Detection and characterisation of *Ureaplasma* spp in men with and without urogenital symptoms

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Detection and characterisation of *Ureaplasma* spp in men with and without urogenital symptoms

by

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Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae

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University of Pretoria
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South Africa

January 2012

The things that will destroy us are:
politics without principle,
pleasure without conscience,
wealth without work,
knowledge without character,
business without morality,
science without humanity, and
worship without sacrifice.

Mahatma Gandhi

Declaration

I, Nontuthuko Excellent Maningi, hereby declare that the work on which this dissertation is based, is original and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree at this or any other university or tertiary education institution or examination body.

.....

Signature of candidate

.....

Date

ACKNOWLEDGEMENTS

First, I want to thank the Almighty, who made all things possible for me.

To my family, especially my mother and my late father who never gave up on me, I wouldn't have done it without their support and encouragement.

I want to thank my supervisor Dr Kock for all the assistance, guidance and encouragement she has given me throughout my MSc.

A special thanks to my co-supervisor Prof AA Hoosen, he was not only a supervisor but also a father to me, he has inspired me from the first day I met him.

I also want to thank the Department of Medical Microbiology, Dr Adam and the NHLS (Tshwane Academic Division) for the opportunity to perform this research and use of their facilities.

I would like to thank my colleagues from the Department of Medical Microbiology for all their help, and putting a smile on my face every day.

I wish to acknowledge the financial grant for this study from the University of Pretoria.

A special thanks to the Medical Research Council (MRC), National Research Foundation (NRF) and University of Pretoria for financial assistance received during my MSc study

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

LIST OF ABREVIATIONS

AIDS	Acquired immune deficiency sendrome
APC	Amino-Acid-Polyamine-Organocation Transporters
ATP	Adenosine triphosphate
BV	Bacterial vaginosis
CE	Conformité Européenne
CDC	Centers for Disease Control and Prevention
CDS	Coding sequences
CLSI	Clinical and Laboratory Standards Institute
CNS	Central nervous system
CSF	Cerebrospinal fluid
CO_2	Carbon dioxide
DNA	Deoxyribose nucleic acid
HGT	Horizontal gene transfer
HIV	Human immunodeficiency virus
IFA	Immunofluorescence assay
IFN-γ	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IOM	International Organization for Mycoplasmology
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-1ra	Interleukin -1 receptor antagonist
Kb	Kilobase pairs
Mabs	Monoclonal antibodies
MATE	Multiple antimicrobial extrusion family transporters
MgPa	Major antigen of *Mycoplasma genitalium*
Min	Minutes
MIC	Minimum inhibitory concentration
MOP	Multiple-oligosaccharidyl-lipid-polysaccharide family
µl	Microlitre
MBA	Multiple banded antigen
M-PCR	Multiplex polymerase chain reaction
mRNA	Messenger ribonucleic acid
NCNGU	Non-chlamydial non-gonoccocal urethritis
NGU	Non-gonoccocal urethritis
NDP	Nucleoside diphosphate
NMP	Nucleoside monophosphate

NSU	Non-specific urethritis
PABs	Polyclonal antibodies
PCR	Polymerase chain reaction
PID	Pelvic inflammatory disease
PRPP	Phosphoribosylprophosphate
RNR	Ribonucleotide reductase
rRNA	Ribosomal ribonucleic acid
PTS	Phosphoenolpyruvate-dependent sugar phosphotransferase transport system
spp	Species
STIs	Sexually transmitted infections
tRNA	Transfer ribonucleic acid
TNF-α	Tumor necrosis factor alpha
UV	Ultraviolet light
VIC	Voltage-gated ion channel
WHO	World Health Organization

LIST OF ARTICLES IN PREPARATION FOR SUBMISSION AND CONFERENCE CONTRIBUTIONS

PUBLICATIONS

Maningi NE, Hoosen AA and Kock MM (2011) Characterization of *Ureaplasma parvum* from symptomatic and asymptomatic men attending a family practice in Pretoria, South Africa. To be submitted for publication to: *American Journal of Sexually Transmitted Disease*.

CONFERENCE PRESENTATIONS

1 **Maningi NE, Hoosen AA and Kock MM** (2009) Prevalence of *Ureaplasma* species in clinical specimens received at NHLS from January to December 2009. Faculty day (15-08-2009), University of Pretoria, Faculty of Health Sciences. (Poster presentation)

2 **Maningi NE, Hoosen AA and Kock MM** (2010) Detection and characterization of *Ureaplasma* species in men with or without urogenital symptoms. Faculty day (18-08-2010), University of Pretoria, Faculty of Health Sciences. (Poster presentation)

3 **Maningi NE, Hoosen AA and Kock MM** (2010) Detection of *Ureaplasma* species and characterization of *U. parvum* in men with or without urogenital symptoms. Research day Medical Research Council (MRC), Cape Town (14-15 October 2010). (Oral presentation)

4 **Kock MM, Maningi NE, Adam A and Hoosen AA** (2011) Characterization of *Ureaplasma parvum* from symptomatic and asymptomatic men attending a family practice in Pretoria, South Africa. International Society for Sexually Transmitted Disease Research, Québec, Canada (10-13 July 2011). (Poster presentation)

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by

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ABSTRACT

Ureaplasma spp colonize the lower genital tract in many healthy men, yet can also cause urethritis and infertility. Since *Ureaplasma* spp are frequently isolated from the normal urogenital tract, it has been suggested that only certain serotypes are associated with diseases. The genus *Ureaplasma* consists of 14 serotypes that can be divided into two biotypes, biotype 1 (*U. parvum*) and biotype 2 (*U. urealyticum*). Biotype 1 includes four serotypes (1, 3, 6 and 14) and biotype 2 includes ten serotypes (2, 4, 5 and 7 to 13).

Identification and characterization of *Ureaplasma* spp are mainly performed by traditional culture methods that are difficult to perform and take two to seven days. Commercial kits have been an alternative method for the quick identification of *Ureaplasma* spp (24 to 48 hrs) but these kits cannot speciate the bacteria. However, polymerase chain reaction (PCR) assays have been developed for characterization and speciation of *Ureaplasma* spp.

In this study, 200 first-void urine specimens were collected, 100 from symptomatic men (discharge/dysuria) and 100 from asymptomatic (without discharge/dysuria) men. The specimens were all cultured on U9 broth then subcultured on A2 agar for confirmation of growth. Antibiotic susceptibility determination was performed on the positive isolates using

the Mycofast evolution 3 kit (ELITech Microbiology, France). Molecular detection of *U. urealyticum* was performed using a commercial kit, the *Ureaplasma urealyticum* real-time PCR kit. Detection and characterization of *U. parvum* was performed using a multiplex Taqman real-time PCR assay targeting the multiple banded antigen genes (MBA).

Culture results of all specimens showed 40% (79/200) urease production on Shepard's U9 broth and of these specimens 35% (28/79) were positive when subcultured on A2 medium, 25% (20/79) were contaminated with either yeast or *Mycoplasma hominis* or both and 39% (31/79) of the U9 positive specimens did not grow when subcultured on Shepard's A2 agar medium.

When determining the susceptibility profile of *Ureaplasma* spp, 32% (9/28) of the isolates did not grow with the kit and four were contaminated. None of the isolates was resistant to all of the antibiotics and all isolates were sensitive to doxycycline, pristinamycin, roxycycline and azithromycin. One isolate was resistant to ciprofloxacin and josamycin but intermediately resistant to ofloxacin and another isolate was resistant to ofloxacin, while a second isolate was only resistant to ciprofloxacin.

Ureaplasma urealyticum was detected in 16% (31/100) of the symptomatic men and 15% (15/100) of the asymptomatic men whereas *Ureaplasma parvum* was detected in 11% (11/100) of the symptomatic men and 18% (18/100) of the asymptomatic men. There was no significant difference between the two groups (p= 01598). The distribution of serotypes did not differ significantly between the two groups (p= 0.309). The predominant serotype was serotype 6 followed by 1, 3 and 14.

In conclusion, the real-time PCR assay was rapid, not prone to contamination and implementation of the assay in diagnostic laboratories will help in the rapid detection and administering of treatment to patients (especially neonates) infected with *Ureaplasma* species. The determination of the susceptibility profiles of *Ureaplasma* spp will assist in the monitoring of the antibiotic resistant profile trends in Pretoria, so that the correct antibiotic regimens can be administered to people with *Ureaplasma* infections.

Keywords: *Ureaplasma parvum*, *Ureaplasma urealyticum*, Multiple banded antigen gene (*mba*), Real-time PCR, Serovars

CHAPTER 1

INTRODUCTION

Ureaplasma species are members of the class *Mollicutes* (Robertson *et al*., 2002). The bacteria frequently colonize healthy sexually active men and women; therefore, it is difficult to associate the bacteria with illness (Zheng *et al*., 1992; De Francesco *et al*., 2009). It is suggested that not all serotypes of *Ureaplasma* spp are disease-associated (Zheng *et al*., 1992; De Francesco *et al*., 2009). The bacteria have virulence factors, such as adhesion structures, multiple banded antigen proteins (MBA) and a urease enzyme that are believed to be pathogenic when the host's immune system is compromised (Waites *et al*., 2009).

Detection of *Ureaplasma* spp in clinical specimens has traditionally relied on growth in special culture media, such as U9 broth, A2, A7 and A8 agar medium (Mallard *et al*., 2005). Although culture is considered the "gold standard", it is labour intensive, takes two to seven days and requires special expertise (Mallard *et al*., 2005). The clinical sensitivity of culture methods remains unclear (Mallard *et al*., 2005; Dhawan *et al*., 2006).

Rapid and sensitive molecular assays that give results the same day have been developed for the diagnosis of *Ureaplasma* spp (Blanchard *et al*., 1993; Mallard *et al*., 2005; Cao *et al*., 2007a, Cao *et al*., 2007b, Xiao *et al*., 2010). These molecular assays are important in the study of the epidemiology of both *Ureaplasma* spp and to evaluate the clinical relevance of the burden of infection in relation to pathogenic potential (Mallard *et al*., 2005).

Ureaplasma spp do not have a cell wall, therefore, they are intrinsically resistant to antimicrobial agents, such as penicillin and cephalosporins. Bacteriostatic agents, such as tetracycline and macrolides as well as bactericidal agents, such as fluoroquinolones are the drugs of choice for the treatment of *Ureaplasma* species (Beeton *et al*., 2009). However, resistance of ureaplasmas to these drugs has been described (Beeton *et al*., 2009).

More studies are needed for the detection of *Ureaplasma* species in South Africa, since there is a high prevalence of human immunodeficiency virus (HIV) infection and the acquired immune deficiency syndrome (AIDS) that decreases the patient's immune system. Data on the prevalence, detection and characterisation of *Ureaplasma* infections in men are limited in South Africa. The aim of this study was to detect genital *Ureaplasma* species and to

characterize *Ureaplasma parvum* from urine specimens from symptomatic and asymptomatic men obtained from a private clinic in Pretoria, South Africa.

The objectives of this study were:

- To collect first-void urine specimens from asymptomatic (no dysuria/discharge) men and first void urine specimens from symptomatic men (dysuria/discharge)
- To isolate *Ureaplasma* species using U9 broth and culture on A2 differential agar plates
- To determine the antimicrobial profile of the *Ureaplasma* positive isolates using a commercial assay (Mycofast Evolution 3 kit)
- To extract DNA from urine using a commercial DNA extraction kit (DNA-Sorb-A)
- To detect *U. urealyticum* using a CE-approved real-time PCR assay
- To detect and characterize the *Ureaplasma parvum* serotypes using a multiplex real-time Taqman PCR assay

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Ureaplasma infections are sexually transmitted infections (STIs) and are an important public health issue in sub-Saharan Africa (WHO, 2001). Given the epidemiological link that exists between STIs and HIV, the high prevalence of *Ureaplasma* infections may increase the HIV infection rate (WHO, 2001). The leading causes of male urethritis are *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in 30% to 50% of cases (Salari and Karimi, 2003). However, genital *Ureaplasma* species are thought to be potential causes of non-chlamydial non-gonoccocal urethritis (NCNGU) in men accounting for 10% to 20% of cases (Morency *et al*., 2001; Pepin *et al*., 2001; Taylor-Robinson, 2002). Genital ureaplasmas are part of the normal flora of the urogenital tract and only cause infection when the immune system is suppressed (De Francesco *et al*., 2009). The genital bacteria are prevalent in sexually active men and women with multiple partners (Polvsen *et al*., 2002). Many studies showed that genital ureaplasmas are more prevalent in women than in men at a rate of about 40% to 80% (Cedillo-Ramirez *et al*., 2000; Ren and Zhu, 2003; Salari and Karimi, 2003). In sexually active men, genital *Ureaplasma* spp have been described to be associated with a number of diseases, such as non-specific urethritis (NSU), infertility, prostatitis and epididymitis (Zeighami *et al*., 2007). In women, *Ureaplasma* species are associated with urethritis, pyelonephritis, pelvic inflammatory disease, endometritis or chorioamnionitis (Pinna *et al*., 2006).

