can either be relative to an external standard curve or to one or more co-amplified internal control mRNAs (Bustin, 2005). The former is based on the use of a dilution series of an external standard, which can be used to generate a standard curve of Ct (threshold cycle) against initial target copy number (Ke *et al*, 2000). The standard curve is commonly generated using a dilution series of at least 5 different concentrations of the standard (Applied

Biosystems, 2003). Genomic DNA (gDNA) and plasmids containing cloned target sequences are commonly used as standards in real-time PCR. Copy numbers of pathogens in specimens can be calculated from the linear regression of that standard curve, with the *y*-intercept giving the sensitivity and the slope giving the amplification efficiency (Bustin, 2005). A slope of –3.3 means that the PCR has an efficiency of 1, or 100% and the amount of PCR product doubles during each cycle. Ideally, the slope should be between –3.3 to –3.8 (Applied Biosystems, 2003). The accuracy of absolute quantification depends entirely on the accuracy of the standards used. In general, standard curves are highly reproducible and allow the generation of specific and reproducible results. However, it should be noted that external standards cannot detect or compensate for inhibitors that may be present in the specimens (Klein *et al*, 2002).

5.1.2 Quantitative detection of *M. genitalium* in clinical studies

The q-PCR assay developed by Yoshida *et al* (2002a), for the detection of *M. genitalium,* was able to quantify 16S rRNA gene concentrations ranging from 10 to 10^7 copies per reaction. The researchers used urine specimens from men with and without urethritis and found *M. genitalium* loads ranging from $2.3x10^2$ to $3.3x10^6$ genome copies/ml in men with NGU, compared to loads of less than $2x10^2$ genome copies/ml in asymptomatic men.

Dupin *et al* (2003) detected and quantified *M. genitalium* in urine specimens of men belonging to 3 clinical groups, namely those with microscopic evidence of urethritis, those with symptoms but without microscopic evidence and asymptomatic men. The concentration of *M. genitalium* in the specimens was significantly higher in the first group than in the other two groups, with a mean concentration of $1.2x10^4$ genome copies/ml urine. This was in agreement with a similar study conducted by Svenstrup *et al* (2005) which indicated a mean concentration of $2.5x10^4$ genome copies/µl. Jensen *et al* (2004a) showed a higher *M. genitalium* DNA load in men with urethral discharge ($2.48 x10^2$ genome copies/ml) compared to those without discharge ($1.3x10^1$). However, there was a broad overlap between these loads where the range for patients with discharge was 6 – $2.2x10^5$ and for those without 1.5 – $1.4x10^4$ genome copies/ml. This overlap was more significant in urethral than in urine specimens. Jensen *et al* (2004a) found that when comparing urethral swab and urine specimens, 28% of *M. genitalium* positive urethral swab specimens contained less than 10 genome copies/ml in the corresponding urine specimens. According to the authors, this could have been ascribed to the centrifugation and treatment steps during processing of urine specimens.

Deguchi *et al* (2002) have used q-PCR to monitor *M. genitalium* loads in men with recurrent NGU. By measuring the bacterial loads before and after treatment with levofloxacin, the researchers showed that the mean initial load of $1.54x10^6$ copies/ml was reduced to less than 50 copies/ml within 7 days after treatment

commenced. However, after 28 days, most of the men had bacterial loads comparable to the initial numbers ($1.54x10^6$ genome copies/ml) and these increased bacterial loads were associated with recurrent symptoms of urethritis (Deguchi *et al,* 2002).

5.1.3 Study problem and aims

The detection of *M. genitalium* in higher numbers in urogenital specimens from men with NGU than in asymptomatic men will strengthen the evidence that this organism is a pathogen and not a passenger. This is one of the modified Henle-Koch postulates as suggested by Taylor-Robinson in 1983.

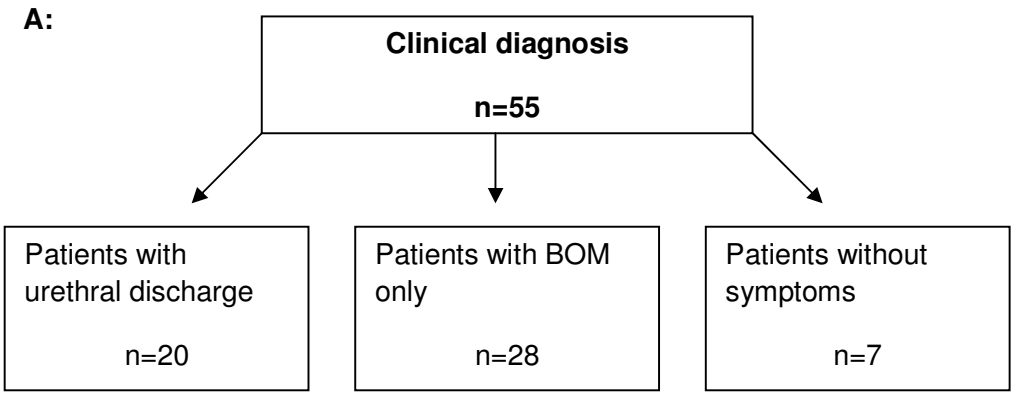
In this study we compared the *M. genitalium* loads in urine specimens from men with signs and symptoms of urethritis with that from men without any urogenital symptoms.

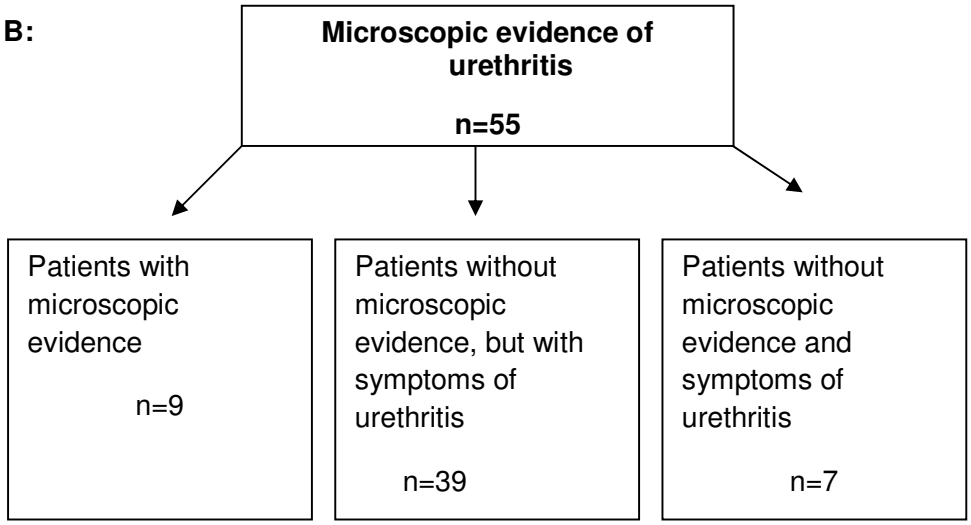
5.2 MATERIALS AND METHODS

5.2.1 Patients and Specimens

First void urine specimens were collected from men presenting to a family practitioner in Pretoria, South Africa, with signs and symptoms of urethritis, as

well as from men without symptoms. Both groups of patients had not received any antibiotic therapy in the preceding month (see also Chapters 3 (Section 3.2.1) and 4 (Section 4.2.1)). In Chapter 3 (Section 3.3.2) a total of 55 specimens tested positive for *M. genitalium* using real-time PCR (Section 3.3.2). The DNA from these 55 specimens was extracted using the Roche AMPLICOR® CT/NG specimen preparation kit (Section 3.2.2) and used for analysis. Based on clinical and microscopic evidence of urethritis the specimens were grouped as follows:





5.2.2 Q-PCR

The quantitative real-time PCR assay for the detection of *M. genitalium* targeting a 190 bp product of the *gap* gene as described by Svenstrup *et al* (2005) was used to determine the bacterial loads in the specimens (refer Chapter 3, Section 3.2.3.2 for detailed q-PCR methodology).

5.2.2.1 CREATING A STANDARD CURVE FOR Q-PCR WITH GENOMIC *M. GENITALIUM* DNA

Genomic DNA from *M. genitalium* DNA (strain G-37; 33530D) was obtained from the American Type Culture Collection (ATCC). A standard curve was prepared using 10^6, 10^5, 10^4, 10^3, 10^2 and 10 copies of the genome. This was done by calculating the mass of the organism's genome using the following equation (Applied Biosystems, 2003):

$$m = (n)\ (1.096 \times 10^{-21}\ g/bp)$$

Where: n = genome size (for *M. genitalium* 580 kbp (Fraser *et al*, 1995)).

m = mass

g = gram

This equation was derived from:

$$m = \left[n \right] \left[\frac{1 \text{ mole}}{6.023X10^{23} \text{ molecules (bp)}} \right] \left[\frac{660 \text{ g}}{\text{mole}} \right] = \left[n \right] \left[\frac{1.096x10^{-21} g}{\text{bp}} \right]$$

Where: n = genome size (bp)

m = mass

Avogadros number = $6,023x10^{23}$ molecules / 1 mole

Average molecular weight (MW) of a double-stranded DNA molecule = 660 g/mole (Applied Biosystems, 2003)

The calculated mass of 6.36×10^{-16} g was converted to 6.36×10^{-7} ng and used to determine the mass of DNA used in each dilution as shown in Table 5.1.

Table 5.1 Mass of genomic DNA needed in different copy number dilutions.

Copy number	X Mass	= Mass of genomic DNA needed (ng)
$1x10^6$	6.36×10^{-7}	6.36×10^{-1}
$1x10^5$		6.36×10^{-2}
$1x10^4$		6.36×10^{-3}
$1x10^3$		6.36×10^{-4}
$1x10^2$		6.36×10^{-5}
$1x10^1$		6.36×10^{-6}

The gDNA concentration needed, to achieve the desired copy number was determined by dividing the mass obtained in Table 5.1 by 5 µl (the volume to be pipetted into each reaction). These values are given in Table 5.2.

Table 5.2 Concentration of genomic DNA needed to achieve required gDNA mass.

Copy number	Mass of gDNA needed (ng)	/ volume in each reaction	Final concentration (ng/µl) of gDNA
$1x10^{6}$	6.36×10^{-1}	5 µl	0.13
$1x10^{5}$	6.36×10^{-2}		$0.13x10^{-1}$
$1x10^{4}$	6.36×10^{-3}		$0.13x10^{-2}$
$1x10^{3}$	6.36×10^{-4}		$0.13x10^{-3}$
$1x10^{2}$	6.36×10^{-5}		$0.13x10^{-4}$
$1x10^{1}$	6.36×10^{-6}		$0.13x10^{-5}$

The serial dilution of the 1 ng/µl gDNA solution was prepared using the following equation:

$$C_1V_1 = C_2V_2$$

Where: C_1 = Stock solution (1 ng/µl gDNA)

C_2 = Value obtained in Table 4.2

V_2 = Final volume of 100 µl (Applied Biosystems, 2003)

As V_1 was calculated to be 13 µl, a volume of 87 µl distilled water was used as diluent to achieve a final volume of 100 µl. The serial dilutions were made by adding 10µl of subsequent dilutions to 90 µl of water.