Genital *Ureaplasma* bacteria were first described by Shepard as the T-strain mycoplasmas because of the unique ability of the bacteria to hydrolyze urea to generate energy (Shepard, 1954; Pinna *et al*., 2006). The discovery was made when Shepard cultivated the bacteria *in vitro* from the urethra of men with non-gonococcal urethritis (Shepard, 1954; Pinna *et al*., 2006). In 1974, the genus *Ureaplasma* was separated into two biovars and the bacteria were considered to infect only humans (Robertson *et al*., 2002). In 2002, the two biovars were classified as two species, *U. urealyticum* and *U. parvum* (Kong and Gilbert, 2004). Each of the species was further characterised into serotypes, with *U. parvum* consisting of four

serotypes 1, 3, 6 and 14 and *U. urealyticum* including serotypes 2, 4, 5 and 7 to 13 (Robertson *et al*., 2002).

The main method of detecting *Ureaplasma* species is by conventional laboratory methods namely serology and culture (Zeighami *et al*., 2007). Culture methods include media, such as the Shepard U9 broth and A2 agar medium (Shepard, 1956; Mallard *et al*., 2005). The bacterium takes two to seven days to grow and is difficult to cultivate because it requires special additives, such as sterols, horse serum, antibiotics, antifungals and urea (Zeighami *et al*., 2007). The *Ureaplasma* colonies are difficult to see on the agar medium and dry quickly (Shepard, 1956; Mallard *et al*., 2005). The disadvantage with both serology and culture methods are that they require experienced staff and the results take long to be available (Pinna *et al*., 2006). In addition, culture methods can only detect the bacteria but cannot distinguish between species (Coa *et al*., 2007a).

New rapid molecular based assays for *Ureaplasma* spp characterisation and identification have been developed to replace conventional culture methods (Xiao *et al*., 2010). Polymerase chain reaction (PCR) assays detect and identify human-associated *Ureaplasma* spp by targeting the 16S rRNA gene and the 16S-23S rRNA intergenic spacer region, the urease gene subunit and multiple banded antigen (*mba*) gene (Blanchard *et al*., 1993; Teng *et al*., 1994; Luki *et al*., 1998; Kong *et al*., 2000; Coa *et al*., 2007a). Real-time PCR assays have been developed to distinguish and to quantify the two *Ureaplasma* species (Coa *et al*., 2007b). The real-time PCR assays are more rapid, specific and sensitive and less subject to contamination compared to conventional PCR assays (Xiao *et al*., 2010).

2.2 History of *Ureaplasma* species

In 1937, pleuropneumonia-like bacteria was first reported to be associated with pathology in humans causing bovine pleuropneumonia (Waites *et al*., 2005). In the 1950s, the pleuropneumonia-like bacteria was given a new name *Mycoplasma* (Waites *et al*., 2005). The genus *Ureaplasma* was originally classified as part of the genus *Mycoplasma* because the bacteria lack cell walls (Waites *et al*., 2005). The genus *Ureaplasma* belongs to the class *Mollicutes* ("soft skin"), which evolved from clostridia-like Gram-positive bacteria by gene deletion (Waites *et al*., 2009). In 1954, Shepard described the genus *Ureaplasma* as the tiny (T)-strain *Mycoplasma* because when cultured on solid agar it showed the typical spherical

colonies on agar, which made them different from other members of the *Mycoplasma* (Shepard, 1954).

In 1974, a new genus and species were proposed, namely *Ureaplasma urealyticum* (Kong and Gilbert, 2004). Using serological analysis it was shown that *U. urealyticum* consists of 14 serotypes (Robertson and Stemke, 1982). The 14 serotypes were divided into two groups known as biovar 1 and biovar 2 (Kong and Gilbert, 2004). In 2002, these biovars were classified as two distinct species, *U. parvum* (biovar 1) and *U. urealyticum* (biovar 2) based on genome size, 16S rRNA gene sequence, the 16S-23S rRNA intergenic spacer region, multi-locus enzyme polymorphism, DNA-DNA hybridization, differential growth responses to manganese and differences in the multiple banded antigen (*mba*) genes (Robertson *et al*., 2002). Serotypes 1, 3, 6 and 14 were named *U. parvum* (previously *U. urealyticum* biovar 1) due to their slightly smaller genome size and *U. urealyticum* previously designated biovar 2 or biovar T960 includes serotypes 2, 4, 5, 7 to 13 (Robertson *et al*., 2002).

2.3 Epidemiology of *Ureaplasma* species

Genital *Ureaplasma* species are part of the normal flora of the urogenital tract of men and women. Human *Ureaplasma* species are found in the cervix or vagina of 40% to 80% of asymptomatic, sexually active women and in about 5% to 20% of asymptomatic, sexually active men (Kafeitzis *et al*., 2004). The bacteria are associated with urogenital tract diseases, although their pathogenesis is not clearly understood (Horner *et al*., 2001). Diseases, such as non-gonococcal urethritis, infertility, chorioamnionitis, stillbirth, premature birth and perinatal periods are associated with ureaplasmal colonization (Kafetzis *et al*., 2004).

The reported prevalence and association of *Ureaplasma* spp with disease are limited, vary and are conflicting between studies (Kim *et al*., 2003). In a study done by Takashi *et al*. (2004) in Japan, the prevalence of *U. urealyticum* (biovar 2) in men with non-gonococcal urethitis (NGU) was 15.8% and 7.8% in men without NGU, whereas the prevalence of *U. parvum* (biovar 1) in men with NGU was 8.5% and 13.5% in men without NGU. The study enrolled 572 men with urethritis and 141 men without urethritis (Takashi *et al*., 2004).

The prevalence of *U. urealyticum* reported in Iran by Salari and Karimi (2003) was 19.2% in patients with symptoms and 7.2% in patients without symptoms. The study enrolled 125 men with urogenital symptoms and 125 men without urogenital symptoms (Salari and Karimi *et al*., 2003).

In South Africa, Sturm and colleagues (2004) conducted a study in Durban, KwaZulu Natal that included 335 men with symptoms of urethral discharge and 100 men without urethral symptoms. The men were tested for different organisms, including *Neisseria gonorrhoeae* and *Chlamydia trachomatis* as established pathogens and *U. urealyticum* as a potential pathogen (Sturm *et al*., 2004). *Ureaplasma urealyticum* was tested using a PCR assay (Sturm *et al*., 2004). The prevalence of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* was 52% (175/335) and 16% (53/335), respectively. The prevalence of *U. urealyticum* in symptomatic men was 36% (121/335); however, in asymptomatic men *N. gonorrhoeae* was not detected and *U. urealyticum* was detected in 30% (30/100) men (Sturm *et al*., 2004).

2.4 Characteristics of *Ureaplasma* species

Ureaplasma species have unique characteristics distinguishing the bacteria from other bacteria (Robertson *et al*., 2002). These characteristics include unique proteins outside the cell responsible for serotype specificity as well as the ability to hydrolyze urea for energy (Robertson *et al*., 2002).

2.4.1 Scientific classification of the *Ureaplasma* species

Ureaplasma species are bacteria that belong to the *Mollicutes* class (Table 2.1) (Bradbury, 2001; Robertson *et al*., 2002). The *Mollicutes* class comprises four orders, five families and two hundred known species, with seventeen of those species known to infect humans (Waites *et al*., 2009). The genus *Ureaplasma* consists of seven species (Robertson *et al*., 1993; Harasawa *et al*., 1996; Stemke and Robertson, 1996; Euzéby, 2011).

Table 2.1: The scientific classification of the *Ureaplasma* species (Bradbury, 2001; Robertson *et al*., 2002; Euzéby, 2011)

Rank	Scientific name
Kingdom	*Bacteria*
Phylum	*Firmicutes*
Class	*Mollicutes*
Order I	*Mycoplasmatales*
Family I	*Mycoplasmataceae*
Genus II	*Ureaplasma*
Species	*U. cati, U. canigenitalium, U. diversum*, *U felinum*, *U. gallorale*, *U. urealyticum* and *U. parvum*

The order *Mycoplasmatales* is comprised of two genera, *Mycoplasma* and *Ureaplasma* (Figure 2.1) (Robertson *et al*., 2002). The genus *Ureaplasma* clusters within the *Mycoplasma pneumoniae* clade of the *Mollicutes* based on 16S rRNA gene homology (Figure 2.1) (Robertson *et al*., 2002).

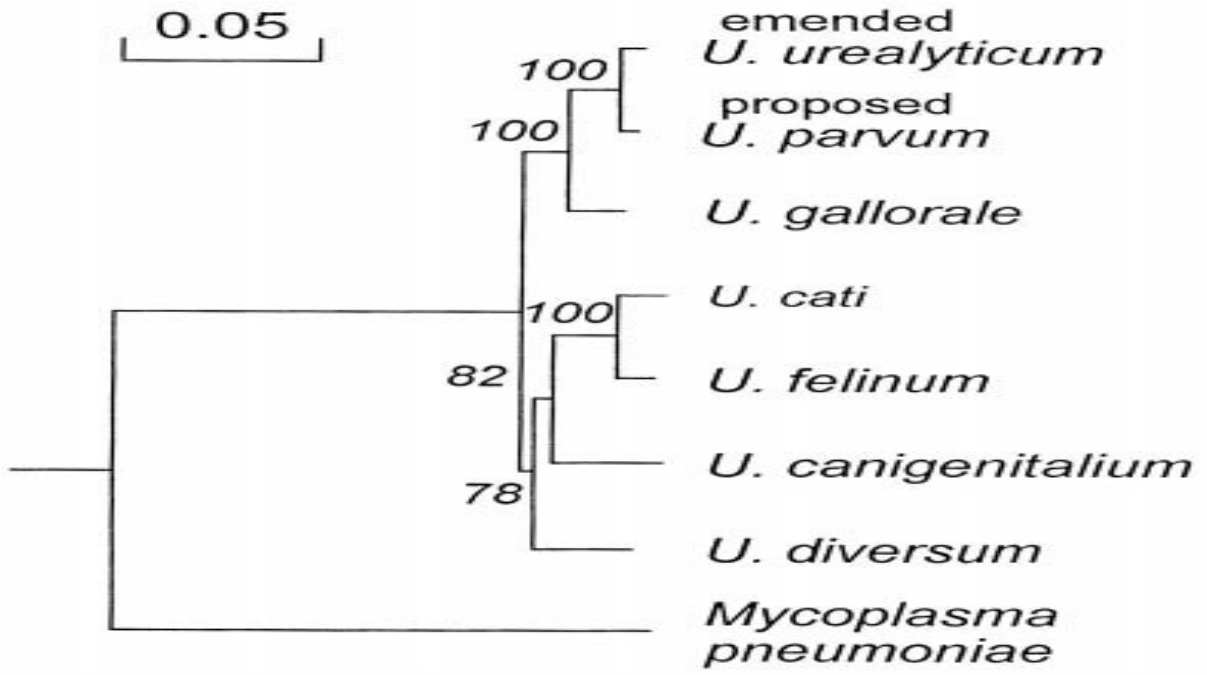

Figure 2.1: Phylogenetic tree revealing the two divergent clusters within the *Ureaplasma* genus. The relationship between the type strains of the emended species *Ureaplasma urealyticum* and *Ureaplasma parvum* are shown (GenBank accession no u00089; Robertson *et al*., 2002)

Ureaplasma parvum and *U. urealyticum* are the only species that cause disease in humans; the other species cause disease in mammals and avian species (Robertson *et al*., 2002). The genus is composed of two clusters with cluster 1 icluding the emended *U. urealyticum* species and the newly proposed *U. parvum* (Robertson *et al*., 2002). Cluster 2 is composed of the rest of the species (Figure 2.1) (Robertson *et al*., 2002). The second cluster is composed of the species *U. gallorale*, which is associated with birds, while *U. cati* and *U. felinum* are associated with cats (Harasawa *et al*., 1990). *Ureaplasma diversum* is associated with bovine animals, while *U. canigenitalium* is associated with dogs (Howard and Gourlay, 1982). *Ureaplasma gallorale* was originally isolated from chickens (Stemke and Robertson, 1996), but belongs to the same cluster as *U. urealyticum* and *U. parvum*; clustering separately from the other animal *Ureaplasma* species (Robertson *et al*., 2002).

2.4.2 Genomics of the genus *Ureaplasma*

The *Ureaplasma* spp, *Mycoplasma genitalium* and *M. hominis* are human *Mollicutes* species, are self replicating with a small genome and share the same niche (Zeighami *et al*., 2007). The genome of *M. genitalium* is the smallest, it is 580 kb in size, with *M. hominis* the second smallest being 696 kb in size (Pereyre *et al*., 2009). The genome of the *Ureaplasma* genus is; however, larger than the other two *Mycoplasma* bacteria with a genome size ranging between 670 kb to 760 kb (Table 2.2) (Pereyre *et al*., 2009). *Ureaplasma parvum* has a smaller genome than *U. urealyticum* with a genome size ranging between 670 kb to 760 kb while the genome size of *U. urealyticum* ranges from 760 kb to 1 170 kb (Ekiel *et al*., 2009).

The models trying to understand the evolution of bacteria with small genomes have suggested that the small genome size is due to the full dependence on the host of the bacteria; obtaining many compounds of metabolism from the host's tissues (Moran, 2002; Ochman and Davalos, 2006). This enables the bacteria to eliminate their own unneeded biosynthesis pathways and genes, therefore reducing the genome size (Moran, 2002). The bacteria also have a fast evolutionary rate because of its reduced genome (Momynaliev, 2007). Although these three *Mollicutes* species all have a reduced genome size, their metabolic mechanisms and pathogenic roles are different (Pereyre *et al*., 2009).

Table 2.2: Comparison of the general features of the *M. hominis* genome with those of *M. genitalium* and *U. parvum* serovar 3 (Pereyre *et al*. 2009)

Features	*M. hominis* (PG21)	*M. genitalium* (G37)	*U. parvum* (serovar 3)
Genome size (bp)	665 445	580 074	751 719
GC content of genome (%)	27.1	31.7	25.5
Gene density (%)	89.8	91.2	91.3
Total number of CDS	537	482	614
Hypothetical proteins	106	2	182
Conserved hypothetical proteins	86	164	107
CDSs with predicted function	345	316	325
Pseudogenes	14	5	0
Average protein length (aa)	369	363	371
Proteins with predicted MW> 300kDa	2	0	2
Predicted lipoprotein	43	18	37
rRNA sets	2	1	2
tRNA	33	36	30
Start codon usage (%)			
AUG	95.1	88.5	92.5
UUG	3.0	4.1	3.0
GUG	1.7	7.4	4.5
Other	0.2	0	0
Stop codon usage (%)			
UUA	83	72	85
UAG	16	27	14
Tryptophan codon usage (%)			
UGA	87	64	87
UGG	13	36	13

The G+C content of *Ureaplasma* DNA is 25%, which results in the highest A+T content compared to the other two *Mollicutes* species' genomes (Table 2.2) (Glass *et al*., 2000). The mutation pressure that causes the A+T enrichment of ureaplasmas decreases the capacity to remove uracil from DNA (Glass *et al*., 2000). Uracil sources in the DNA are caused by mis-incoporation of dUTP by DNA polymerase and spontaneous deamination of deoxycytidine residues (Glass *et al*., 2000). The bias of the mutation pressure towards increased A+T reflects the elimination of genes encoding the DNA repair enzymes (Moran, 2002). The two enzymes that repair these mismatches are dUPase, which prevents dUTP from being

misincoporated into the DNA and the other enzyme is uracil-DNA glycosylase, which removes uracil residues from DNA (Glass *et al*., 2000). These two enzymes are not found in ureaplasmas and this make these bacteria to be biased to a high A+T content (Glass *et al*., 2000).

Phylogenomic studies have recently reported the occurrence of horizontal gene transfer (HGT) among mycoplasmas sharing the same host (Pereyre *et al*., 2009). However, according to Sirand-Pugnet *et al*. (2007) horizontal gene transfer is limited in the minimal bacteria. Pereyre and colleagues (2009) assessed the horizontal gene transfer between *M. hominis* and *U. parvum* and found five clusters of genes from the *M. hominis* genome that had their closest homologs in the urogenital pathogen *U. parvum*, which belongs to the phylogenetically distant "*Pneumoniae*" group. This analysis suggested the occurrence of HGT between *M. hominis* and *U parvum* (Pereyre *et al*., 2009).

A comparison of the three minimal *Mollicutes* genomes showed that 247 coding sequences (CDSs) are common to all three genomes (Pereyre *et al*., 2009). *Mycoplasma hominis* has 220 specific CDSs with *M. genitalium* having 172 CDSs and *U. parvum* having 280 CDSs (Pereyre *et al*., 2009) (Figure 2.2A). The *M. hominis* genome shares 24 CDSs with *M. genitalium* and 46 CDSs with *U. parvum* (Pereyre *et al*., 2009). *Mycoplasma genitalium* and *U. parvum* share 41 CDSs that are not found in *M. hominis* (Pereyre *et al*., 2009). These CDSs encode proteins that are involved in major cellular functions, such as DNA metabolism, protein synthesis, nucleotide synthesis, transport, binding of substrates, fatty acid and phospholipids metabolism (Figure 2.2B) (Pereyre *et al*., 2009). Some *U. parvum* specific genes are associated with cytadherence, virulence and the gene encoding the MBA protein (UU375). The MBA is a major antigen recognized during infection of humans and five MBA N-terminal paralogs (UU172, UU189, UU483, UU487, UU526) exist (Pereyre *et al*., 2009).

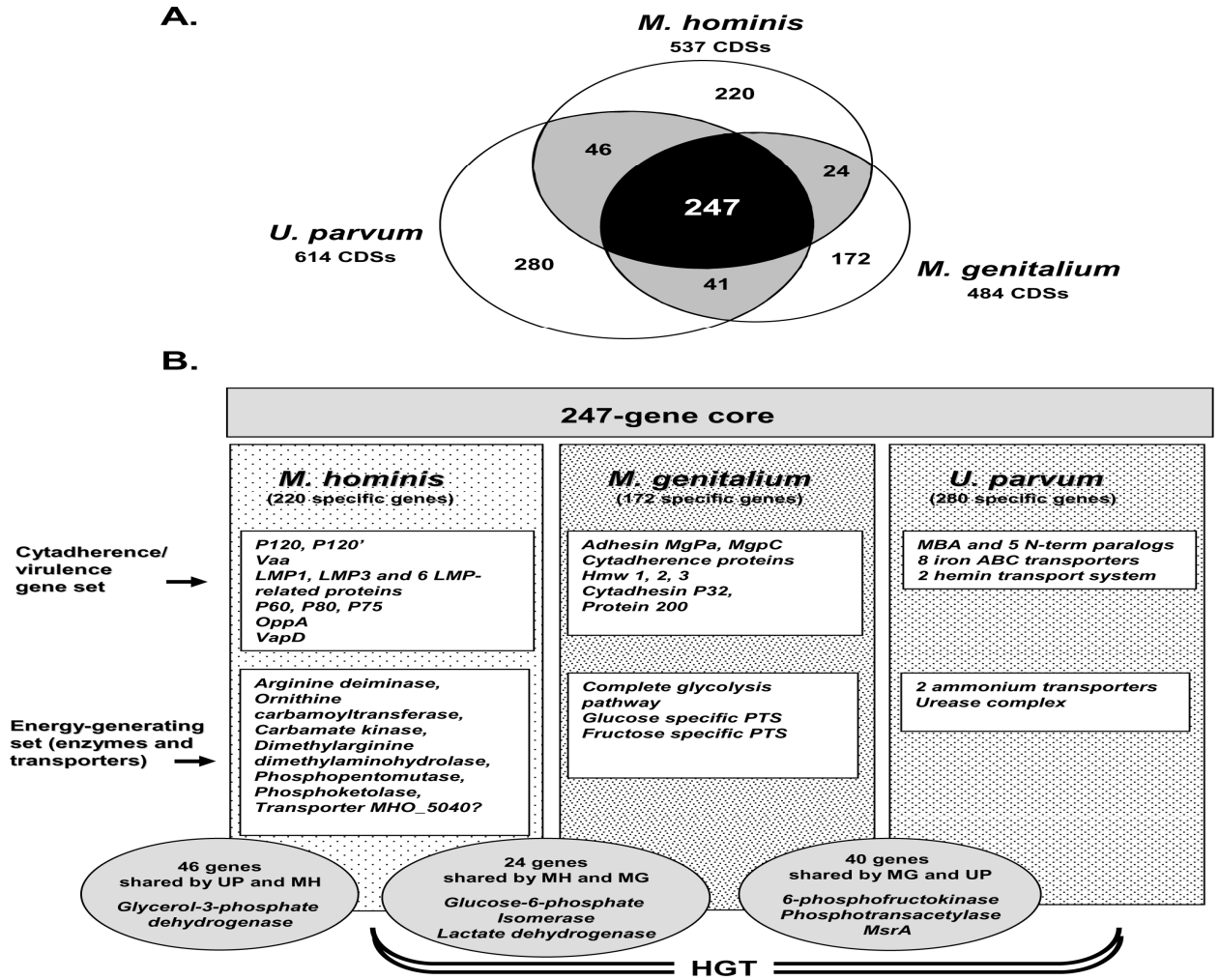

Figure 2.2: **(A) The number of specific and shared CDSs for the genomes of *M. hominis* PG21, *M. genitalium* G37, and *U. parvum* serovar 3. (B) A schematic focus on the known species-specific proteins involved in cytadherence/virulence and energy-generating pathways (Pereyre *et al*., 2009).**

Ureaplasma parvum has 280 specific genes (Pereyre *et al*., 2009). This includes eight iron transporters (UU022, UU023, UU025, UU357, UU358, UU400, UU515, UU516) (Figure 2.2), nine putative ABC substrate-binding protein-iron (UU024, UU027, UU028, UU069, UU071, UU359, UU360, UU401, UU517) and two hemin transporters (UU070, UU399) that are not found in *M. genitalium* or *M. hominis* (Pereyre *et al*., 2009).

Figure 2.3 hows an integrated view of the metabolism and transporters of *M. hominis*, *M. genitalium* and *U. parvum*. The metabolic products are shown in black. Putative proteins present in *M. hominis* are shown in green; proteins absent from *M. hominis* are shown in red. ‘‘MG’’ in purple boxes indicates that the gene encoding the corresponding protein is present in the *M. genitalium* G37 genome. ‘‘UP’’ in blue boxes means that the gene encoding the

corresponding protein is present in the *U. parvum* genome. Transporters are coloured according to their substrates: yellow, cations; green, anions and amino-acids; orange, carbohydrates; purple, multidrug and metabolic end product efflux. Arrows indicate the direction of substrate transport (Pereyre *et al*., 2009).

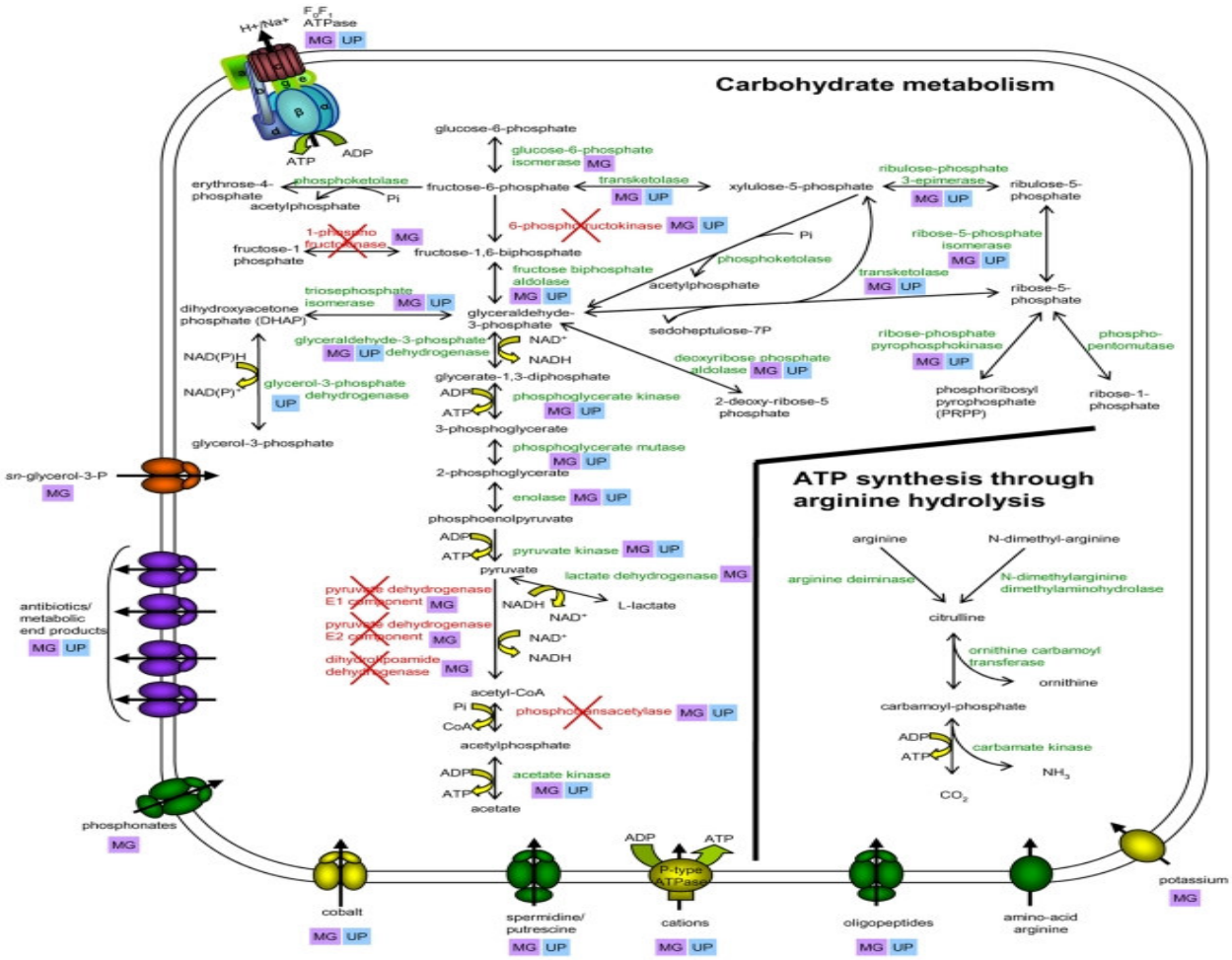

Figure 2.3: **Comparison of carbohydrate and arginine-metabolism pathways and transporters encoded by *M. hominis* PG21, *M. genitalium* G37, and *U. parvum* serovar 3 (Pereyre *et al*., 2009)**

Ureaplasma specific genes are involved in the major energy generating metabolic pathway, which generates ATP through urea hydrolysis. The seven components of the urease complex (UU428 to UU434) are the ammonium transporters Amt-1 and Amt-2 (UU133, UU219) and the atypical delta subunit of the F0F1-ATPase (UU133, UU134) (Figure 2.3) (Pereyre *et al*., 2009).