5.2.2.2 Q-PCR ASSAY

The PCR master mix was prepared as follows: 0.5 µM each primer, 0.2 µM each probe and 2 µl of LightCycler® FastStart DNA Master^{PLUS} Hybridization Probes (Roche Diagnostics) (containing hotstart *Taq* DNA polymerase, reaction buffer, $MgCl_2$ and dNTP mix (with dUTP instead of dTTP)). The volume of each reaction was adjusted with molecular grade water to 15 µl of master mix. Five µl of the appropriate standards, specimens and a negative control (water) was added. The 20 µl (final volume) reaction mixture was pipetted into glass capillaries that were filled by pulse centrifugation in a microcentrifuge (Mikro 200R, Hettich). The capillaries were loaded into the LightCycler 1.5 (Roche Diagnostics), and amplification started. The amplification conditions were enzyme activation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 8 seconds and extension at 72°C for 8 seconds. The temperature transition rate was 20°C/s and FRET from mg-gap-669FL to

mg-gap-700LC was measured in channel F2/F1 after annealing. A melting curve analysis was performed after completion of the amplification by heating samples to 95EC (20°C/s) without holding, followed by cooling to 50°C (20°C/s) for 15 seconds and slowly reheating to 95°C (0.1°C/s). A T_m of 68°C was expected for *M. genitalium* amplicons (Svenstrup *et al,* 2005).

LightCycler software 3.5 was used to generate a standard curve of the prepared standards. This was obtained by plotting the PCR cycle number against the log concentration of the standard dilutions. The graph was used by the LightCycler software to calculate the concentration of *M. genitalium* DNA in the unknown specimens. The slope of the log-linear portion of the calibration graph was calculated and expected to be between -3.3 and -3.8 (Bustin *et al*, 2009). The efficiency of the amplification was calculated using the formula: Efficiency = $-1+10^{(-1/\text{slope})}$ (Bustin *et al*, 2009). The theoretical maximum of 1.00 (or 100%) indicates that the amount of product doubles with each cycle.

5.2.3 STATISTICAL ANALYSIS

The Mann-Whitney test was used to calculate the p values to determine the relation between the median DNA load in specimens from men with and without urethritis. All statistical analyses were two-tailed and were performed with the significance set at a p of <0.05. OpenEpi was used for calculations.

5.3 RESULTS

5.3.1 Specimens tested

The bacterial loads in the 55 urine specimens that tested positive for *M. genitalium* with real-time PCR in Chapter 3 were determined.

5.3.2 Standard curve for q-PCR with genomic *M. genitalium* DNA

A standard dilution series (10^6 to 10^1 genome copies/µl) of the DNA of *M. genitalium* type strain G37 (ATCC 33530D) was amplified with the DNA from the specimens in the LigtCycler (Figure 5.2). Fluorescence curves could be detected from 10^6 to 10^2 *M. genitalium* genome copies/µl. The standard curve is shown in Figure 5.3. The slope of the standard curve was -3.543, resulting in a calculated efficiency of 91.5%. This indicated that the number of PCR products increased by 1.9 copies after each cycle.

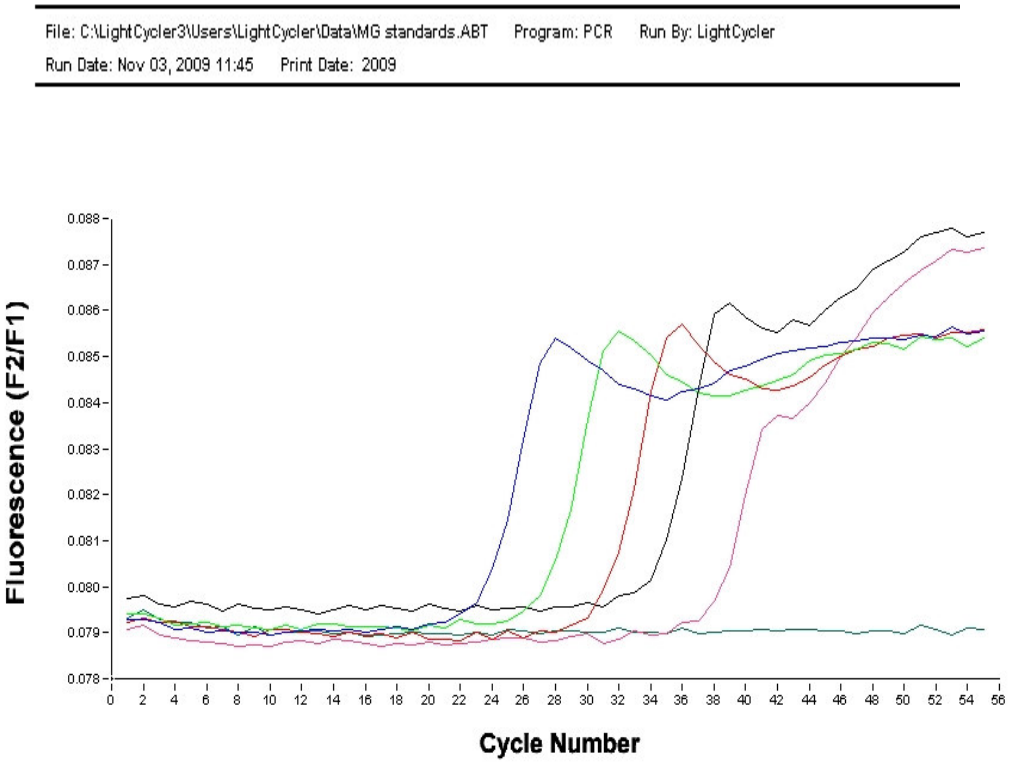


Figure 5.1 PCR amplification of serial 10-fold dilutions of the *M. genitalium gap* gene.

- $1x10^6$copies/µl
- $1x10^5$copies/µl
- $1x10^4$copies/µl
- $1x10^3$copies/µl
- $1x10^2$copies/µl
- Water

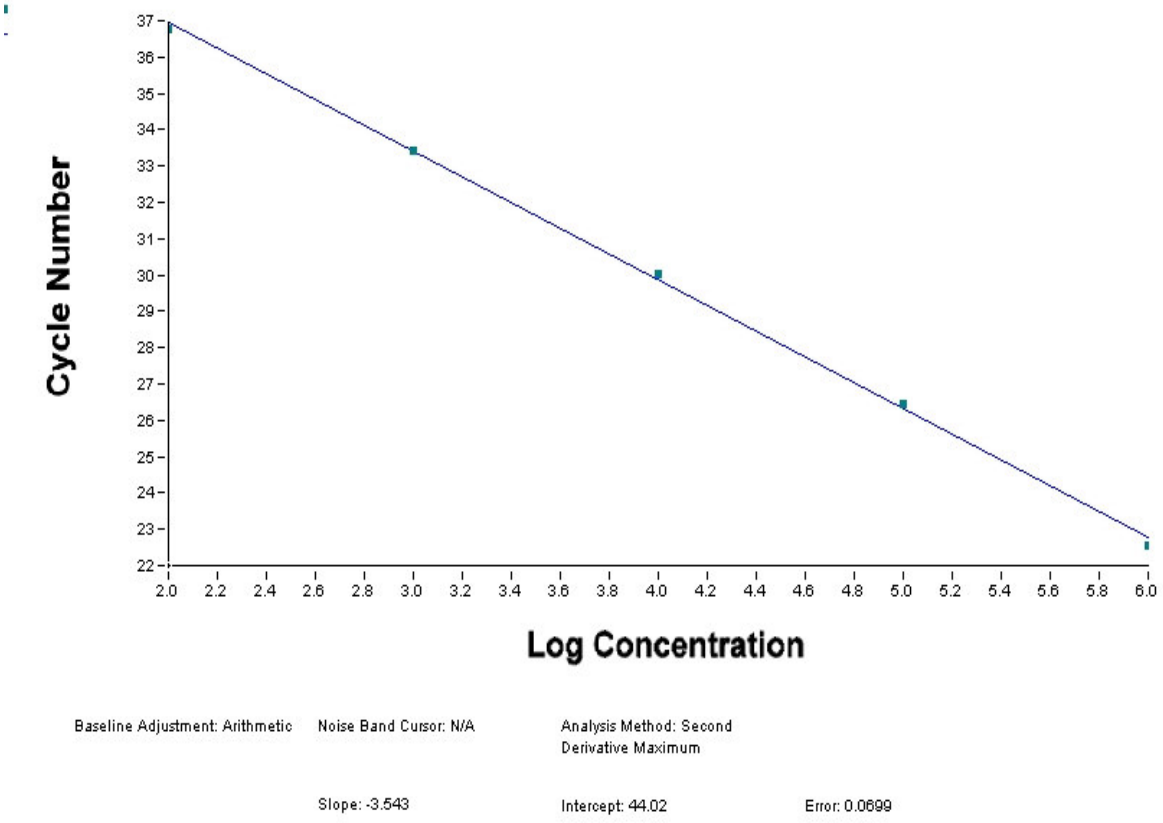


Figure 5.2 Standard curve obtained from amplification of dilution series (10^6-10^1) of *M. genitalium* DNA

5.3.3 *Mycoplasma genitalium* loads in clinical specimens

Using the standard curves obtained, the *M. genitalium* load in each specimen was determined. The bacterial loads as well as crossing points are shown in Table 5.3.

The bacterial loads obtained in *M. genitalium* positive specimens from men with urethral discharge (20), men with BOM (28) and men without symptoms (7) were compared. The highest number of organisms (mean bacterial load: $5.3x10^3$ copies/µl) was seen in men with urethral discharge, followed by those with BOM (mean bacterial load: $6.5x10^2$ copies/µl) and those without symptoms (mean bacterial load: $4.7x10^1$ copies/µl). There were significant differences between the different groups ($p<0.001$ between the discharge and BOM groups, and $p = 0.02$ between the BOM and asymptomatic groups).

The *M. genitalium* loads in the 9 urine specimens with microscopic evidence of urethritis were compared to the 39 specimens without microscopic evidence but with signs of urethritis and the 7 specimens from asymptomatic men. The highest bacterial load was seen in specimens from men with microscopic evidence of urethritis (mean bacterial load: $8.2x10^3$ copies/µl). This was significantly higher ($p<0.001$) than the loads in the specimens from men with urethritis symptoms but without microscopic evidence (mean: $1.3x10^3$ copies/µl). These patients in turn had significantly higher bacterial loads ($p<0.001$) than the asymptomatic patients (mean: $4.7x10^1$ copies/µl). In Figure 5.3 the difference in bacterial loads amongst the *M. genitalium* positive patient groups is depicted.