2.4.3 Morphological and physiological characteristics of the *Ureaplasma* species

The ureaplasmas are pleomorphic, nonmotile and lack a cell wall (Waites and Talkington, 2005). The bacterium has three organelles: cell membrane, ribosomes and a circular double-stranded deoxyribonucleic acid (DNA) molecule (Waites and Talkington, 2005). *Ureaplasma* does not use glucose, carbohydrates or arginine to generate adenosine triphosphate (ATP) (Waites and Talkington, 2005). The generation of ATP occurs by the use of the enzyme, urea hydrolase, to hydrolyse urea through a chemiosmotic mechanism (Waites and Taylor-Robinson, 2007). The metabolism of urea by *Ureaplasma* contributes to the pathogenicity of the bacteria (Mallard *et al*., 2005). The organism grows optimally under facultative anaerobic environmental conditions at 36°C to 38°C, at a pH of 6.0 to 6.5 (Shepard and Lunceford, 1976).

2.4.3.1 Synthesis of nucleic acids in *Ureaplasma* species

There are two pathways for the synthesis of DNA and those are *de novo* and the salvage pathways (Glass *et al*., 2000). The synthesis of DNA precursors by the *de novo* pathway uses different enymes for many reactions with small molecules, such as the amino acid, phosphoribosylprophosphate (PRPP), CO_2, ATP and NH_3 to manufacture ribonucleoside monophosphates (NMPs) (Nordlund and Reichard, 2006). The NMPs are phosphorylated to ribonucleoside diphosphates (dNDPs) and further reduced to deoxyribonucleoside diphosphates (dNDPs) by the enzymes ribonucleotide reductase (RNR) (Nordlund and Reichard, 2006).

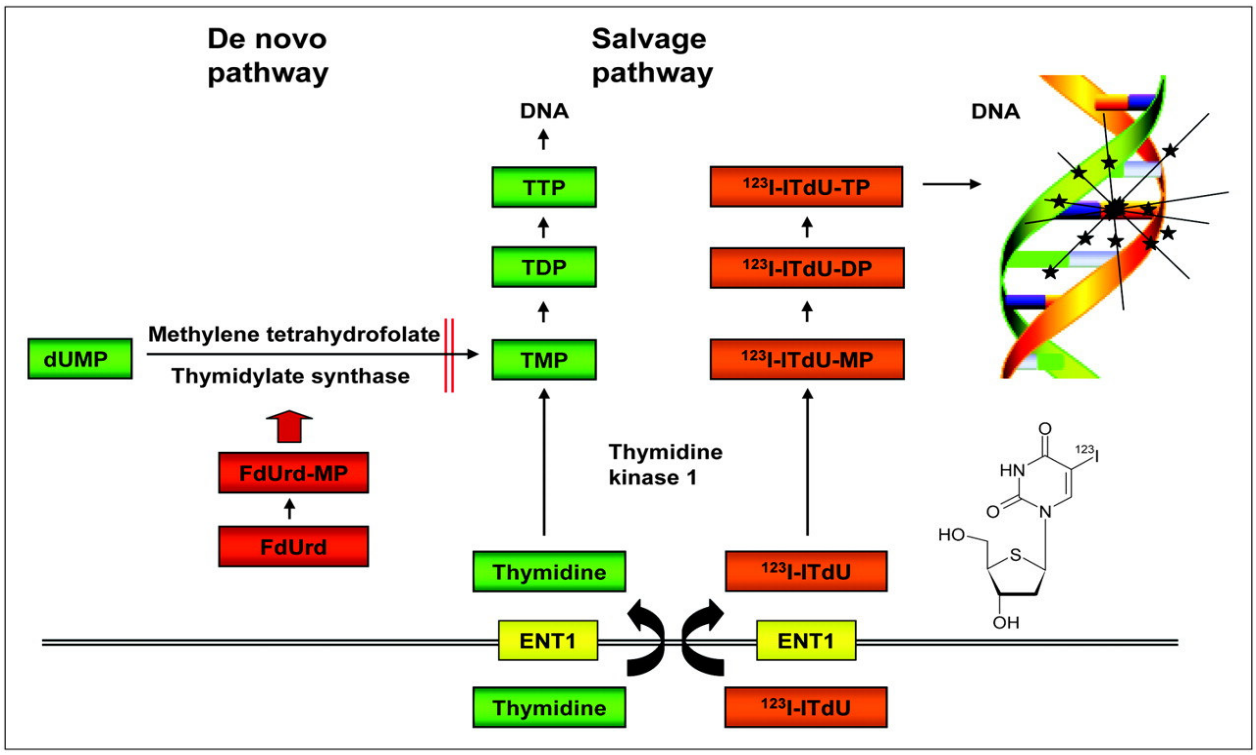

Figure 2.4: **A schematic presentation of the nucleoside synthesis, with the salvage pathway on the left and the *de novo* pathway on the right (Nordlund and Reichard, 2006)**

Ureaplasma species do not have genes that code for the *de novo* pathway that synthesize the RNA and DNA precursors (Glass *et al*., 2000). Therefore, the bacteria have to import all their DNA precursors or have a mechanism for converting ribonucleosides to deoxyribonucleosides (Glass *et al*., 2000). Ureaplasmas rely on the salvage pathway for the synthesis of DNA precursors (Glass *et al*., 2000). The salvage pathway synthesizes DNA by recycling bases from degraded DNA or RNA and reattaching these bases to the ribose unit (Figure 2.4) (Nordlund and Reichard, 2006).

2.4.3.2 The metabolic pathway of the *Ureaplasma* species

Ureaplasma species have a small genome size; as a result, most of their nutrients necessary for growth are imported from the host or from other bacteria (Sudhakar and Subramani, 2007). To achieve this, several transporters are required and ureaplasmas have 30 different transporters, which represent many different transporter families (Sudhakar and Subramani, 2007). Even though ureaplasmas have many different transport systems, some transport systems are missing, such as transporters for nucleotides, nickel and urea (Sudhakar and

Subramani, 2007). The missing transporters may be found among the hypothetical *U. urealyticum* proteins that have five or more predicted transmembrane regions (Sudhakar and Subramani, 2007). Transport systems are involved in the uptake of nutrients, ions, excretion of end products of metabolism and quorum sensing between cells and the environment (Figure 2.5) (Glass *et al*., 2000). Transporters transport metabolites and nutrients, such as amino-acids, polypeptides, deoxyribonucleosides or deoxyribonucleoside precursors, inorganic metal ions of cobalt, copper, iron, potassium and magnesium that function as cofactors for many enzymes (Sudhakar and Subramani, 2007).

In Figure 2.5, an integrated view of the main transporters and metabolism elements of an *U. urealyticum* cell is represented, deduced from the set of genes for which Glass *et al*. (2000) could predict functions. *Ureaplasma* proteins are shown in red and metabolic products in black. Question marks indicate enzymes/transporters not identified that would be required to complete pathways and missing enzyme and transporter names are shown in green italics. The various transporters are coloured according to the substrates they transport: yellow, cations; green, anions and amino acids; orange, carbohydrates; purple, multidrug and metabolic end product efflux. The direction of substrate transport is indicated by arrows. The transporters shown are as follows: (1) an F-type ATPase; (2) two Amt ammonium transporters; (3) P-type ATPase cation transporters; (4) a K^+ channel from the voltage-gated ion channel (VIC) superfamily; (5) a Magnesium MgtE transporter; (6) XasA a glutamate: GABA antiporter from the amino-acid-polyamine-organocation (APC) transporter superfamily; (7) two multidrug antimicrobial extrusion family transporters (MATE); (8) a ptsH element of a phosphoenolpyruvate-dependent sugar phosphotransferase transport system (PTS); and (9) an extensive collection of ABC transporters. For the ABC-type transporters rectangles are shown for the substrate-binding protein, diamonds for the membrane-spanning permeases and circles for the ATP-binding subunits (Glass *et al*., 2000)

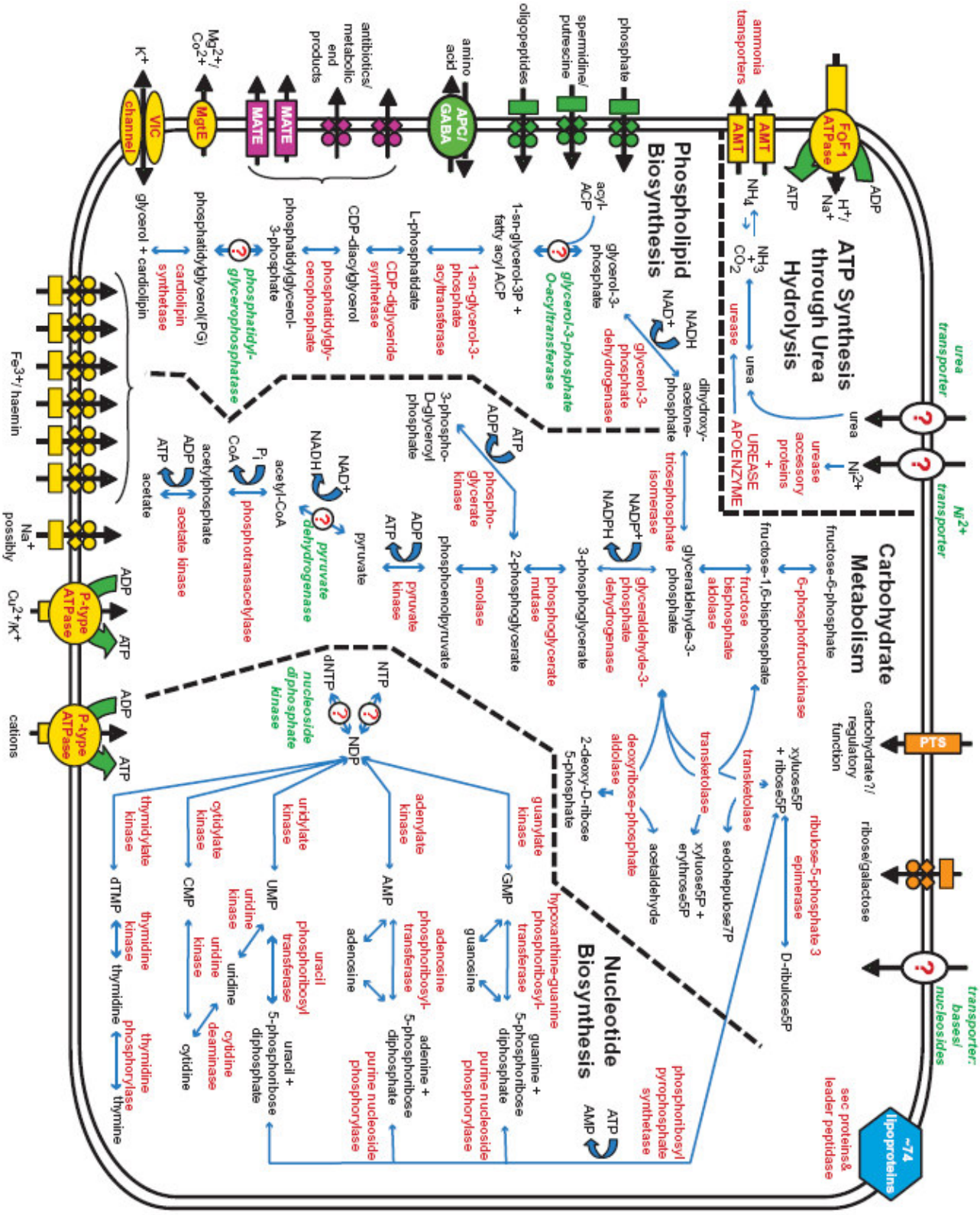

Figure 2.5: **Schematic presentation of the main transporters and metabolism elements of the *U. urealyticum* cells (Glass *et al.*, 2000)**

2.4.3.2.1 Ammonium transporters

Glass and colleagues (2000) have identified genes coding for the ammonia transporters that are energy-dependent in *Ureaplasma* spp. Ammonium transporters are a family of proteins, which transport ammonium ions across the cell membrane (Glass *et al*., 2000).

2.4.3.2.2 F-ATPase complex of transporters

The F-ATPases (F_1F_0-ATPases) are the prime producers of ATP, using the proton gradient generated by oxidative phosphorylation (Glass *et al*., 2000). The ATP molecules are synthesised through the formation of an electrochemical gradient through urea hydrolysis (Sudhakar and Subramani, 2007).

2.4.3.2.3 P-ATPase type cation transporters

The P-ATPases (E1E2-ATPases) are found in the plasma membranes of the bacteria and function to transport a variety of different ions across membranes (Sudhakar and Subramani, 2007). *Ureaplasma urealyticum* has several P-type ATPases including CopA, which is a copper-importing P-type ATPase, PacL that is a cation transport P-type ATPase and a Ca^{2+} ATPase, which catalyses a calcium ion antiport (Sudhakar and Subramani, 2007). Some enzymes of the P-ATPase family have been reported to flip phospholipids from the monolayer of the bilayer membrane to the other monolayer (Sudhakar and Subramani, 2007).

2.4.3.2.4 Voltage-gated ion channel

This channel usually consists of homotetrameric structures with each α-subunit possessing six transmembrane spanners (Sudhakar and Subramani, 2007). Potassium (K^+) channels have been shown to form tetramers that may function to maintain the membrane potential in the early stationary phase of growth (Sudhakar and Subramani, 2007).