Table 5.3 ***Mycoplasma genitalium*** **concentrations in first void urine specimens from men with and without signs and symptoms of urethritis**

	Based on clinical diagnosis (n=55)			**Based on microscopic evidence of urethritis (n=55)**		
	Patients with urethral discharge (n=20)	**Patients with BOM only (n=28)**	**Patients without urogenital symptoms (n=7)**	**Patients with microscopic evidence (n=9)**	**Patients without microscopic evidence, but with symptoms of urethritis (n=39)**	**Patients without microscopic evidence or symptoms of urethritis (n=7)**
Bacterial load range (copies/µl)	2.9×10^2 – 2.5×10^4	4.9×10^1 – 2.9×10^3	2.5×10^1 – 6.6×10^1	1.4×10^3 – 2.5×10^4	4.9×10^1 – 1.8×10^4	2.5×10^1 – 6.6×10^1
Mean bacterial load (copies/µl)	5.3×10^3	6.7×10^2	$4.6X10^1$	8.2×10^3	1.3×10^3	$4.6X10^1$
Crossing point range (°C)	27.6 – 33.5	30.2 – 36.3	35.7 – 37.0	27.6 – 31.2	37.9 – 36.3	35.7 – 37.0
Mean crossing point (°C)	30.6	33.3	36.2	29.6	32.9	36.2

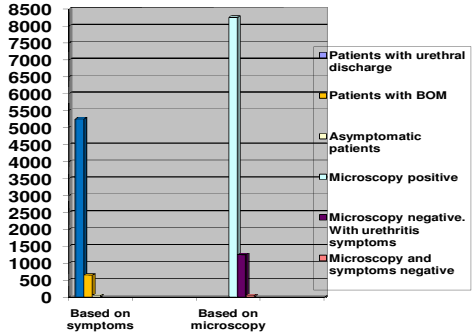


Figure 5.3 Differences in bacterial loads amongst the *M. genitalium* positive patients.

5.4 DISCUSSION

Several studies have demonstrated that *M. genitalium* is more closely associated with symptomatic urethritis than with asymptomatic urethritis, particularly in patients with urethral discharge (Horner *et al*, 2001). The detection of the organism in higher numbers in specimens from patients with NGU than in those without would contribute to the evidence that *M. genitalium is* a cause of NGU.

In this study a q-PCR assay was used to determine the *M. genitalium* DNA load in men with and without urethritis based on either microscopy or clinical signs and symptoms. Genomic DNA from the *M. genitalium* ATCC G-37 strain was serially diluted to obtain a standard curve from which the bacterial loads in the specimens could be calculated. The assay amplified the *gap* gene, which appears once in the genome (Svenstrup *et al*, 2005). This gene was used to determine the genome copies in the urine specimens obtained from men that tested positive for *M. genitalium*.

This q-PCR assay was used to determine the *M. genitalium* bacterial load in men with and without symptoms of urethritis. In the patients with discharge, a mean bacterial load of $5.3x10^3$ genome copies/µl was detected, followed by $6.7x10^2$ genome copies/µl in patients with BOM, and $4.6X10^1$ genome copies/µl in asymptomatic patients. The concentration of *M. genitalium* in patients with urethral discharge was significantly

higher than in those patients with BOM ($p<0.001$) and significantly more organisms was detected in patients with urethritis symptoms than in those without ($p = 0.02$).

Using microscopic diagnosis of urethritis, i.e >5PMNLs/hpf, results obtained were similar to those based on clinical signs of urethritis. In the specimens where microscopy confirmed urethritis, significantly higher ($p<0.001$) *M. genitalium* loads were detected than in the patients with symptoms of urethritis ($8.2x10^3$ genome copies/µl) but without microscopic evidence ($1.3x10^3$ genome copies/µl). Both these groups carried significantly higher bacterial loads ($p<0.001$) than the asymptomatic group of patients ($4.6X10^1$ genome copies/µl).

Yoshida *et al* (2002a) developed the first q-PCR assay for quantification of *M. genitalium* and found higher DNA loads of the organism in urine specimens from men with urethritis (diagnosed with microscopy) than in those from asymptomatic men. Unfortunately, only two asymptomatic men were examined for comparative evaluation in the study by Yoshida *et al* (2002a). Using a Taqman assay targeting the *M. genitalium* 16S rRNA gene, the researchers found bacterial loads ranging from $2.3x10^2$ to $3.3x10^6$ genome copies/ml in urine specimens from men with NGU, compared to $2.0x10^2$ and <5x10 genome copies/ml in the two asymptomatic men.

Svenstrup *et al* (2005) detected *Mycoplasma genitalium* loads ranging from 1 to 2.5 $x10^4$ genome copies/µl when the *M. genitalium* load in urethral specimens of

symptomatic men attending an STD clinic in Sweden was determined, using the LightCycler assay. Low copy numbers were seen for the asymptomatic *M. genitalium* positive men, (2 – 12 genome copies/µl). This was similar to the results obtained in this study ($4.9x10^2$ – $2.5 x10^4$ genome copies/µl in symptomatic men and $2.5 x10^1$ – $6.6 x10^1$ genome copies/µl in asymptomatic men).

Jensen *et al* (2004a) made use of a Taqman 5' nuclease q-PCR assay to examine *M. genitalium* positive men and to correlate the DNA load to symptoms and signs of urethritis. Similar to this study, a strong association between high *M. genitalium* DNA load and the presence of discharge was shown. The mean *M. genitalium* DNA load in urethral swabs from patients with a discharge was $2.48x10^2$ genome copies/ml compared to $1.3x10^1$ genome copies/ml in specimens from patients without discharge (Jensen *et al*, 2004a). Although these values are less than those found in this study, it was noted by Jensen *et al* that the *M. genitalium* DNA load was significantly higher ($p = 0.0038$) in urine specimens than in the urethral swab specimens. Jensen *et al* (2004a) could not show a significant association with a higher *M. genitalium* DNA load in men with and without a visible discharge when testing urine specimens, although a trend was observed.

Dupin *et al* (2003) used FRET hybridizing probes in a LightCycler assay targeting a 115 kDa gene (associated with adhesion) of *M. genitalium* to detect and quantify the organism in male patients with urethritis in France. Patients were grouped as i) those

with urethritis (based on microscopic evidence); ii) those without microscopic evidence of urethritis but with urethral symptoms and iii) asymptomatic men. The rate of detection was significantly higher in the group with microscopic evidence of urethritis ($p<0.001$) than in the other groups and the mean observed concentration of *M. genitalium* was $1.2x10^4$ genome copies/ml (Dupin *et al*, 2003)

In this study the *M. genitalium* load in patients with urethritis based on urethral discharge and in those with urethritis diagnosed with microscopy were compared. The results indicated higher bacterial loads in patients with urethritis diagnosed with microscopy ($8.2x10^3$ vs $5.3x10^3$ genome copies/µl) although this was not significant ($p = 0.3$). As discussed in Chapter 4, microscopy is not the method preferred by family practitioners to diagnose urethritis and it was shown in this study that a diagnosis based on signs and symptoms of urethritis can be used. Specimens from patients with discharge had the highest *M. genitalium* loads ($5.3x10^3$ genome copies/µl), followed by those with BOM ($6.7x10^2$ genome copies/µl), while a few copies ($4.6x10^1$ genome copies/µl) were detected in asymptomatic men.

Quantitative PCR assays can be employed to determine clinical response to antimicrobial treatment (Hamasuna *et al*, 2005). The longitudinal evaluation of *M. genitalium* loads in urine specimens of men with urethritis during antimicrobial treatment may be a good alternative for estimating susceptibilities in the absence of convenient culture methods.

This study indicated a strong association between *M. genitalium* bacterial load and urethritis in men. The results showed that as the number of organisms increased, the severity of the symptoms increased likewise; an indication of the role that *M. genitalium* plays in disease progression.

The spread of *M. genitalium* in the population may be facilitated by the absence of a reliable rapid and accurate diagnostic test. Deguchi *et al* (2002) has shown that *M. genitalium* can persist in hosts even after treatment with levofloxacin for 13 days, implicating that the remaining *M. genitalium* bacteria may multiply causing recurrent and even chronic NGU. This suggested a strong relation between the increase in the number of organisms and disease progression. Men without any symptoms of urethritis may still carry low numbers of *M. genitalium* which can be transmitted to their sexual partners.

CHAPTER 6
OVERALL DISCUSSION & CONCLUSION

This study was undertaken to address the question whether *Mycoplasma genitalium* is a pathogen or a passenger in the genital tract of humans. This question, relating to various organisms that occur as commensal and/or pathogens, has faced researchers in the past and in addressing it, Koch's postulates usually apply.

The Koch postulates, developed in the 19th century, state that if a given organism is established as the causative agent of a specific disease, it is implied that the disease can be treated and/or controlled by elimination of the organism or by preventing contact with the organism. In order to prove this, the organism has to be isolated from the host, and after growth in pure culture, has to induce disease in a new host. Fulfilling these postulates can be very difficult or impossible for some pathogens especially for those that cannot be isolated in pure culture. Furthermore, viruses cannot be propagated without cells, and some diseases may be due to different pathogens (Jensen, 2006).

As a consequence of the shortcomings of Koch's postulates for certain organisms, revisions have been proposed. Taylor-Robinson (1983) suggested some modifications for mycoplasmas before they can be regarded as pathogens. These include:

i. the detection of the pathogen more frequently and/or in larger numbers from patients with disease than from those without infection,

ii. clinical and microbiological cure following treatment with antimicrobial agents,

iii. the ability to be transmitted between hosts.

In this study, we investigated the association of *M. genitalium* with adult male urethritis. In order to achieve this, a convenient research molecular diagnostic assay was used to determine the prevalence of the organism in men with and without clinical symptoms of urethritis, and finally, the bacterial load of the organism calculated in men harbouring the organism.

Culture techniques for *M. genitalium* have achieved some success, but it has limitations, as it requires elaborate media, is time-consuming, and has low yields. Therefore, laboratory diagnosis depends on the use of nucleic acid amplification tests (NAATs) and in order to do this, researchers have targeted a few genes. Various specimen types, including first void urine (FVU), endourethral swabs, cervical swabs and vaginal swabs can be used to detect *M. genitalium* infection in males and females, but the specimen type that is most convenient, is usually the one that is least invasive.

In this study, we compared three NAATs, namely a commercial PCR assay, an in-house adapted real-time PCR and a research-only TMA test for the detection of *M. genitalium* targeting different genes. 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. All three assays were highly specific (98-99%) for the detection of *M. genitalium.* However, where q-PCR and TMA demonstrated high sensitivities (96% and 100%), the sensitivity of the conventional PCR assay was low (78%). The TMA performed slightly better than the q-PCR assay, and both performed better than the commercial (conventional) PCR assay. The choice between these two assays will be based on ones' research aims. The TMA assay is convenient for epidemiological studies, as urine can be collected into a transport tube and kept stable for 28 days at room temperature, and four of the most common urethral pathogens can be tested for from the same tube. The real-time PCR assay can be used for diagnostic purposes and for quantitative studies. The establishment of these highly sensitive assays for the detection of *M. genitalium* is important as *M. genitalium* infection is often missed, if not ignored, due to the lack of convenient diagnostic methods.

After showing that the TMA assay was a suitable for the detection of *M. genitalium*, it was used to determine the occurrence of urethral pathogens in urine specimens obtained from symptomatic and asymptomatic South African men attending a family practice in Pretoria. The presence of urethral pathogens was evaluated according to the diagnosis of urethritis based on clinical signs and symptoms and the diagnosis of urethritis based on microscopic evidence.

As expected, there was a high burden of urethritis pathogens amongst men presenting with urethritis be it a discharge or only complaints of BOM. *M. genitalium* was the commonest pathogen detected (17.3%) from all patients studied and was found in a

higher percentage of men with NGU than *C. trachomatis* (12.3%). Amongst men with visible discharge, *Neisseria gonorrhoeae* was the most frequently (44.6%) detected pathogen. All four pathogens investigated were found significantly less in the asymptomatic group (*N. gonorrhoeae* $p<0.001$; *C. trachomatis* $p = 0.04$; *T. vaginalis* $p = 0.03$; *M. genitalium* $p = 0.04$). This finding shows an association of *M. genitalium* with urethritis in men.

Despite the use of the sensitive TMA assay used, no pathogen was detected in 57% of all patients studied. However, we did not test for other possible less likely causes of urethritis e.g. genital herpes, adenoviruses and ureaplasmas.

N. gonorrhoeae, *C. trachomatis* and *M. genitalium* were detected significantly more in men with urethral discharge, while only *N. gonorrhoeae* and *C. trachomatis* were significantly more associated with urethritis based on microscopic criteria. There was no significant difference for the detection of *M. genitalium* or *T. vaginalis* whether there was microscopic evidence of urethritis or not. The value of the Gram-stained urethral smear for the management of men with urethritis has been consistently shown to be of limited value.

Results from this study were similar to those obtained in previous South African studies, with similar prevalences for *N. gonorrhoeae* and *C. trachomatis*. *M. genitalium* and *T. vaginalis* were detected in higher numbers in this study, which may be due to the more

sensitive assay used for detection, or may reflect a true increase in prevalence. The necessity of epidemiological studies of this nature for the proper understanding of the aetiology of adult male urethritis was shown, and required if infected persons are to be treated effectively. This is especially relevant in countries like South Africa where syndromic management is used for the treatment of male urethritis.

It was shown that *M. genitalium* was closely associated with symptomatic urethritis, particularly in patients with urethral discharge. Detection of the pathogen in urine specimens from asymptomatic men was not significant. The subsequent detection of the organism in higher numbers in urogenital specimens from men with NGU than in asymptomatic men strengthens the evidence that this organism is a pathogen and not a passenger. This is one of the modified Koch postulates as suggested by Taylor-Robinson in 1983.

Mycoplasma genitalium bacterial loads in urine specimens from men with signs and symptoms of urethritis were compared with those from men without any symptoms. The real-time PCR assay that was evaluated in this study was used to create a *M. genitalium* DNA standard curve from which the bacterial loads in the specimens could be determined. There was significantly higher numbers of *M. genitalium* in patients with urethral discharge than in those with BOM ($p<0.001$), and significantly more organisms in patients with urethritis symptoms than in those without ($p = 0.02$). Based on microscopic diagnosis of urethritis, similar results were obtained. In the specimens where microscopy confirmed urethritis, significantly higher *M. genitalium* loads were

seen than in the patients with symptoms of urethritis but without microscopic evidence ($p<0.001$). Both these groups carried significantly higher bacterial loads than the asymptomatic group of patients ($p<0.001$). This study has shown that there is a strong association with *M. genitalium* and clinical and microscopic urethritis. As the number of organisms increased, the severity of the symptoms increased, indicating the role of the organism in disease progression.

In conclusion, by applying the modified Koch postulates, it was shown that *Mycoplasma genitalium* is by no means a passenger, but rather a significant urethritis pathogen that should be taken into account for diagnosis and when designing treatment of adult male urethritis

REFERENCES

Alvarez RA, Blaylock MW, Baseman JB. Surface localized glyceraldehyde-3-phosphate dehydrogenase of *Mycoplasma genitalium* binds mucin. *Mol Microbiol* 2003, **48**: 1417-1425.

Anagrius C, Lore B, Jensen JS. *Mycoplasma genitalium*: prevalence, clinical significance, and transmission. *Sex Transm Infect* 2005, **81**: 458-462.

Applied Biosystems. *Creating standard curves with genomic DNA or plasmid DNA templates for use in quantitative PCR.* 2003, 1-9.

Arber W. Genetic variation: molecular mechanisms and impact on microbial evolution. *FEMS Microbiol Rev* 2000, **24**: 1-7.

Ballard RC, Fehler HG, Htun Y, Radebe F, Jensen JS, Taylor-Robinson D. Coexistence of urethritis with genital ulcer disease in South Africa: influence on provision of syndromic management. *Sex Transm Infect* 2002, **78**: 274-277.

Baseman JB, Daly KL, Trevino LB, Drouillard DL. Distinctions among pathogenic human mycoplasmas. *Isr J Med Sci* 1984, **20**: 866-869.

Baseman JB, Dallo SF, Tully JG, Rose DL. Isolation and characterization of *Mycoplasma genitalium* strains from the human respiratory tract. *J Clin Microbiol* 1988*,* **26**: 2266-2269.

Baseman JB, Reddy SP, Dallo SF. Interplay between mycoplasma surface proteins, airway cells and the protean manifestations of mycoplasma-mediated human infections. *Am J Respir Crit Care Med* 1996, **154**: S137-S144.

Baseman JB, Cagle M, Korte JE, Herrera C, Rasmussen WG, Baseman JG, Shain R, Piper JM. Diagnostic assessment of *Mycoplasma genitalium* in culture-positive women. *J Clin Microbiol* 2004, **42**: 203-211.

Bizarro CV & Schuck DC. Purine and pyrimidine nucleotide metabolism in Mollicutes. *Gen Mol Biol* 2007*,* **30**: 190-201.

Björnelius E, Lidbrink P, Jensen JS. *Mycoplasma genitalium* in non-gonococcal urethritis - a study in Swedish male STD patients. *Int J STD AIDS* 2000, **11**: 292-296.

Björnelius E, Anagrius E, Goys G, Carlberg H, Jahannisson G, Lidbrink P, Moi H, Jensen JS. *Mycoplasma genitalium*: when to test and treat: present status in Scandinavia [abstract 0388]. In: Program and abstracts of the 15th Biennal

meeting of the International society for Sexually Transmitted Diseases Research (Ottawa, Canada). 2003: 137.

Björnelius E, Anagrius E, Bojs G, Carlberg H, Johannisson G, Johansson E, Moi H, Jensen JS, Lidbrink P. Antibiotic treatment of symptomatic *Mycoplasma genitalium* infection in Scandinavia: a controlled clinical trial. *Sex Transm Infect* 2008*,* **84**: 72-76.

Black V, Magooa P, Radebe F, Myers M, Pillay C, Lewis D. The detection of urethritis pathogens among patients with the male urethritis syndrome, genital ulcer syndrome and HIV voluntary counseling and testing clients: should South Africa's syndromic management approach be revised? *Sex Transm Inf* 2008, **84**: 254-258.

Blanchard A, Hamrick W, Duffy L, Baldus K, Cassell GH. Use of the polymerase chain reaction for detection of *Mycoplasma fermentans* and *Mycoplasma genitalium* in the urogenital tract and amniotic fluid. *Clin Infect Dis* 1993, **17** Suppl 1: S272-279.

Blaylock MW, Musatovova O, Baseman JG, Baseman JB. Determination of infectious load of *Mycoplasma genitalium* in clinical samples of human vaginal cells. *J Clin Microbiol* 2004*,* **42**: 746-752.

Bradshaw CS, Tabrizi SN, Read TR, Garland SM, Hopkins CA, Moss LM, Fairley CK. Etiologies of nongonococcal urethritis: bacteria, viruses, and the association with orogenital exposure. *J Infect Dis* 2006a, **193**: 336-345.

Bradshaw CS, Jensen JS, Tabrizi SN, Read TRH, Garland SM, Hopkins CA, Moss LM, Fairley CK. Azithromycin failure in *Mycoplasma genitalium u*rethritis. *Emerg Infect Dis* 2006b, **12**: 1149-1152.

Bradshaw CS, Fairley CK, Lister NA, Chen S, Garland SM, Tabrizi SN. *Mycoplasma genitalium* in men who have sex with men at male-only saunas. *Sex Transm Infect* 2009, **85**: 432-435.

Brown DR, Whitcomb RF, Bradbury JM. Revised minimal standards for description of new species of the class Mollicutes (division Tenericutes). *Int J Syst Evol Microbiol* 2007, **57**: 2703–2719.

Burgos R, Pich OQ, Ferre-Navarro M, Baseman JB, Querol E, Piñol J. *Mycoplasma genitalium* P140 and P110 adhesins are reciprocally stabilized and required for cell adhesion and terminal-organelle development. *J Bacteriol* 2006, **188**: 1827-8637.

Burgos R, Pich OQ, Querol E, Piñol J. Functional analysis of the *Mycoplasma genitalium* MG312 protein reveals a specific requirement of the MG312 N-terminal domain for gliding motility. *J Bacteriol* 2007*,* **189**: 7014-7023.

Busolo F, Camposampiero D, Bordignon G, Bertollo G. Detection of *Mycoplasma genitalium* and *Chlamydia trachomatis* DNAs in male patients with urethritis using the polymerase chain reaction. *New Microbiol* 1997, **20**: 325-332.

Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 2005*,* **25**: 169–193.

Bustin SA, Benes V, Nolan T, Pfaffl MW. Quantitative real-time RT-PCR – a perspective *J Mol Endocrinol* 2005, **34**: 597–601.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE Guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009**, 55**: 611–622.

Carlsen KH, Jensen G, Dohn B, Jensen JS. *Mycoplasma genitalium* PCR – does freezing of specimens effect sensitivity? [abstract P1.70]. In: Program and abstracts of the 18th Biennal meeting of the International Society for Sexually Transmitted Diseases Research (London, UK). 2009: 210

Casin I, Vexiau-Robert D De La Salmonière P, Eche A, Grandry B, Janier M. High prevalence of *Mycoplasma genitalium* in the lower genitourinary tract of women attending a sexually transmitted disease clinic in Paris, France. *Sex Transm Dis* 2002, **29**: 353–359.