2.4.3.2.5 Magnesium ion transporter

Magnesium transporters are proteins that transport magnesium across the cell membrane (Sudhakar and Subramani, 2007). In the bacteria, Mg^{2+} is mainly supplied by the CorA protein and where the CorA protein is absent, by the MgtE protein (Glass *et al*., 2000).

2.4.3.2.6 Amino acid-polyamine-organocation transporters

The APC superfamily of transport proteins includes members that function as solute that are cation symporters and solute antiporters (Glass *et al*., 2000). The APC are found in bacteria, archaea, yeasts and fungi and they vary in length being as small as 350 residues and as large as 850 residues (Glass *et al*., 2000).

2.4.3.2.7 Multiple-oligosaccharidyl-lipid-polysaccharide family

The MOP superfamily includes four distantly related families, amongst which is the MATE ubiquitous family, which is responsible for the export of drugs from the bacterial cell (Sudhakar and Subramani, 2007). All members of the MOP superfamily function as catalyst of multidrug efflux of their substrate by a cation antiport (Sudhakar and Subramani, 2007).

2.4.3.2.8 Phosphoenolpyruvate-dependent sugar phosphotransferase transport system

The PTS is a carbohydrate transport system in *Ureaplasma* that catalyzes the phosphorylation of incoming sugar substrates simultaneous with their translocation across the cell membrane (Sudhakar and Subramani, 2007). It plays an important role in the acquisition of sugars essential for growth and energy (Sudhakar and Subramani, 2007).

2.4.3.2.9 Iron transporters

Among the 19 ABC transporters, six are different iron (Fe^{3+}) and/or haemin transporters (Glass *et al*., 2000). The Fe^{3+} ion is essential for the function of enzymes, but the low solubility of Fe^{3+} at physiological pH forced the ureaplasmas to develop special mechanisms to acquire essential amounts of iron (Glass *et al*., 2000). It is speculated that ureaplasmas

increased its capacity to import iron by increasing the number of iron transporter genes (Sudhakar and Subramani, 2007).

2.5 Pathogenesis and virulence of *Ureaplasma* infections

Pathogenesis of *Ureaplasma* is determined by the presence of virulence factors, such as adhesins, human IgA protease, phospholipase A and C, urease, multiple banded antigen protein and hydrogen peroxide (Viscardi and Hasday, 2009). However, the existence of the interrelationships between the presence of these virulence factors in *Ureaplasma* and the incidence of diseases in humans has not yet been demonstrated (Momynaliev *et al*., 2007). The difficulty in the clarification of these interrelationships may be due to macro and microgenomic heterogeneity of the bacteria (Momynaliev *et al*., 2007). It is believed that a combination of variable strain-specific genes in the *Ureaplasma* with known virulence factors determine the development of pathological processes on the mucosal surface of the human urogenital tract (Momynaliev *et al*., 2007).

The adhesins allow the attachment of the bacteria to the genital and respiratory mucosal cell (Viscardi and Hasday, 2009). The attachment allows the bacteria to withstand the clearing action of urination by the host (Waites *et al*., 2005). After attachment, the bacteria primarily reside on the mucosal membrane of the urogenital tract of the host (Waites *et al*., 2005). After the bacteria have attached, immunoglobulin A (IgA) protease is secreted to cleave the IgA 1 allowing the bacteria to evade the host's immune defence (Viscardi and Hasday, 2009). Inside the cell, the bacteria secrete products, such as urease, hydrogen peroxide and phospholipids, which injure the mucosal cells (Waites *et al*., 2005). Urease production by *Ureaplasma* spp causes a breakdown of urea to ammonia and carbonate and this carbonate spontaneously decomposes to form another molecule of ammonia and carbonic acid, which results in an increase in pH (Viscardi and Hasday, 2009). An increase in extracellular pH stimulates the pathogenecity of *Ureaplasma* spp by damaging mucosal cells (Viscardi and Hasday, 2009). The ureaplasmal multiple banded antigen (MBA) contains both serovar-specific and cross-reactive epitopes and it is the predominant antigen recognized during ureaplasmal infections in humans (Viscardi and Hasday, 2009). It exhibits highly variable sizes *in vitro* and in isolates *in vivo*, suggesting that antigen size variation may be another mechanism through which the bacteria evade host defences (Viscardi and Hasday, 2009).

2.6 Host defence mechanism to *Ureaplasma* species

When ureaplasmas invade the mucosal barrier, the host secretes antibody IgA to prevent the invasion; however, this process can fail because the bacteria has IgAse to break down the IgA antibody (Waites *et al*., 2005). Failure of IgA to prevent the bacteria from entering the cell can lead to the production of cytokines that trigger the recruitment and activation of phagocytic cells that engulf and kill the bacteria (Barton *et al*., 2003).

2.7 Mode of transmission of genital *Ureaplasma* species

Ureaplasma spp are transmitted sexually (genital-to-genital or oral-to-genital), depending on the number of sexual partners and the frequency of sexual intercourse (Drew and Sherrard, 2008). Colonization rates may increase to more than 80% in sexually active individuals (Drew and Sherrard, 2008). Children acquire *Ureaplasma* by direct contact vertically from the mother at either birth or *in utero* (Drew and Sherrard, 2008). Some people acquire it nosocomially through transplanted tissues (Murray *et al*., 2007). Vertical transmission of ureaplasmas has been reported to range from 18% to 88% and isolation rates vary inversely with gestation age (Kafetzi *et al*., 2004; Pinna *et al*., 2006).

2.8 Clinical manifestation of *Ureaplasma* species

It has been difficult to associate specific disease with *Ureaplasma* spp due to the high colonization rate of the bacteria in the urogenital tract (Ryan and Ray, 2004). Members of the *Ureaplasma* species cause urethritis in men, which is characterized by burning urine, white or cloudy discharge and the feeling of passing urine frequently, irritation in the penis, tenderness and staining of the underwear (Table 2.3) (Bradshaw *et al*., 2006).

Table 2.3: **Conditions/diseases that are generally caused by or associated with *Ureaplasma* species (Volgmann *et al*., 2005; Waites *et al*., 2005; Waites *et al*., 2009)**

Disease	*Ureaplasma spp*
Male urethritis	+
Urinary calculi	+
Prostatitis	±
Spermocystitis	+
Epididymitis	±
Pelvic inflammatory disease	±
Cervicitis	±
Bacterial Vaginosis	±
Infertility	±
Chorioamnionitis	+
Spontaneous abortion	+
Prematurity/low birth weight	+
Intrauterine growth retardation	±
Extragenital disease	+
Neonatal meningitis	±
Respiratory distress syndrome (RDS)	±

+: causal role; ±: significant association and/or strong suggestive evidence, but causal role not proven

Ureaplasmas are unlikely to gain access to the prostate during an acute ureaplasmal infection but when the bacteria do gain access, they cause acute urethra-prostatitis (Bradshaw *et al*., 2006). *Ureaplasma* species are associated with infection stones (Bradshaw *et al*., 2006). Production and subsequent release of ammonia in the urinary tract causes precipitation of magnesium ammonium phosphate, better known as struvite and possibly result in struvite stones, which has only been shown in rats (Waites *et al*., 2005).

Ureaplasma species have been shown to cause infertility in men (Table 2.3) (Waites *et al*., 2009). It is believed that colonization of ureaplasmas can lead to disturbances in spermatogenesis, sperm function and sperm transport (Volgmann *et al*., 2005; Waites *et al*., 2009). The bacterium has been reported to reduce both sperm motility and count and may increase sperm abnormality (Waites *et al*., 2009). It has also been found that ureaplasma infections can lead to the deterioration of semen density and sperm vitality (Waites *et al*., 2009). However, some studies have established that the presence of ureaplasmas in sperm specimens has no real effect on the semen quality (Waites *et al*., 2005; Gdoura *et al*., 2008).

In women, ureaplasmas colonize the vagina and cervix and the bacteria are associated with preterm labour, spontaneous abortion and chorioamnionitis (Kim *et al*., 2003; Taylor-Robinson, 2007). In the neonate, ureaplasmas are associated with manifestations, such as congenital pneumonia and neonatal meningitis (Table 2.3) (Waites *et al*., 2009). Ureaplasmas have been associated with pneumonia and chronic lung disease in newborn babies (Schelonka and Waites, 2007).

The association of *Ureaplasma* spp with bacterial vaginosis (BV) is conflicting amongst studies (Table 2.3) (Waites *et al*., 2005). Keane and colleagues (2000) detected no difference in the occurrence of *M. genitalium* and *Ureaplasma* spp in women with or without bacterial vaginosis. However, Cedillo-Ramirez and colleagues (2000) isolated *M. hominis* and *Ureaplasma* spp from 17% and 53% respectively in women with BV, compared to 2% and 3% respectively from women without BV.

Mycoplasmas may be co-factors in the progression of HIV disease in the human body (Jian-Ru *et al*., 2011). Jian-Ru and colleagues (2011) conducted a study in China on the prevalence of *U. urealyticum* and *M. hominis* in 497 men with HIV/AIDS. The study showed that *M. homins* and *U. urealyticum* are prevalent in HIV infected male patients. The prevalence of *M. hominis* and *U. urealyticum* was 29.2% and 27.2% respectively. The high prevalence of mycoplasmas and ureaplasmas in HIV infected patients indicated that these bacteria might be co-factors in the progression of HIV disease (Jian-Ru *et al*., 2011).

2.9 Laboratory detection of *Ureaplasma* species

Detection of *Ureaplasma* species is based on phenotypic and genotypic methods. The method that is commonly used for the detection of *Ureaplasma* species is culture by using both liquid and solid media (Pinna *et al*., 2006). However, because the *Ureaplasma* species are fragile to adverse conditions, culture methods are difficult to perform and take five days (Pinna *et al*., 2006). Molecular methods have been developed to solve the problem of culture and have shown to be very sensitive and rapid (Nelson *et al*., 1998).

2.9.1 *In vitro* growth of *Ureaplasma* species

Phenotypic investigation of *Ureaplasma* species is based on growth in Shepard's U9 broth and growth on Shepard's A2, A7 and A8 agar media (Waites *et al*., 2005). The specimen is first grown in Shepard's U9 broth, which is supplemented with horse serum as a source of sterols, yeast extracts, urea (substrate), antibiotics and antifungals to inhibit other microorganisms and phenol red as a pH indicator (Shepard, 1954). If the U9 broth with *Ureaplasma* spp is incubated at 36ºC for 48 hours, urease in the bacteria hydrolyzes urea in the broth to ammonia, which makes the media alkaline and changes the colour from yellow to pink or red due to the presence of phenol red indicator in the media (Pinna *et al*., 2006). The *Ureaplasma* spp deplete urea in the medium fast, which leads to the death of the bacteria (Pinna *et al*., 2006). Broth culture should be examined for colour changes twice a day for up to seven days because of the rapid death phase of the bacteria in culture (Pinna *et al*., 2006). It is therefore important to subculture the bacteria on Shepard's A2, A7 and A8 agar, which are selective media for ureaplasmas and *M. hominis* (Pinna *et al*., 2006). The growth on agar media is examined for small, irregular (roughly spherical) colonies, which grow downward into the agar using a stereomicroscope at 20X or 60X magnification (Figure 2.6) (Waites *et al*., 2005).

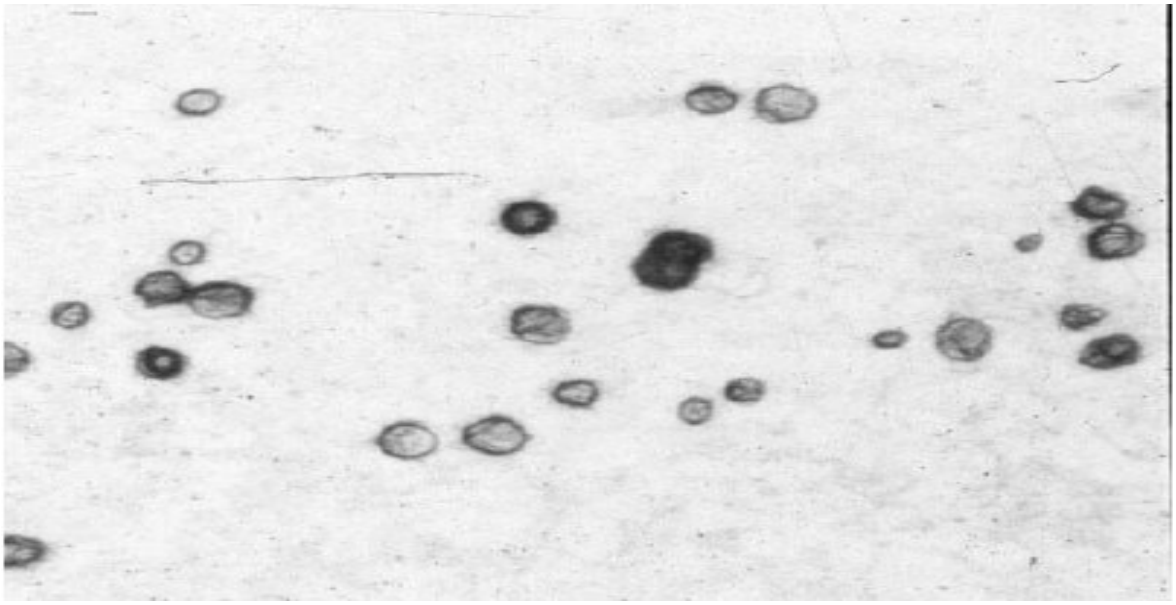

Figure 2.6: Typical colony morphology of *Ureaplasma* species on A8 solid media (Waites *et al*., 2005)

Culture methods are considered as the "gold standard" but have limited identification ability and the organism can be positively identified to genus level only by observing colony morphology and urease production (Waites *et al*., 2005). The "gold standard" method is time-consuming, since the bacterium is difficult to isolate and requires two to seven days incubation before the results are available to the clinicians (Gdoura *et al*., 2007). The culture methods are expensive, since it requires special media and is normally used in reference laboratories (Waites *et al*., 2005).