Centers for Disease Control and Prevention (CDC). Update to CDC's sexually transmitted diseases treatment guidelines 2006, **55**(RR11): 1-94.

Chalker VJ, Jordan K, Ali T, Ison C. Real-time PCR detection of the mg219 gene of unknown function of *Mycoplasma genitalium* in men with and without non-gonococcal urethritis and their female partners in England. *J Med Microbiol* 2009, **58**: 895-899.

Chen CY, Steiner BM, Chi KH, Ballard RC. Evaluation of potential *Mycoplasma genitalium* targets for real-time PCR testing using urine specimens. [abstract WP-104]. In: Program and abstracts of the 16th Biennal meeting of the International Society for Sexually Transmitted Diseases Research (Amsterdam, The Netherlands). 2005: 303.

Chernesky M, Jang D, Smieja M, Portillo E, Ewert R, Pritchard C, Maceachern D, Doucette C, MacDonald A, Kapala J, Sumner J, Hill C. Validation of the Aptima Combo 2 assay for the detection of *Chlamydia trachomatis* and *Neisseria*

gonorrhoeae in SurePath liquid-based Pap test samples taken with different collection devices. *Sex Transm Dis* 2009, **36**: 581-583.

Chong S, Jang D, Song X, Mahony J, Petrich A, Barriga P, Chernesky M. Specimen processing and concentration of *Chlamydia trachomatis* added can influence false-negative rates in the LCx assay but not in the APTIMA Combo 2 assay when testing for inhibitors. *J. Clin. Microbiol* 2003, **41:** 778–782.

Clausen HF, Fedder J, Drasbek M, Nielsen PK, Toft B, Ingerslev HJ, Birkelund S, Christiansen G. Serological investigation of *Mycoplasma genitalium* in infertile women. *Hum Reprod* 2001, **16**: 1866-1874.

Cohen CR, Manhart LE, Bukusi EA, Astete S, Brunham RC, Holmes KK, Sinei SK, Bwayo JJ, Totten PA. Association between *Mycoplasma genitalium* and acute endometritis. *Lancet* 2002, **359**: 765–766.

Collier AM, Carson JL, Hu PC, Hu SS, Huang CH, Barile MF, Attachment of *Mycoplasma genitalium* to the ciliated epithelium of human fallopian tubes. In: *Recent advances in Mycoplasmology* 1990, eds G Stanek, GH Cassel, JG Tully, RF Whitcomb. Gustav Fisher Verlag, Stuttgard, 730-732.

Crucitti T, van Dyck E, Tehe A, Abdellati S, Vuylsteke B, Buve A, Laga M. Comparison of culture and different PCR assays for detection of *Trichomonas vaginalis* in self collected vaginal swabs specimens. *Sex Trans Infect* 2003,**79**: 393-398.

Dallo SF,Chavoya A, Su CJ, Baseman JB. DNA and protein sequence homologies between the adhesins of *Mycoplasma genitalium* and *Mycoplasma pneumoniae. Infect Immun* 1989, **57**: 1059-1065.

Deguchi T, Komeda H, Yasuda M, Tada K, Iwata H, Asano M, Ezaki T, Kawada Y. *Mycoplasma genitalium* in non-gonococcal urethritis. *Int J STD AIDS* 1995a, **6**: 144-145.

Deguchi T, Gilroy CB, Taylor-Robinson D. Comparison of two PCR-based assays for detecting *Mycoplasma genitalium* in clinical specimens. *Eur J Clin Microbiol Infect Dis* 1995b, **14**: 629-631.

Deguchi T & Maeda S. *Mycoplasma genitalium*: Another important pathogen of nongonococcal urethritis. *J Urol* 2002, **167**: 1210-1217.

De Jongh M, Dangor Y, Adam A, Hoosen AA. Gonococcal resistance: evolving from penicillin, tetracycline to the quinolones in South Africa – implications for treatment guidelines *Int J STD AIDS* 2007, **18**: 697-699.

De Jongh M, Le Roux M, Adam A, Caliendo AM, Hoosen AA. Co-Infection with *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Trichomonas vaginalis* in symptomatic South African men with urethritis: Implications for syndromic management. *Open Trop Med J* 2009, **2**: 13-16.

Desai K & Robson HG. Comparison of the Gram-stained urethral smear and first-voided urine sediment in the diagnosis of nongonococcal urethritis. *Sex Transm Dis* 1982, **9**: 21-25.

Dhandayuthapani S, Blaylock MW, Bebear CM, Rasmussen WG, Baseman JB. Peptide methionine sulfoxide reductase (MsrA) is a virulence determinant in *Mycoplasma genitalium*. *J Bacteriol* 2001, **183**: 5645-5650.

Department of National Health Directorate HIV/AIDS and STDs. Protocols for the management of a person with sexually transmitted diseases (STD), South Africa. Protocol no 1 1996, Pretoria: DOH.

Dolapci I, Tekeli A, Ozsan M, Yaman O, Ergin S, Elhan A. Detecting of *Mycoplasma genitalium* in male patients with urethritis symptoms in Turkey by polymerase chain reaction. *Saudi Med J* 2005, **26**: 64-68.

Dupin N, Bijaoui G, Schwarzinger M, Ernault P, Gerhardt P, Jdid R, Hilab S, Pantoja C, Buffet M, Escande JP, Costa JM. Detection and quantification of *Mycoplasma genitalium* in male patients with urethritis. *Clin Infect Dis* 2003, **37**: 602-605.

Dutro SM, Hebb JK, Garin CA, Hughes JP, Kenny GE, Totten PA. Development and Performance of a Microwell-Plate-Based Polymerase Chain Reaction Assay for *Mycoplasma genitalium*. *Sex Transm Dis* 2003, **30**: 756-753.

Eastick K, Leeming JP, Caul EO, Horner PJ, Millar MR. A novel polymerase chain reaction assay to detect *Mycoplasma genitalium*. *Mol Pathol* 2003, **56**: 25-28.

Edberg A, Jurstrand M, Johansson E, Wikander E, Höög A, Ahlqvist T, Falk L, Jensen JS, Fredlud H. A comparative study of three different PCR assays for detection of *Mycoplasma genitalium* in urogenital specimens from men and women. *J Med Microbiol* 2008, **57**: 304-309.

Espy MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA, Yao JDC, Wengenack NL, Rosenblatt JE, Cockerill III FR, Smith TF. Real-Time PCR in clinical microbiology: applications for routine laboratory testing. *Clin Microbiol Rev* 2006, **19**: 165-256.

Falk L, Fredlund H, Jensen JS. Tetracycline treatment does not eradicate *Mycoplasma genitalium*. *Sex Transm Inf* 2003, **79**: 318-319.

Falk L, Fredlund H, Jensen JS. Symptomatic urethritis is more prevalent in men infected with *Mycoplasma genitalium* than with *Chlamydia trachomatis*. *Sex Transm Inf* 2004*,* **80**: 289-293.

Falk L, Fredlund H, Jensen JS. Signs and symptoms of urethritis and cervicitis among women with or without *Mycoplasma genitalium* or *Chlamydia trachomatis* infection. *Sex Transm Infect* 2005, **81**: 73-78.

Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb JF, Dougherty BA, Merrick JM. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* 1995, **269**: 496-512.

Fraser CM, Gocayne JD, White O, Adams MD, Clayton RA, Fleischmann RD, Bult CJ, Kerlavage AR, Sutton G, Kelley JM, Fritchman RD, Weidman JF, Small KV, Sandusky M, Fuhrmann J, Nguyen D, Utterback TR, Saudek DM, Phillips CA, Merrick JM, Tomb JF, Dougherty BA, Bott KF, Hu PC, Lucier TS, Peterson SN, Smith HO, Hutchison CA 3rd, Venter JC. The minimal gene complement of *Mycoplasma genitalium. Science* 1995, **270**: 397-403.

Freundt EA, Whitcomb RF, Barile MF, Bové JM, Cottew GS, Ernø H, LemckeR, Maniloff J, Neimark H, Razin S, Taylor-Robinson D, Tully JG, Whittlestone P. Proposal of minimal standards for descriptions of new species of the Class Mollicutes. *Int J Syst Bacteriol* 1979, **29**: 172-80.

Furr PM & Taylor-Robinson D. Microimmunofluorescence technique for detection of antibody to *Mycoplasma genitalium*. *J Clin Pathol* 1984, **37**: 1072-1074.

Gambini D, Decleva I, Lupica L, Ghislanzoni M, Cusini M, Alessi E. *Mycoplasma genitalium* in males with nongonococcal urethritis: prevalence and clinical efficacy of eradication. *Sex Transm Dis* 2000, **27**: 226-229.

Gaydos CA, Quinn TC, Willis D, Weissfeld A, Hook EW, Martin DH, Ferraro DV, Schachter J. Performance of the APTIMA Combo 2 Assay for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in female urine and endocervical swab specimens. *J Clin Microbiol* 2003*,* **41:** 304-309.

Gaydos CA &, Quinn TC Urine nucleic acid amplification tests for the diagnosis of sexually transmitted infections in clinical practice. *Curr Opin Infect Dis* 2005, **18**: 55-66.

Gaydos C, Maldeis NE, Hardick A, Hardick J, Quinn TC. *Mycoplasma genitalium* compared to *Chlamydia*, Gonorrhea and *Trichomonas* as an etiologic agent of urethritis in men attending STD clinics. *Sex Transm Infect* 2009, **85**: 438-440.

Gdoura R, Kchaou W, Chaari C, Znazen A, Keskes L, Rebai T, Hammami A. *Ureaplasma urealyticum, Ureaplasma parvum, Mycoplasma hominis* and *Mycoplasma genitalium* infections and semen quality of infertile men. *BMC Infect Dis* 2007, **7**: 129.

Gen-Probe Inc. *APTIMA® urine specimen collection kit for male and female urine specimens.* Package insert 2001, BX 0123-01 Rev. B.

Gen-Probe Inc. *APTIMA® General Purpose Reagents (GPR) 250 Kit.* Package insert 2006, 500106 Rev. A.

Gen-Probe Inc. *APTIMA COMBO 2® Assay*. Package Insert 2009, 501798 Rev. A.

Glass JI, Assad-Garcia N, Alperovich N, Yooseph S, Lewis MR, Maruf M, Hutchison III CA, Smith HO, Venter JC (2006). Essential genes of a minimal bacterium. *Proc Natl Acad Sciences* 2006, **103:** 425-430.

Göbel UB, Geiser A, tanbridge EJ. Oligonucleotide probes complementary to variable region of ribosomal RNA discriminate between Mycoplasma species. *J Gen Microbiol* 1987, **133**: 1969-1974.

Goulet M, Dular R, Tully JG, Billowes G, Kasatiya S. Isolation of *Mycoplasma pneumoniae* from the human urogenital tract. *J Clin Microbiol* 1995, **33**: 2823-2825.

Grześko J, Elias M, Mączyńska B, Kasprzykowska U, Tlaczala M, Goluda M. Occurrence of *Mycoplasma genitalium* in fertile and infertile women. *Fertil Steril* 2009, **91**: 2376-2380.

Gunson RN, Collins TC, Carman WF. Practical experience of high throughput real time PCR in the routine diagnostic virology setting. *J Clin Virol* 2006, **35**: 355-367.

Haddow LJ, Bunn A, Copas AJ, Gilson R, Prince M, Ridgway GL, Sadiq ST. Polymorph count for predicting non-gonococcal urethral infection: a model using *Chlamydia*

trachomatis diagnosed by ligase chain reaction. *Sex Transm Infect* 2004, **80**: 198-200.

Hamasuna R, Osada Y, Jensen JS. Antibiotic susceptibility testing of *Mycoplasma genitalium* by TaqMan 5' nuclease real-time PCR. *Antimicrob Agents Chemother* 2005*,* **49**: 4993-4998.

Hamasuna R, Osada Y, Jensen JS. Isolation of *Mycoplasma genitalium* from first-void urine specimens by coculture with Vero cells. *J Clin Microbiol* 2007*,* **45**: 847-850.

Hannan PC. Comparative susceptibilities of various AIDS-associated and human urogenital tract mycoplasmas and strains of *Mycoplasma pneumoniae* to 10 classes of antimicrobial agent *in vitro*. *J Med Microbiol* 1998, **47**: 1115-1122.

Hardick J, Giles J, Hardick A, Hsieh H, Quinn T, Gaydos C. Performance of the Gen-Probe transmission-mediated amplification research assay compared to that of a multitarget real-time PCR for *Mycoplasma genitalium* detection. *J Clin Microbiol* 2006a*,* **44**: 1236-1240.

Hardick A, Hardick J, Wood BJ, Gaydos C. Comparison between the Gen-Probe Transcription-Mediated Amplification *Trichomonas vaginalis* research assay and real-time PCR for *Trichomonas vaginalis* detection using a Roche LightCycler instrument with female self-obtained vaginal swab samples and male urine samples. *J Clin Microbiol* 2006b, **44**: 4197-4199.

Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res* 1996*,* **6**: 986–994.

Himmelreich R, Plagens H, Hilbert H, Reiner B, Herrmann R. Comparative analysis of the genomes of the bacteria *Mycoplasma pneumoniae* and *Mycoplasma genitalium*. *Nucleic Acids Res* 1997, **25**: 701-712.

Hjorth SV, Björnelius E, Lidbrink P, Falk L, Dohn B, Berhelsen L, Ma L, Marrtin DH, Jensen JS. Sequence-based typing of *Mycoplasma genitalium* reveals sexual transmission. *J Clin Microbiol* 2006, **44**: 2078-2083.

Hooton TM, Roberts MC, Roberts PL, Holmes KK, Stamm WE, Kenny GE. Prevalence of *Mycoplasma genitalium* determined by DNA probe in men with urethritis. *Lancet* 1988, **1**: 266-268.

Hopps HE & Del Giudice RA. Cell culture models as ancillary tools in the isolation and characterization of mycoplasmas. *Isr J Med Sci* 1984, **20**: 927-930.

Horner PJ, Gilroy CB, Thomas BJ, Naidoo RO, Taylor-Robinson D. Association of *Mycoplasma genitalium* with acute non-gonococcal urethritis. *Lancet* 1993, **342**: 582-585.

Horner PJ & Taylor-Robinson D. *Mycoplasma genitalium* and non-gonococcal urethritis. *Lancet* 1994, **343**: 790-791.

Horner PJ, Thomas B, Gilroy CB, Egger M, Taylor-Robinson D. Role of *Mycoplasma genitalium* and *Ureaplasma ureolyticum* in acute and chronic nongonococcal urethritis. *Clin Infect Dis* 2001, **32**: 995-1003.

Huppert JS, Mortensen JE, Reed JL, Kahn JA, Rich KD, Hobbs MM. *Mycoplasma genitalium* detected by transcription mediated amplification is associated with *Chlamydia trachomatis* in adolescent women. *Sex Transm Dis* 2008, **35**: 250-254.

Hussain AI, Robson WLM, Kelly R, Reid T, Gangem JD. *Mycoplasma penetrans* and other mycoplasmas in urine of human immunodeficiency virus-positive children. *J Clin Microbiol* 1999, **37**: 1518-523.

Hyman HC, Yogen D, Razin S. DNA Probes for detection and identification of *Mycoplasma pneumoniae* and *Mycoplasma genitalium*. *J Clin Microbiol* 1987, **25**: 726-728.

Inamine JM, Loechel S, Hu PC. Analysis of the nucleotide sequence of the P1 operon of *Mycoplasma pneumoniae*. *Gene* 1988, **73**: 175-183.

Inamine JM, Loechel S, Collier AM, Barile MF, Hu PC. Nucleotide sequence of the MgPa (mgp) operon of *Mycoplasma genitalium* and comparison to the P1 (mpp) operon of *Mycoplasma pneumoniae*. *Gene* 1989, **82**: 259-267.

International Committee on Systematic Bacteriology (ICSB) - Subcommittee on the Taxonomy of Mollicutes. Revised minimum standards for description of new species of the class Mollicutes (division Tenericutes). *Int J Syst Bacteriol* 1995, **45**: 605-612.

Ishihara S, Yasuda M, Ito S, Maeda S, Deguchi T. *Mycoplasma genitalium* urethritis in men. *Int J Antimicrob Agents* 2004, **24S**: S23-S27.

Jang D, Chernesky M, Luinstra K, Chong S, Smieja M, Cai W, Hayhoe B, MacRitchie C, Main C. The APTIMA Combo 2® assay detected more women infected with *Chlamydia trachomatis* than the AMPLICOR® assay by testing cervical and vaginal swab specimens and urine. [abstract TP-060]. In: Program and abstracts of the 16th Biennal meeting of the International Society for Sexually Transmitted Diseases Research (Amsterdam, The Netherlands). 2005: 214.

Janier M, Lassau F, Casin I, Grillot P, Scieux C, Zavaro A, Chastang C, Bianchi A, Morel P. Male urethritis with and without discharge: a clinical and microbiological study. *Sex Transm Dis* 1995, **22**: 244-252.

Janier M, Lassau F, Casin I. *Mycoplasma genitalium* in male urethritis. *Int J STD AIDS* 1997, **8**: 534.

Jensen JS, Uldum SA, Sondergard-Andersen J, Vuust J, Lind K. Polymerase chain reaction for detection of *Mycoplasma genitalium* in clinical samples. *J Clin Microbiol* 1991*,* **29**: 46-50.

Jensen JS, Orsum R, Dohn B, Uldum S, Worm AM, Lind K. *Mycoplasma genitalium*: a cause of male urethritis? *Genitourin Med* 1993, **69**: 265-269.

Jensen JS, Blom J, Lind K. Intracellular location of *Mycoplasma genitalium* in cultured Vero cells as demonstrated by electron microscopy. *Int J Exp Pathol* 1994, **75**: 91-98.

Jensen JS, Hansen HT, Lind K. Isolation of *Mycoplasma genitalium* strains from the male urethra. *J Clin Microbiol* 1996, **34**: 286-291.

Jensen JS, Borre MB, Dohn B. Detection of *Mycoplasma genitalium* by PCR amplification of the 16S rRNA gene. *J Clin Microbiol* 2003, **41**: 261-266.

Jensen JS, Bjornelius E, Dohn B, Lidbrink P. Use of TaqMan 5' nuclease real-time PCR for quantitative detection of *Mycoplasma genitalium* DNA in males with and

without urethritis who were attendees at a sexually transmitted disease clinic. *J Clin Microbiol* 2004a, **42**: 683-692.

Jensen JS, Bjornelius E, Dohn B, Lidbrink P. Comparison of first void urine and urogenital swab specimens for detection of *Mycoplasma genitalium* and *Chlamydia trachomatis* by polymerase chain reaction in patients attending a sexually transmitted disease clinic. *Sex Transm Dis* 2004b, **31**: 499-507.

Jensen JS. *Mycoplasma genitalium* infections. *Dan Med Bull* 2006*,* **53**: 1-27.

Jensen JS, Bradshaw CS, Tabrizi SN, Fairley CK, Hamasuna R. Azithromycin treatment failure in *Mycoplasma genitalium*-positive patients with nongonococcal urethritis is associated with induced macrolide resistance. *Clin Infect Dis* 2008, **47**: 1546-1553.

Johannisson G, Enström Y, Löwhagen GB, Nagy V, Ryberg K, Seeberg S, Welinder-Olsson C. Occurrence and treatment of *Mycoplasma genitalium* in patients visiting STD clinics in Sweden. *Int J STD AIDS* 2000, **11**: 324-326.

Jordan JJ. Real-time detection of PCR products and microbiology. *New Technologies for Life Sciences: A Trends Guide* 2000, 61-66.

Jurstrand M, Jensen JS, Magnuson A, Kamwendo F, Fredlund H. A serological study of the role of *Mycoplasma genitalium* in pelvic inflammatory disease and ectopic pregnancy. *Sex Transm Infect* 2007, **83**: 319-323.

Ke LD, Chen Z, Yung WK. A reliability test of standard-based quantitative PCR: exogenous vs endogenous standards. *Mol Cell Probes* 2000, **14**: 127–135.

Keane FE, Thomas BJ, Gilroy CB, Renton A, Taylor-Robinson D. The association of *Chlamydia trachomatis* and *Mycoplasma genitalium* with non-gonococcal urethritis: observations on heterosexual men and their female partners. *Int J STD AIDS* 2000, **11**: 435-439.

Klein D. Quantification using real-time PCR technology: applications and limitations. *TRENDS Mol Med* 2002, **8**: 257-260.

Krause DC & Balish MF. Structure, function, and assembly of the terminal organelle of *Mycoplasma pneumoniae. FEMS Microbiol Lett* 2001, **198**: 1-7.

Lackey PC, Ennis DM, Cassell GH, Whitley RJ, Hook EW. The etiology of nongonnococcal urethritis. *Clin Infect Dis* 1995, **21**: 759.

Landis SJ, Stewart IO, Chernesky MA. Value of the gram-stained urethral smear in the management of men with urethritis. *Sex Transm Dis* 1988, **15**: 78-84.

Lee SR, Chung JM, Kim YG. Rapid one stepdetection of pathogenic bacteria in urine with sexually transmitted disease (STD) and prostatitis patient by multiplex PCR assay (mPCR). *J Microbiol* 2007, **45**: 453-459.

Leung A, Eastick K, Haddon LE, Horn CK, Ahuja D, Horner PJ. *Mycoplasma genitalium* is associated with symptomatic urethritis. *Int J STD AIDS* 2006, **17**: 285-288.