2.9.2 Commercial kits for the *in vitro* growth of *Ureaplasma* species

Commercial diagnostic test kits for the detection, quantification and susceptibility testing of *Ureaplasma* spp have become available and have made the routine *Ureaplasma* diagnosis much easier (Govender *et al*., 2010). Some of the available kits are the Mycofast evolution 3 (bioMérieux, France), Mycoview (Ivagen, China) and MycoDuo (Bio-Rad, France) kits.

The Mycofast evolution 3 (bioMérieux, France) kit identifies, enumerates and performs antibiotic susceptibility of *U. urealyticum* and *M. hominis* from urogenital specimens (Figure 2.7). The kit is based on the natural resistance of *U. urealyticum* to lincosamide and the erythromycin resistance of *M. hominis* as well as the hydrolysis of ammonia by the urease enzyme of *U. urealyticum* (bioMérieux, France). Growth of the bacteria is indicated by a colour change of the medium by the pH indicator (Phenol red) after 24 to 72 hours of incubation.

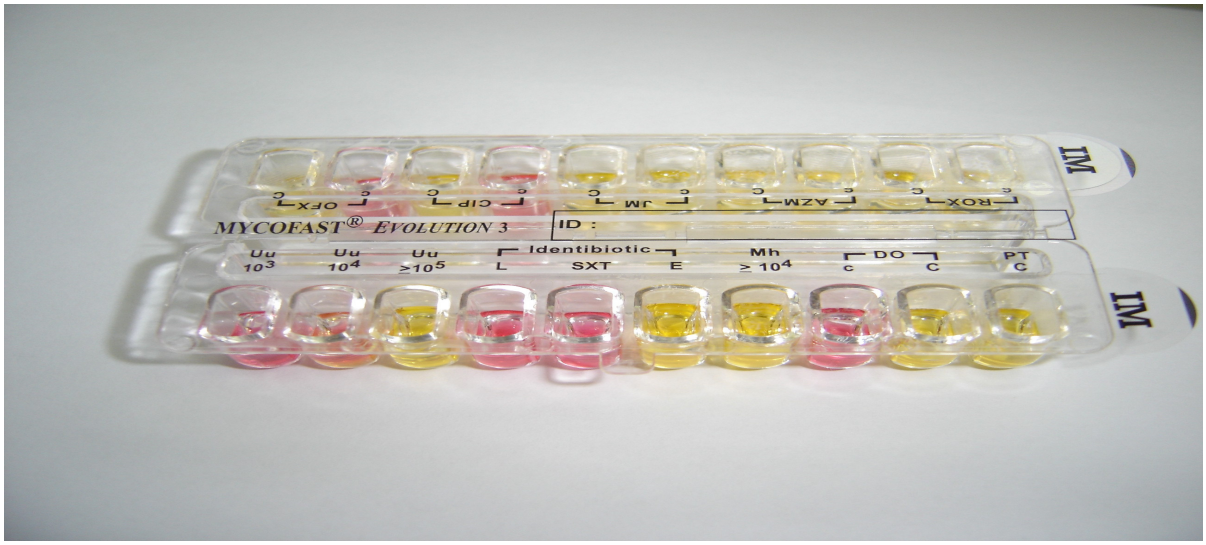

Figure 2.7: **Mycofast Revolution 3 strip positive results are indicated by a pink colour while negative results are indicated by a yellow colour**

The susceptibility test includes seven antibiotics at two different concentrations. The results are available within 24 to 48 hours (Abele-Horn *et al*., 1996; Clegg *et al*., 1997). The performance of this kit is similar to standard media (Pinna *et al*., 2006).

The MycoDuo (Bio-Rad, France) kit enables the identification, culture and differential titration of *Ureaplasma* species and *M. hominis*. The kit is based on the urea and arginine metabolism and the results are indicated by a colour change from yellow to pink due to the pH indicator in the medium (Figure 2.8) (Govender *et al*., 2010).

Figure 2.8 (a) **Figure 2.8 (b)**

Figure 2.8: The MycoDuo kit: with 2.8 (a) showing a *Ureaplasma* spp positive result for a high titre and 2.8 (b) showing a *Ureaplasma* spp positive result at a low titre (Govender *et al*., 2010)

The results are available in 24 to 48 hours. This kit does not perform susceptibility testing (Govender *et al*., 2010). The overall agreement between the Takara PCR kit and the MycoDuo assay of *Ureaplasma* spp was determined to be 96% by Govender *et al*. (2010).

The MycoView test kit enables identification and differential titration of *U. urealyticum* and *M. hominis* from different urogenital specimens. The kit is also based on selective media and enzymatic colour changes. The performance of the kit is almost the same as the Mycofast evolution 3 kit, except that the antimicrobial susceptibility test includes nine antimicrobial drugs, whereas the Mycofast evolution 3 kit includes seven drugs (Pinna *et al*., 2006).

Commercial kits are easier to use compared to traditional culture methods and have shorter incubation times (Abele-Horn *et al*., 1996, Cunliffe *et al*., 1996, Govender *et al*., 2010). The

kits are useful in laboratories that do not specialise in *Ureaplasma* detection (Pinna *et al*., 2006). However, problems, such as contamination with the use of the kits, have been encountered (Pinna *et al*., 2006).

2.9.3 Immunotyping of *Ureaplasma* species

Serological methods are important for the typing of *Ureaplasma* species in the diagnostic laboratories (Daxboeck *et al*., 2003). Monoclonal and polyclonal antibodies enable the detection of the multiple-banded antigens responsible for serotype specificity on the cell surface (Vancutsem *et al*., 2008). The serotyping methods are time-consuming, difficult to interpret; results are inconclusive because of multiple cross-reactions, are not reproducible and have a poor discriminating capacity when used with samples containing two or more serotypes (Vancutsem *et al*., 2008; Xiao *et al*., 2010). To overcome these problems an enzyme-linked immunosorbent assay was developed using monoclonal antibodies (Mabs) for the serotyping of *Ureaplasma* strains and promising results have been obtained using reference strains (Xiao *et al*., 2010). However, the lack of standardization and availability of serotyping assays limit the number of laboratories that use these assays for the serotyping of *Ureaplasma* species (Xiao *et al*., 2010).

2.9.4 Molecular detection and characterisation assays for the genus *Ureaplasma*

Molecular assays have been used as an alternative method for the identification of the slow-growing, fastidious *Ureaplasma* species (Robertson *et al*., 1993). Molecular assays include both conventional and real-time PCR assays. These methods are reported to be rapid and accurate (Nelson *et al*., 1998). Molecular assays are more frequently used in research laboratories than diagnostic laboratories in South Africa. This is due to the high cost of molecular assays compared to traditional culture methods. Detection of *Ureaplasma* species using polymerase chain reaction assays takes two to four hours in comparison with two to seven days by the conventional culture methods (De Francesco *et al*., 2009).

Commercial PCR kits, such as Takara's *Mycoplasma* PCR detection kit (Takara Bio Inc, Japan) have been used and it has been shown to be rapid and accurate for the detection and identification of *Mycoplasma* and *Ureaplasma* species (Govender *et al*., 2010). Govender and colleagues (2010) compared the kit with the Mycoview and Mycoplasma Duo kits and the

Takara PCR kit showed 100% sensitivity and 100% specificity. The Takara PCR kit cannot differentiate between *U. urealyticum* and *U. parvum*. The *Ureaplasma urealyticum* real-time PCR kit (Zhangai Diagnostics, China) detects and quantifies ureaplasmas in one hour. The PrimerDesign™ Genesig kit (Biosearch Technologies Inc, UK) was designed for the detection and quantification of *U. urealyticum*. It is based on TaqMan chemistry and it targets the urease complex (*ure*G) gene (Shangai Diagnostics, China). The kit also has uracil-N-glycosylase to prevent carry over contamination and contains 150 tests compared to the *Ureaplasma urealyticum* real-time PCR kit (Shangai Diagnostics, China) that contains 25 tests; however, both kits contain an internal control.

2.9.4.1 Conventional PCR assays for the detection of *Ureaplasma* species

Conventional PCR assays detect and identify human *Ureaplasma* spp targeting the 16S rRNA gene (Mallard *et al*., 2005), 16S-23S rRNA intergenic spacer region (Cultrera *et al*., 2006; Kong *et al*., 2000), the urease (*ure*) gene (Blanchard, 1990) and the *mba* gene (Robertson *et al*., 2000). Conventional PCR assays have been widely investigated for the detection of *Ureaplasma* species with sensitivity values ranging from 90% to 95% and in every investigation PCR positive, culture negative results were found (Kong *et al*., 2000; Colaizy *et al*., 2003; Mallard *et al*., 2005). All of the PCR-based methods reported so far have lacked the capacity for the complete characterization of all 14 *Ureaplasma* serotypes (Mallard *et al*., 2005).

2.9.4.2 Real-time PCR assays for *Ureaplasma* species detection

Real-time PCR assays are accurate and rapid techniques for the use in the clinical diagnostic setting and for research applications (Colaizy *et al*., 2003). The assays have been widely investigated and have been shown to be useful for the simultaneous detection and serotype characterization of ureaplasmas in clinical specimens (Coa *et al*., 2007b; Xiao *et al*., 2010). The genes targeted for the detection and characterization of serotypes of *Ureaplasma* species are the urease (Coa *et al*., 2007b) and multiple banded antigen (Coa *et al*., 2007a; Xiao *et al*., 2010) genes.

Yi and colleagues (2005) in South Korea developed a real-time PCR assay targeting the urease gene to detect *Ureaplasma* spp and to determine the serotypes simultaneously. Eighty-

seven clinical specimens (amniotic fluid, cord blood, vaginal swab) were tested for *Ureaplasma* spp using culture, conventional PCR and real-time PCR. When compared with conventional PCR, sensitivity and specificity of the real-time PCR were 89.5% and 98.5% respectively, while the values of culture were 47.4% and 100%, respectively. Of the 18 clinical specimens that were found positive and speciated by the real-time PCR, *U. parvum* was 66.7% and *U. urealyticum* was 33.3%. This study showed that the real-time PCR assay can be useful for the simultaneous detection and biovar discrimination of *Ureaplasma* spp in clinical specimens and a further study to quantify *Ureaplasma* spp could be facilitated on the basis of this method.

Cao and colleagues (2007a) developed a multiplex real-time PCR assay based on TaqMan chemistry for the simultaneous detection and characterization of *U. parvum* serovars targeting the *mba* gene. Two serotypes were successfully detected in one reaction and the detection rate of ureaplasmas was rapid compared to the conventional culture method (Cao *et al*., 2007a). In the same year, Cao and colleagues (2007b) developed a real-time Taqman PCR assay for the quantitative detection and differentiation of *Ureaplasma* spp targeting the urease gene. The assay proved to be rapid (1.5 hours), specific and with convenient differentiation of the *Ureaplasma* spp (Cao *et al*., 2007b).

Yu and colleagues (2008) conducted a study on 98 symptomatic and 236 asymptomatic men to determine the association of *M. genitalium* and *U. urealyticum* with gonococcal urethritis in a sexually transmitted infection clinic in Hong Kong. Two real-time PCR assays were used to differentiate and to detect *U. urealyticum* and *M. genitalium*. The real-time PCR assay detecting *U. urealyticum* was specifically designed to target the urease gene by using two TaqMan minor groove binding primer and probe sets. *Mycoplasma genitalium* was detected by a TaqMan minor groove binding real-time PCR assay targeting a conserved region of the major antigen of *Mycoplasma genitalium* (MgPa) adhesion gene. The real-time PCR assays detected *U. urealyticum* in 22% (22/98) symptomatic men and 20% (47/236) asymptomatic men. *Mycoplasma genitalium* was detected in 10% (10/98) symptomatic men and 2% (5/236) asymptomatic men with no significant difference. The real-time PCR assays were rapid, easy to perform and not prone to contamination (Yu *et al*., 2008).

Xiao and colleagues (2010) designed primers and probes for the detection and discrimination of *U. parvum* and *U. urealyticum*, including the 14 serotypes by real-time PCR targeting the MBA gene. The sensitivity and specificity of the assays were 96.9% and 79.0% respectively compared to the culture method. This was the first described real-time PCR assay to discriminate all 14 serotypes and the assay provided a quick and reliable means for investigation of the epidemiology and pathogenecity of ureaplasmas at the serotype level (Xiao *et al*., 2010).

The sensitivity of real-time PCR assays for the detection of *Ureaplasma* spp ranges from 90% to 100% compared with culture methods, which is about 80% (Colaizy *et al*., 2003). Given the high sensitivity of PCR, false positivity is common compared to culture methods due to the fastidious nature of the bacteria (Colaizy *et al*., 2003). Real-time PCR assays are easier to perform, there is no handling of the amplification product and it needs small quantities of DNA to amplify (Yi *et al*., 2005; Cao *et al*., 2007a). The assays help in the early initiation of appropriate treatment and control of infection (Cao *et al*., 2007a; Xiao *et al*., 2010).