Lewis D, Tshelane S, Khanyile N, Pillay C. Surveillance of Sexually Transmitted Infections in Gauteng province, South Africa; 2000-2006 [abstract]. In: Program and abstracts of the 17th Biennal meeting of the International Society for Sexually Transmitted Diseases Research (Seattle, USA) 2007.

Levett PN, Brandt K, Olenius K, Brown C, Montgomery K, Horsman GB. Evaluation of three automated nucleic acid amplification systems for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in first-void urine specimens. *J Clin Microbiol* 2008, **46**: 2109-2111.

Lind K, Lindhardt BO, Schutten HJ, Blom J, Christiansen C. Serological cross-reactions between *Mycoplasma genitalium* and *Mycoplasma pneumoniae. J Clin Microbiol* 1984, **20**: 1036-1043.

Lowe P, O'Loughlin P, Evans K, White M, Bartley PB, Vohra R. Comparison of the Gen-Probe APTIMA Combo 2 Assay to the AMPLICOR CT/NG assay for detection of

Chlamydia trachomatis and *Neisseria gonorrhoeae* in urine samples from Australian men and women. *J Clin Microbiol* 2006, **44**: 2619-1621.

Luo D, Xu W, Liang G Wang S, Wang Z, Bi Z, Zhu W. Isolation and identification of *Mycoplasma genitalium* from high risk populations of sexually transmitted diseases in China. *Chin Med J (Engl)* 1999, **112**: 489-492.

Luo D, Zhu W, Zhang X, Chen X, Wang S, Wang Z, Liang G, Sun H, Xu W. Molecular epidemiologic study of *Mycoplasma genitalium* infection in high risk populations of sexually transmitted diseases in China. *Chin Med J (Engl)* 2000, **113**: 1015-1018.

Ma L & Martin DH. Single-nucleotide polymorhisms in the rRNA operon and variable numbers of tandem repeats in the lipoprotein gene among *Mycoplasma genitalium* strains from clinical specimens. *J Clin Microbiol* 2004. **42**: 4876-4878.

Ma L, Taylor S, Jensen JS, Myers L, Lillis R, Martin DH. Short tandem repeat sequences in the *Mycoplasma genitalium* genome and their use in a multilocus genotyping system. *BMC Microbiol* 2008, **8**: 130.

Maeda S, Tamaki M, Nakano M, Uno M, Deguchi T, Kawada Y. Setection of *Mycoplasma genitalium* in patients with urethritis. *J Urol* 1998, **159**: 405-407.

Maeda SI, Tamaki M, Kojima K, Yoshida T, Ishiko H, Yasuda M, Deguchi T. Association of *Mycoplasma genitalium* persistence in the urethra with recurrence of nongonococcal urethritis. *Sex Transm Dis* 2001, **28**: 472-476.

Mahony J, Chong S, Jang D, Luinstra K, Faught M, Dalby D, Sellors J, Chernesky M. Urine specimens from pregnant and nonpregnant women inhibitory to amplification of *Chlamydia trachomatis* nucleic acid by PCR, ligase chain reaction, and transcription-mediated amplification: identification of urinary substances associated with inhibition and removal of inhibitory activity. *J Clin. Microbiol* 1998, **36**: 3122–3126.

Manhart LE, Critchlow CW, Holmes KK, Dutro SM, Eschenbach DA, Stevens CE, Totten PA. Mucopurulent cervicitis and *Mycoplasma genitalium*. *J Infect Dis* 2003, **187**: 650–657.

Manhart LE, Holmes KK, Hughes JP, Houston LS & Totten PA. *Mycoplasma genitalium* among young adults in the United States: An emerging sexually transmitted infection. *Am J Public Health* 2007, **97**: 1118-1125.

Manhas A, Sethi S, Sharma M, Wanchu A, Kanwar AJ, Kaur K, Mehta SD. Association of genital mycoplasmas including *Mycoplasma genitalium* in HIV infected men with nongonococcal urethritis attending STD & HIV clinics. *Indian J Med Res* 2009, **129**: 305-310.

Martin DH. Nongonococcal Urethritis: new views through the prism of modern molecular microbiology. *Curr Infect Dis Rep* 2008, **10**: 128-132.

Martin DH & Bowie WR. Urethritis in males. In: *Sexually transmitted diseases* 3rd edn 1999, eds K Holmes, PA Mardh, PF Sparling, SM Lemon, WE Stamm, P Piot, JN Wasserheit, McGraw-Hill, New York, 833-834.

Martin DH, Cammarata C, Van Der Pol B, Jones RB, Quinn TC, Gaydos CA, Crotchfelt K, Schachter J, Moncada J, Jungkind D, Turner B, Peyton C. Multicenter evaluation of Amplicor and automated Cobas Amplicor CT/NG tests for *Neisseria gonorrhoeae*. *J Clin Microbiol* 2000, **38**: 3544–3549.

McGowin CL, Popov VL, Pyles RB. Intracellular *Mycoplasma genitalium* infection of human vaginal and cervical epithelial cells elicits distinct patterns of inflammatory cytokine secretion and provides a possible survival niche against macrophage-mediated killing. *BMC Microbiol* 2009, **9**: 139.

Mena L, Wang X, Mroczkowski TF, Martin DH. *Mycoplasma genitalium* infections in asymptomatic men and men with urethritis attending a sexually transmitted diseases clinic in New Orleans. *Clin Infect Dis* 2002, **35**: 1167-1173.

Mernaugh GR, Dallo SF, Holt SC, Baseman JB. Properties of adhering and nonadhering populations of *Mycoplasma genitalium*. *Clin Infect Dis* 1993, **17**: S69-S78.

Moi H, Reinton N, Moghaddam A. *Mycoplasma genitalium* is associated with symptomatic and asymptomatic non-gonococcal urethritis in men. *Sex Transm Infect* 2009a, **85**: 15-18.

Moi H, Reinton N, Moghaddam A. *Mycoplasma genitalium* in women with lower genital tract inflammation. *Sex Transm Infect* 2009b, **85**: 10-14.

Mombach JCM, Lemke N, da Silva MN, Ferreira RA, Isaia E, Barcellos CK. Bioinformatics analysis of mycoplasma metabolism: Important enzymes, metabolic similarities, and redundancy. *Comp Biol Med* 2006, **36**: 542-552.

Moncada J, Schachter J, Hook EW 3rd, Ferrero D, Gaydos C, Quinn TC, Willis D, Weissfeld A, Martin DH. The effect of urine testing in evaluations of the sensitivity of the Gen-Probe Aptima Combo 2 assay on endocervical swabs for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*: the infected patient standard reduces sensitivity of single site evaluation. *Sex Transm Dis* 2004, **31**: 273-277.

Morency P, Dubois MJ, Grésenguet G, Frost E, Mâsse B, Deslandes S, Somsé P, Samory A, Mberyo-Yaah F, Pépin J. Aetiology of urethral discharge in Bangui, Central African Republic. *Sex Transm Infect* 2001, **77**: 125-129.

Morozumi M. Hasegawa K, Kobayashi R, Inoue N, Iawata S, Kuroki H, Kawamura N, Nakayama E, T. Tajima, Shimizu K, Ubukata K. Emergence of macrolide-resistant *Mycoplasma pneumoniae* with a 23S rRNA gene mutation. *Antimicrob Agents Chemother* 2005, **49**: 2302–2306.

Morrison-Plummer J, Lazell A, Baseman JB. Shared epitopes between *Mycoplasma pneumoniae* major adhesin protein P1 and a 140-kilodalton protein of *Mycoplasma genitalium*. *Infect Immun* 1987, **55**: 49-56.

Moxon ER, Rainey PB, Nowak MA, Lenski RE. Adaptive evolution of highly mutable loci in pathogenic bacteria. *Curr Biol* 1994, **4**: 24-33.

Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH. *Manual of Clinical Microbiology* Vol 1 8th edn 2003, ASM Press, Washington, D.C.

Musatovova O, Dhandayuthapani S, Baseman JB. Transcriptional starts for cytoadherence-related operons of *Mycoplasma genitalium*. *FEMS Microbiol Lett* 2003, **229**: 73-81.

Musatovova O, Herrera C, Baseman JB. Proximal region of the gene encoding cytadherence-related protein permits molecular typing of *Mycoplasma genitalium* clinical strains by PCR-restriction fragment length polymorphism. *J Clin Microbiol* 2006, **44**: 598-603.

Palmer HM, Gilroy CB, Furr PM, Taylor-Robinson D. Development and evaluation of the polymerase chain reaction to detect *Mycoplasma genitalium*. *FEMS Microbiol Lett* 1991, **61**: 199-203.

Pépin J, Sobéla F, Deslandes S, Alary M, Wegner K, Khonde N, Kintin F, Kamuragiye A, Sylla M, Zerbo PJ, Baganizi E, Koné A, Kane F, Mâsse B, Viens P, Frost E. Etiology of urethral discharge in West Africa: the role of *Mycoplasma genitalium* and *Trichomonas vaginalis*. *Bull World Health Organ* 2001, **76**: 355-362.

Pépin J, Labbé A-C, Khonde N, Deslandes S, Alary M, Dzokoto A, Asamoa-Adu C, Méda H, Frost E.. *Mycoplasma genitalium*: an organism commonly associated with cervicitis among west Africa sex workers. *Sex Transm Infect* 2005, **81**: 67-72.

Pereyre S, Renaudin H, Bébéar C, Bébéar CM. *In vitro* activities of the newer quinolones garenoxacin, gatifloxacin and gemifloxacin against human mycoplasmas. *Antimicrob Agents Chemother* 2004, **48**: 3165-3168.

Perez G, Skurnick JH, Denny TN, Stephens R, Kennedy CA, Regivick N, Nahmias A, Lee FK, Lo S, Wang H, Weiss SH, Louria DB. Herpes Simplex Type II and *Mycoplasma genitalium* as risk factors for heterosexual transmission: report from the heterosexual HIV transmission study. *Int J Infect Dis* 1998, **3**: 5-11.

Peterson SN, Bailey CC, Jensen JS, Borre MB, King ES, Bott KF, Hutchison CA 3rd. Characterization of repetitive DNA in the *Mycoplasma genitalium* genome: possible role in the generation of antigenic variation. *Proc Natl Acad Sci U S A* 1995, **92**: 11829-11833.

Pham-Kanter GB, Steinberg MH, Ballard RC. Sexually transmitted diseases in South Africa. *Genitourin Med* 1996, **72**: 160-171.

Pich OQ, Burgos R, Ferrer-Navarro M, Querol E, Piñol J. Role of *Mycoplasma genitalium* MG218 and MG317 cytoskeletal proteins in terminal organelle organization, gliding motility and cytadherence. *Microbiol* 2008, **154**: 3188-3198.

Pollack JD, Myers MA, Dandekar T, Herrmann R. Suspected utility of enzymes with multiple activities in the small genome *Mycoplasma* species: the replacement of the missing “household” nucleoside diphosphate kinase gene and activity by glycolytic kinases. *J Integ Biol* 2002, **6**: 247-258.

Prescott LM, Harley JP, Klein DA. *Microbiology*, 6th edn 2005, McGraw-Hill, New York.

Razin S & Jacobs E. Mycoplasma adhesion. *J Gen Microbiol* 1992, **138**: 407-422.

Razin S, Yogev D, Naot Y. Molecular biology and pathogenicity of mycoplasmas. *Microbiol Mol Biol Rev* 1998, **62**: 1094-1156.

Reddy SP, Rasmussen WG, Baseman JB. Molecular cloning and characterization of an adherence-related operon of *Mycoplasma genitalium*. *J Bacteriol* 1995, **177**: 5943-5951.

Ross JDC & Jensen JS. *Mycoplasma genitalium* as a sexually transmitted infection: implications for screening, testing, and treatment. *Sex Transm Infect* 2006, **82**: 269-271.

Rottem S. Interaction of mycoplasmas with host cells. *Physiol Rev* 2003, **83**: 417-432.

Samra Z, Borin M, Bukowsky Y, Lipshitz Y, Sompolynski D. Non-occurrence of *Mycoplasma genitalium* in clinical specimens. *Eur J Clin Microbiol Infect Dis* 1988, **7**: 49-51.

Sasaki Y, Shintani M, Shimada T, Watanabe H, Sasaki T. Detection and discrimination of *Mycoplasma pneumoniae* and *Mycoplasma genitalium* by the *in vitro* DNA amplification. *Micobiol Immunol* 1992, **36**: 21-27.

Seto S, Layh-Schmitt G, Kenri T, Miyata M. Visualization of the attachment organelle and cytadherence proteins of *Mycoplasma pneumoniae* by immunofluorescence microscopy. *J Bacteriol* 2001, **183**: 1621-1630.

Scholar & Pratt . *The antimicrobial drugs.* 2nd edn 2000, Oxford University Press, USA.

Simms I, Eastick K, Mallinson H, Thomas K, Gokhale R, Hay P, Herring A, Rogers PA. Associations between *Mycoplasma genitalium*, *Chlamydia trachomatis*, and pelvic inflammatory disease. *Sex Transm Infect* 2003, **79**: 154–156.

Sperker B, Hu P, Herrmann R. Identification of gene products of the P1 operon of *Mycoplasma pneumoniae*. *Mol Microbiol* 1991, **5**: 299-306.

Stary A, Schuh E, Kerschbaumer M, Götz B, Lee H. Performance of transcription-mediated amplification and Ligase chain reaction assays for detection of chlamydial infection in urogenital samples obtained by invasive and noninvasive methods. *J Clin Microbiol* 1998*,* **36:** 2666-2670.

Stein MA & Baseman JB. The evolving saga of *Mycoplasma genitalium*. *Clin Microbiol Newsletter* 2005, **28**: 41-48.

Sturm PDJ, Moodley P, Khan N, Ebrahim S, Govender K, Connolly C, Sturm AW. Aetiology of male urethritis in patients recruited from a population with a high HIV prevalence. *J Antimicro* Ag 2004, **24S**, S8-S14.

Su H, Hutchison III CA, Giddings MC. Mapping phosphoproteins in *Mycoplasma genitalium* and *Mycoplasma pneumoniae*. *BMC Microbiol* 2007*,* **7**: 63.

Svenstrup HF, Fedder J, Abraham-Peskir J, Birkelund S, Christiansen G. *Mycoplasma genitalium* attaches to human spermatozoa. *Hum Reprod* 2003, **18**: 2103-2109.

Svenstrup HF, Jensen JS, Bjornelius E, Lidbrink P, Birkelund S, Christiansen G. Development of a quantitative real-time PCR assay for detection of *Mycoplasma genitalium*. *J Clin Microbiol* 2005, **43**: 3121-3128.

Svenstrup HF, Jensen JS, Gevaert K, Birkelund S, Christiansen G. Identification and characterization of immunogenic proteins of *Mycoplasma genitalium***.** *Clin Vaccine Immunol* 2006, **13**: 913-922.

Taylor-Robinson D. The role of mycoplasmas in non-gonococcal urethritis: a review. *Yale J Biol Med* 1983, **56**; 537-543.

Taylor-Robinson D, Tully JG, Barile MF. Urethral infection in male chimpanzees produced experimentally by *Mycoplasma genitalium. Br J Exp Pathol* 1985a, **66**: 95-101.

Taylor-Robinson D, Furr PM, Hanna NF. Microbiological and serological study of non-gonococcal urethritis with special reference to *Mycoplasma genitalium*. *Genitourin Med* 1985b, **61:** 319-324.

Taylor-Robinson D. The history and role of *Mycoplasma genitalium* in sexually transmitted diseases. *Genitourin Med* 1995, **71**: 1-8.

Taylor-Robinson D & Bébéar C. Antibiotic susceptibilities of mycoplasmas and treatment of mycoplasmal infections. *J Antmicrobiol Chemother* 1997, **40:** 622-630.

Taylor-Robinson D & Furr PM. Update on sexually transmitted mycoplasmas. *Lancet* 1998, **351**(S3): 12-15.

Taylor-Robinson D & Horner PJ. The role of *Mycoplasma genitalium* in non-gonococcal urethritis. *Sex Transm Inf* 2001, **77:** 229-321.

Taylor-Robinson D, Jensen JS, Fehler G, Radebe F, Ballard RC. Observations on the microbiology of urethritis in black South African men. *Int J STD AIDS*. 2002, **13**: 323-325.

Taylor-Robinson D, Renton A, Jensen JS, Ison CA, Filatova E, Dmitriev G, Akovbian V. Association of *Mycoplasma genitalium* with acute non-gonococcal urethritis in Russian men: a comparison with gonococcal and chlamydial urethritis. *Int J STD AIDS* 2009, **20**: 234-237.

Tosh AK, Van Der Pol B, Fortenberry JD, Williams JA, Katz BP, Batteiger BE, Orr DP. *Mycoplasma genitalium* among adolescent women and their partners. *J Adolesc Health* 2007, **40**: 412-417.

Totten PA, Schwartz MA, Sjöström KE, Kenny GE, Handsfield HH, Weiss JB, Whittington WL. Association of *Mycoplasma genitalium* with nongonococcal urethritis in heterosexual men. *J Infect Dis* 2001, **183**: 269-276.

Tully JG, Whitcomb RF, Clark HF, Williamson DL. Pathogenic mycoplasmas: cultivation and vertebrate pathogenicity of a new spiroplasma. *Science* 1979, **195**: 892-894.

Tully JG, Taylor-Robinson D, Cole RM, Rose DL. A newly discovered mycoplasma in the human urogenital tract. *Lancet* 1981, **I**: 1288-1291.

Tully JG, Taylor-Robinson D, Rose DL, Cole RM, Bove JM. *Mycoplasma genitalium*, a new species from the human urogenital tract. *Int J Syst Bacteriol* 1983, **33**: 387-396.

Tully JG, Taylor-Robinson D, Rose DL, Furr PM, Graham CE, Barile MF. Urogenital challenge of primate species with *Mycoplasma genitalium* and characteristics of infection induced in chimpanzees. *J Infect Dis* 1986, **153**: 1046-1054.

Tyagi S, & Kramer FR. Molecular beacons - probes that fluoresce upon hybridization. *Nat. Biotechnol* 1996, **14**: 303–308.

Ueno PM, Timenetsky J, Centonze VE, Wewer JJ, Cagle M, Stein MA, Krishnan M, Baseman JB. Interaction of *Mycoplasma genitalium* with host cells: evidence for nuclear localization. *Microbiol* 2008, **154**: 3033-3041.

Uno M, Deguchi T, Komeda H, Hayasaki M, Iida M, Nagatani M, Kawada Y. *Mycoplasma genitalium* in the cervices of Japanese women. *Sex Transm Dis* 1997, **24**: 284–286.

Van de Wijgert J, Altini L, Jones H, de Kock A, Young T, Williamson AL, Hoosen A**,** Coetzee N. Two methods of self-sampling compared to clinician sampling to detect reproductive tract Infections in Gugulethu, South Africa. *Sex Transm Dis* 2006, **33**: 516-523.

Waites KB, Katz B, Schelonka RL. Mycoplasmas and ureaplasmas as neonatal pathogens. *Clin Microbiol Rev* 2005*,* **18**: 757-789.

Wang RY, Grandinetti T, Shih JW. *Mycoplasma genitalium* infection and host antibody immune response in patients infected by HIV, patients attending STD clinics and in healthy blood donors. *FEMS Immunol Med Microbiol* 1997, **19**: 237-245.

Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP. Continous fluorescence monitoring of rapid cycle DNA amplification. *Biotechniques* 1997, **22**: 130–138.

Wolff BJ, Thacker WL, Schwartz SB, Winchell JM. Detection of macrolide resistance in *Mycoplasma pneumoniae* by real-time PCR and high-resolution melt analysis. *Antimicrob Agents Chemother* 2008, **52**: 3542–3549.

Wroblewski JKH, Manhart LE, Dickey KA, Hudspeth MK & Totten PA. Comparison of transcription-mediated amplification and PCR assay results for various genital specimen types for detection of *Mycoplasma genitalium*. *J Clin Microbiol* 2006, **44**: 3306-3312.

Yasuda M, Maeda S, Deguchi T. *In vitro* activity of fluoroquinolones against *Mycoplasma genitalium* and their bacteriological efficacy for treatment of *M. genitalium*-positive nongonococcal urethritis in men. *Clin Infect Dis* 2005, **41**: 1357-1359.

Yokoi S, Maeda S, Kubota Y, Tamaki M, Mizutani K, Yasuda M, Ito S, Nakano M, Ehara H, Deguchi T. The role of *Mycoplasma genitalium* and *Ureaplasma urealyticum biovar 2* in postgonococcal urethritis. *Clin Infect Dis* 2007, **45**: 866-871.

Yoshida T, Deguchi T, Ito M, Maeda S, Tamaki M, Ishiko H. Quantitative detection of *Mycoplasma genitalium* from first-pass urine of men with urethritis and asymptomatic men by real-time PCR. *J Clin Microbiol* 2002a, **40**: 1451-1455.

Yoshida T, Maeda S, Deguchi T, Ishiko H. Phylogeny-based rapid identification of mycoplasmas and ureaplasmas from urethritis patients. *J Clin Microbiol* 2002b, **40**: 105-110.

Yu JT, Tang WY, Lau KH, Chong LY, Lo KK, Wong CK, Wong MY. Role of *Mycoplasma genitalium* and *Ureaplasma urealyticum* in non-gonococcal urethritis in Hong Kong. *Hong Kong Med J* 2008, **14**: 125-129.

Zhang R & Lin Y. DEG 5.0, a database of essential genes in both prokaryotes and eukaryotes. *Nucl Acid Res* 2009, **37**: D455-458.