2.10 Treatment of *Ureaplasma* species

Ureaplasma species are naturally resistant to all beta-lactams and cephalosporins because of their lack of a cell wall; however, agents that target protein synthesis are effective for the treatment of *Ureaplasma* species (Bébéar and Kempf, 2005). Ureaplasmas are susceptible to macrolides, streptogramin combinations and ketolides but they are intrinsically resistant to lincosamides (Bébéar and Kempf, 2005). Doxycycline, tetracycline (in adults) and erythromycin (pregnant women and neonates) remain the drugs of choice for the treatment of *Ureaplasma* species (Waites *et al*., 2008). Fluoroquinolones and chloramphenicol are alternative drugs used for the management of *U. urealyticum* infections (Waites *et al*., 2008). Tetracycline is, however, associated with bone and teeth deposition during calcification, whereas chloramphenicol causes bone marrow toxicity, therefore, the doses of these drugs should be monitored (Mims *et al*., 2004). Fluoroquinolones, such as ciprofloxacin have less side effects than intravenous erythromycin (Aujard *et al*., 2005) but the high level of quinolone resistance in *Ureaplasma* is a major problem, which makes ciprofloxacin not a good drug for treatment (Waites *et al*., 2009).

2.11 Antimicrobial susceptibility tests for *Ureaplasma* species

Antimicrobial susceptibility testing is routinely performed in diagnostic laboratories to determine the optimal antimicrobial treatment regimen for patients with *Ureaplasma* infections (Waites *et al*., 2009). Susceptibility testing has been a big challenge for diagnostic laboratories because currently there are no standardized methods for *Ureaplasma* antibiotic susceptibility (Waites *et al*., 2009). The *Mycoplasma* chemotherapy working team of the International Organisation for Mycoplasmology (IOM) is working on guidelines for the susceptibility testing of mycoplasmas and ureaplasmas (Duffy and Waites, 2008). Most laboratories rely on commercial kits as discussed in section 2.9.2. The kits are based on the broth dilution method, which uses pH changes to show susceptibility or resistance of an organism towards a certain antibiotic (Dosa *et al*., 1999). The disc diffusion method cannot be used because *Ureaplasma* species and *M. hominis* grow slowly and the antibiotic concentration equilibrates throughout the agar medium before colonies appear (Dosa *et al*., 1999). The E-test method is widely used to test susceptibilities of quinolones, tetracycline and macrolides of *Ureaplasma* spp (Figure 2.9) (Ngan *et al*., 2004).

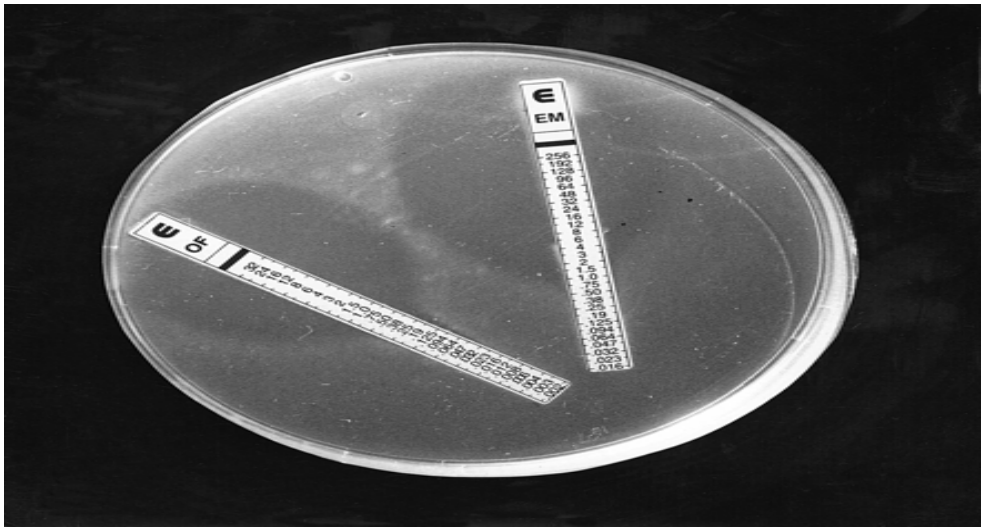

Figure 2.9: **Etest MICs of ofloxacin (OF) and erythromycin (EM) for a clinical isolate of *Ureaplasma urealyticum* (Dosa *et al*., 1999)**

The method can only be used if there is a small number of test isolates since it is expensive (Ngan *et al*., 2004). In 1999, Dosa and colleagues showed that the performance of the Etest is similar to broth dilution for the MIC determination of *Ureaplasma* spp. Different studies have validated the performance of the Etest for susceptibility testing of slow-growing organisms, such as mycoplasmas (Citron *et al*., 1991; Waites *et al*., 1997; Dosa *et al*., 1999).

The Etest is an agar-based MIC method, which uses a plastic strip with a preformed exponential concentration gradient of an antibiotic (Dosa *et al*., 1999).

2.12 Antimicrobial drug resistance

Bacteria resist antimicrobials by certain mechanisms that include intrinsic (natural) resistance, changing the cell membrane, which leads to impermeability and active efflux of the antibiotics (Mims *et al*., 2004). The bacteria can also change the antibiotic target, produce enzymes that inactivate the antibiotic and change the metabolic pathway (Ryan and Ray, 2004).

Tetracyclines are the first line treatment of human urogenital infections caused by *Ureaplasma* species (Bébéar and Kempf, 2005). The drug acts as a protein synthesis inhibitor. It inhibits the binding of aminoacyl tRNA to the mRNA-ribose complex (Mims *et al*., 2004). Roberts and Kenny first described *Ureaplasma* isolates resistant to tetracycline in 1986. The *tet*M gene on a transposable element usually codes for resistance of bacteria to tetracycline (Mims *et al*., 2004). Tetracycline is pumped out of the resistant cells (Mims *et al*., 2004).

Fluoroquinolones are a synthetic class of antibiotics, which inhibit the activity of DNA topoisomerase (gyrase); the enzyme involved with the decoiling, supercoiling and sealing of gaps of bacterial DNA during replication (Mims *et al*., 2004). Bacterial resistance to fluoroquinolones results from amino acid substitution at Asp112Glu in *gyr*A and Ala125Thr, Ala136Thr, Ser83Leu and Asp82Asn in *par*C (Duffy *et al*., 2006; Beeton *et al*., 2009).

Erythromycin and clarithromycin resistance have been reported amongst *Ureaplasma* species (Beeton *et al*., 2009). In a study done by Beeton *et al*. (2009) a mutation on the L4 protein caused by a six base pair deletion led to the loss of arginine and glutamine at residue numbers 66 and 67. Methyltransferase enzyme modification on macrolides has not yet been identified in ureaplasmas (Beeton *et al*., 2009).

2.13 Prevention and control of *Ureaplasma* species

Ureaplasma species are frequently carried in the lower urogenital tract in individuals who are asymptomatic and sexually active, therefore, the use of barrier protection methods, such as condoms are of little or no benefit because the bacteria act as opportunistic normal flora (Waites *et al*., 2005). Since the organisms can occur *in utero* by ascending infection and through intact foetal membranes, delivery of infants by Caesarian section has not prevented colonization in the respiratory tract (Waites *et al*., 2005). There are no current vaccines available for the prevention of *Ureaplasma* infection (Waites *et al*., 2005).

2.14 Summary

Members of the *Ureaplasma* genus are the smallest, free-living, self-replicating prokaryotic cells with double-stranded DNA genomes (Harvey *et al*., 2007). *Ureaplasma* spp are part of the Gram-positive bacteria; however, lacking a bacterial cell wall, which means when a Gram-stain is performed it will take on the colour of the counterstain rather than the crystal violet (Kong *et al*., 2004).

Genital ureaplasmas are a common inhabitant of the genital tract of sexually active men and women; however, approximately 50% of cases of non-chlamydial, non-gonococcal urethritis in men are caused by genital ureaplasmas (Salari and Karimi, 2003). It accounts for 15% of male infertility (Salari and Karimi, 2003).

Macrolides are the most promising antimicrobial agents available for the treatment of infectious mycoplasmas and ureaplasmas (Bébéar, *et al*., 2000). It is important that *in vitro* susceptibility results should be obtained if a tetracycline is used due to the possibility of resistance to this class of antimicrobials (Neal *et al*., 1994).

Detection of *Ureaplasma* species in clinical specimens rely on specific culture techniques (Mallard *et al*., 2005). Although culturing is considered as the “gold standard”, it has shown many limitations (Stellrecht *et al*., 2004). Cultivating *Ureaplasma* spp in the laboratory is time-consuming (two to seven days for growth), lacks sensitivity and requires repeated microscopic observations (Teng *et al*., 1994). It is a relatively expensive technique because it

requires special media, a high degree of expertise and special handling of specimens (Dhawan *et al*., 2006).

Polymerase chain reaction assays have been developed for the detection of ureaplasmas targeting the urease, multiple banded antigen (MBA) and 16S rRNA genes, which are more sensitive and specific when compared to culture methods (Mallard *et al*., 2005). The assays proved to be equal to if not superior to the traditional selective culture techniques (Petrikkos *et al*., 2006). Species can be identified within eight hours and the bacteria can be identified to serotype level (Coa *et al*., 2007a; Coa *et al*., 2007b, Xiao *et al*., 2010). The PCR assays are highly specific, easy to perform and requires minimum preparation of the clinical specimen (Mallard *et al*., 2005). In this study, the aim was to detect *Ureaplasma* species and to characterize *Ureaplasma parvum* in men with and without urogenital symptoms using culture and real-time PCR assays.

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CHAPTER 3

Characterization of *Ureaplasma parvum* Isolates from the Urethra of Men Attending a Family Practice

In the following chapter, the editorial style of the *FEMS Immunology and Microbiology* was followed.

3.1 ABSTRACT

Background: The genus *Ureaplasma* colonizes human mucosal surfaces, such as the urogenital tract of men and women. In adult men, it has been implicated in the aetiology of non-gonococcal urethritis and infertility. Although its pathogenesis is not yet fully understood, it has been suggested that certain serotypes are associated with disease. This study undertook to detect genital *Ureaplasma* species and to characterize *U. parvum* in men with and without urogenital symptoms.

Methods: Two hundred first-void urine specimens were collected from symptomatic (100) and asymptomatic (100) men attending a private clinic. All specimens were cultured in U9 broth and subcultured on A2 agar medium for confirmation of growth. All isolates were tested for susceptibility using the Mycofast Evolution 3 kit. DNA was extracted from all specimens and amplified using a multiplex TaqMan polymerase chain reaction assay targeting the multiple-banded antigen gene for the detection and serotyping of *U. parvum*. *Ureaplasma urealyticum* was detected by a commercial real-time PCR kit.

Results: A total of 28 specimens 16/100 symptomatic and 12/100 asymptomatic men were positive (difference not significant). All isolates were susceptible to doxycycline, pristinamycin, roxycycline and azithromycin. One *Ureaplasma* isolate from an asymptomatic male was resistant to ciprofloxacin and josamycin and intermediately resistant to ofloxacin, while another isolate also from an asymptomatic male was resistant to ofloxacin. An isolate from a symptomatic male was resistant to ciprofloxacin. Molecular assays showed no significant difference (p=0.16) between the *U. parvum* isolated from symptomatic 11% and asymptomatic 18% men as well as for *U. urealyticum* from symptomatic 16% and asymptomatic 15/100 men (p=0.86). Four men (two from each group) were colonized by

both *Ureaplasma* species. The predominant serotype was 6, followed by types 1, 3 and 14 with no significant difference between symptomatic and asymptomatic men (p=0.309).

Conclusions: This is the first report of circulating *U. parvum* serotypes in South Africa. The prevalence rate of *Ureaplasma* species in this study was relatively lower and no significant differences were found between symptomatic and asymptomatic men for either *Ureaplasma* species. Serotype 6 was the most common type detected in this study and this differs from reports from the developed countries, where serotype 3 was reported as the most common. Macrolides and tetracyclines remain effective drugs for treatment of *Ureaplasma* infections.

Keywords: *Ureaplasma parvum*, *Ureaplasma urealyticum*, Multiple banded antigen gene (*mba*), Real-time PCR, Serovars

3.2 INTRODUCTION

Ureaplasma species are members of the class *Mollicutes* (Waites *et al*., 2005). The genus *Ureaplasma* is grouped in the class *Mollicutes* based on the production of the enzyme urease and a small genome size (Waites *et al*., 2005). Ureaplasmas are one of the groups of bacteria that colonize the mucosa of the human urogenital tract and are found in 70% of pregnant women (Kafeitzis *et al*., 2004). *Ureaplasma* species are the leading cause of non-gonococcal urethritis in men and women (De Francesco *et al*., 2009). A high rate of *Ureaplasma* infections is mostly seen in sexually active men with a low socioeconomic status and those men with multiple partners (Povlsen *et al*., 2002).

Urogenital ureaplasmas are seperated into 14 different serotypes based on genotypic characteristics (Volgmann *et al*., 2005). The 14 serotypes were further classified into two different species, which include *U. parvum* that consists of serotypes 1, 3, 6 and 14 and *U. urealyticum*, which consists of the remaining serotypes 2, 4, 5, 7, 8, 9, 10, 11, 12 and 13 (Robertson *et al*., 2002; Waites *et al*., 2009). It is believed that not all the serotypes are associated with the disease syndrome because ureaplasmas are frequently isolated from healthy individuals (Waites *et al*., 2009).

Ureaplasmas lack a cell wall and therefore cannot be treated with beta-lactam agents or glycopeptides that are known to target the cell wall (Bébéar and Kempf, 2005). In addition, these organisms cannot be treated with sulphonamides or trimethoprim because they do not synthesize folic acid (Waites and Taylor-Robinson, 2007). *Ureaplasma* species are resistant to lincosamides but susceptible to macrolides (Beeton *et al*., 2009). Tetracycline remains a drug of choice for *Ureaplasma* species; however, resistance to tetracycline has been described since the 1980s and is mediated by the acquisition of the *tet*M gene (Bébéar and Kempf, 2005). Resistance of *Ureaplasma* species to tetracycline is estimated to be 10%, while it is up to 40% in the case of *Mycoplasma hominis*, although this differs for different regions (Waites and Taylor-Robinson, 2007).

Antimicrobial susceptibility testing for *Ureaplasma* species is important for serious conditions, such as urethritis, infertility and for evaluation of new drugs for *in vitro* activities and to check for the development of resistance (Waites *et al*., 2005). The main methods for antimicrobial susceptibility testing of *Ureaplasma* species are the broth and agar dilution

methods (Waites, 2001). Most diagnostic laboratories rely on commercial kits for susceptibility testing, which takes 24 to 48 hours (Waites and Taylor-Robinson, 2007).

Ureaplasma serotypes have varying degrees of virulence and pathogenicity (Kim *et al*., 2003). The multiple banded antigen protein is a major virulence factor of ureaplasmas (Waites *et al*., 2009). The MBA is the predominant antigen recognized during infection of *Ureaplasma* spp and is involved in the host inflammation response (Waites *et al*., 2009).

Culture methods are the "gold standard" for the detection of ureaplasmas but cannot distinguish between the serotypes (Zeighami *et al*., 2007). Typing of *Ureaplasma* species has always been done by polyclonal or monoclonal antibody using immunofluorescence and agar growth inhibition and serology (Shepard and Lunceford, 1978; Stemke and Robertson, 1981; Xiao *et al*., 2010). These antibody-based assays are time-consuming and difficult to interpret (Yoshida *et al*., 2005; Xiao *et al*., 2010).

Molecular methods, such as real-time PCR assays have been developed for the rapid detection and identification of ureaplasmal DNA in clinical specimens targeting genes, such as the 16S rRNA (Mallard *et al*., 2005), urease genes (Cao *et al*., 2007b) and multiple banded antigen genes (Cao *et al*., 2007a; Xiao *et al.,* 2010). Cao and colleagues (2007a and 2007b) developed two real-time PCR assays targeting the multiple banded antigen (*mba*) and urease genes to simultaneously detect, quantify and serotype *Ureaplasma* species. The assays proved to be rapid in serotyping *U. parvum*, which offer advantages over conventional serotyping methods. Xiao and colleagues (2010) designed primers and probes for the detection and discrimination of *U. parvum* and *U. urealyticum* and the 14 serotypes by real-time PCR targeting the *mba* gene. The sensitivity and specificity of the assays were 96.9% and 79.0% respectively. This was the first described real-time PCR assay to discriminate all 14 serotypes and the assay provided a quick and reliable means for investigation of epidemiology and pathogenecity of ureaplasmas at the serotype level (Xiao *et al*., 2010).

The purpose of this study was to use conventional and commercial kits for culture and compare results with molecular assays for *U. urealyticum* identification and typing of *U. parvum* in men with and without urogenital symptoms from first-void urine. The culture method was used as a "gold standard" to compare with a multiplex real-time PCR assay for the detection of the MBA genes as described by Cao *et al*. (2007a) and with a *U. urealyticum*

commercial kit. Susceptibility testing was performed on culture positive specimens using a Mycofast Evolution 3 kit (ELITech Microbiology, France).

3.3 MATERIALS AND METHODS

3.3.1 Study population and clinical specimens

The study was approved by the Ethics Committee, Faculty of Health Sciences, University of Pretoria (S23/2010). Clinical specimens were collected from both 100 symptomatic (discharge/dysuria) and 100 asymptomatic (non-discharge/non-dysuria) men attending a family practitioner in Pretoria, South Africa. First-void urine was obtained from each participant. The specimens were collected within a period of two months (March to April, 2010) and were stored and transported on ice to the laboratory the day after collection.

3.3.2 Cultivation of *Ureaplasma* species

An aliquot of 100 µl of each urine specimen was centrifuged (Labnet International, Edison, USA) at 3000 *x g* for 5 min and the sediments were inoculated using a sterile filtertip pipette in to a tube of Shepard's U9 broth (Diagnostic Media Products, South Africa). The U9 broth tubes were incubated at 37°C for 24 to 48 h in a CO_2 incubator (HF 212 UV, Shanghai Lishen Scientific Equipment, China) and were checked twice daily for a colour change. Growth of *Ureaplasma* bacteria result in the production of ammonia from the urea, with a resultant increase in pH just above 6.8, which changes the colour of the indicator from yellow to red (Shepard and Lunceford, 1978). According to Walky *et al*. (2009), *Ureaplasma* species have a generation time of approximately 1 h, resulting in the cultures being positive in two to four days.

As soon as the colour change was observed, the broth was subcultured onto Shepard's A2 agar medium. Shepard's A2 agar medium plates were incubated at 37°C for 48 to 72 h in a 5% CO_2 incubator. Colonies were visualised on the agar surface using a low-power microscope (AR Instruments, USA) (10X objective). The ureaplasmas appeared as small, irregular (roughly spherical) colonies into the agar.

A specimen was considered positive for *Ureaplasma* spp if a clear colour change was observed in Shepard's U9 broth and/or typical morphological growth was observed on the A2 agar medium surface. After subculturing, the remainder of the U9 cultures was aliquoted into 1 ml volumes and stored at -80°C for future analysis.

3.3.3 Antimicrobial susceptibility testing of *Ureaplasma* species

The Mycofast evolution 3 test kit (ELITech Microbiology, France) was used with some modifications for the identification and for antimicrobial susceptibility of genital ureaplasmas to seven antibiotics. The test is based on the specific metabolic property (urease activity) and natural resistance (resistance to lincosamide) of ureaplasmas. Specimens that were culture positive on Shepard's A2 agar medium were tested for antimicrobial susceptibility. In brief, the reagents stored at 4ºC (Just Refrigeration Products, Durban) were allowed to reach room temperature (25°C ± 5°C). Shepard's A2 positive isolates were inoculated into a transport medium (UMMt) using a swab. Each inoculated mycoplasma transport medium (UMMt) was then transferred into a new lyophilised growth medium (UMMlyo), the mixture was shaken and 100 µl from the mixture was transferred to each of the 20 wells of the tray. In well numbers 6 and 7, 50 µl of growth activator was added. Two drops of paraffin oil were put in all the wells to create an anaerobic environment for the organisms to grow and all the wells were covered with adhesive film. The colour change was observed due to alkalisation of the medium from yellow to red after 24 h and 48 h of incubation at 37ºC. The wells provided information about the presence or absence of *Mycoplasma hominis* and *Ureaplasma* species and antimicrobial susceptibilities to doxycycline (lower 4 µg/ml and higher 8 µg/ml), josamycin (lower 1 µg/ml and higher 4 µg/ml), ofloxacin (lower 1 µg/ml and higher 4 µg/ml), ciprofloxacin (lower 1 µg/ml and higher 2 µg/ml), azithromycin (lower 0.5 µg/ml and higher 4 µg/ml), roxithromycin (lower 1 µg/ml and higher 4 µg/ml) and pristinamycin (2 µg/ml).

The isolates were characterized as being sensitive, intermediate, or resistant to the antibiotics according to the following criteria:

- Sensitive when its growth was inhibited by the higher and lower critical concentrations of the antibiotic.
- Intermediate when its growth was inhibited by the higher critical concentration of the antibiotic, but not the lower critical concentration.

- Resistant when its growth was not inhibited by either the higher or lower critical concentration of the antibiotic.

3.3.4 Total genomic DNA extraction

An aliquot of 100 µl of urine was used for the extraction of DNA using the DNA-Sorb-A extraction kit (Sacace Biotechnology, Italy). In brief, for each specimen an aliquot of 100 µl of urine and 300 µl of lysis solution (Sacace Biotechnology, Italy) containing an enzyme that destroys the cell was added in a 1.5 ml polypropylene tube (Merck, Germany) and the tube was vortexed (Labnet International, Edison, NJ USA) and incubated at 66°C for 5 min in a heating block (Labnet International, Edison, NJ, USA). After the incubation, the tubes were centrifuged (Labnet International, Edison, NJ, USA) at 12 000 to 16 000 *x g* for 7 to 10 sec and the supernatant was transferred into new tubes for further DNA extraction. The sorbent solution (Sacace Biotechnologies, Italy) was vortexed (Labnet International, Edison, NJ USA) vigorously for 30 sec before 20 µl was transferred to each tube containing only the supernatant; the tubes were vortexed for 7 sec and were incubated for 3 min at room temperature (25°C ± 5ºC). All tubes were centrifuged for 30 sec at 5000 *x g* and using a filter tip micropipette the supernatant was removed carefully and discarded from each tube without disturbing the pellet. An aliquot of 500 µl of washing solution was added into each tube and the tubes were vortexed and centrifuged for 30 sec at 1 000 *x g*. The supernatant was discarded, the washing step was repeated and the tubes were incubated with the caps open at 65°C for 5 to 10 min for evaporation. The pellet was resuspended in 100 µl of the DNA-eluent (Sacace Biotechnology, Italy) and incubated for 5 min at 65°C and vortexed periodically. The tubes were centrifuged at 12 000 *x g* for 10 min. The supernatant contained the DNA ready for further analysis. The DNA concentration was measured using a Nanodrop spectrophotometer (NanoDrop Technologies Inc, USA) and the DNA was stored at -20ºC until further analysis.

3.3.5 Real-time PCR for the detection of *U. urealyticum*

A real-time PCR reaction for the detection of *mba* genes was performed using the Taqman *U. urealyticum* real-time PCR kit according to the manufacturer's instructions (Shanghai ZJ Bio-Tech, China). The DNA prepared from clinical specimens was amplified and tested for the presence of *U. urealyticum* using the LightCycler® 480 (Roche Diagnostics, Germany). In brief, each reaction tube contained a final mastermix volume of 40.4 µl and the mastermix

consisted of 4 µl of the prepared DNA template and 36.4 µl of the master mix (35 µl reaction mix, 0.4 µl enzyme mix and 1 µl internal control) (Shanghai ZJ Bio-Tech, China). The amplification and detection of the *U. urealyticun* was performed using the LightCycler®480 (Roche Diagnostics, Germany) with the following cycling conditions: 37°C for 2 min (1 cycle), 94°C for 2 min (1 cycle), followed by 40 cycles of 93°C for 5 sec and 60°C for 30 sec. Fluorescence was measured during annealing at 60°C in the FAM (530 nm) channel for *U. urealyticum* amplification and in the VIC/JOE (560 nm) channel for the internal control (IC). The results were said to be positive when the cycle threshold (C_T) in the FAM channel was less or equal to 38.

3.3.6 Multiplex Real-time PCR assays for the detection of *U. parvum* serovars

A multiplex real-time PCR reaction for the detection of the *mba* genes of *U. parvum* serovars was done using a modified method of the protocol described by Cao *et al.* (2007a) (Table 3.1). Each reaction included a forward primer, a reverse primer and a TaqMan probe mixture of serotype 3 and 6 in one tube and a mixture of serotypes 1 and 14 in another tube containing the same DNA sample (Roche Diagnostics, Germany).

Table 3.1: The oligonucleotide sequences of the primers and Taqman probes for the detection of *Ureaplasma parvum* serotypes 1, 3, 6 and 14 of *U. parvum* (Cao *et al*., 2007a)

Primers/Probes	Oligonucleotide Sequence (5'-3')	T_m (°C)
UP F	GTATTTGCAATCTTTATATGTTTTCG	54
UP R	TCCAGCTCCAACTAAGGTAAC	54
UP FP 1	FAM-TGTAAGATTGCTAAATC-BHQ	63
UP FP 3	HEX-TGTAAGATTACCAAATC- BHQ	63
UP FP 6	FAM-AGTGTCATATTTTTTACTAC- BHQ	64
UP FP 14	HEX-TCTTAGCTATGACATTAG-BHQ	64

UP F: *U. parvum* forward primer, UP R: *U. parvum* reverse primer, UP FP: *U. parvum* forward probe 1, 3, 6 and 14

FAM: Carboxyfluorescein

HEX: Carboxy-2',4, 4', 5', 7, 7'-hexachlorofluorescein

BHQ: Black hole quencher

Amplification was done in a final volume of 20 µl consisting of 4 µl of the LightCycler® Taqman® Master reaction mix (Roche Diagnostics, Germany), 0.5 µl of the forward primer (20 µM) and 0.5 µl of the reverse primer, 0.5 µl of each of the two *U. parvum* Taqman probes (Table 3.1), 5 µl of DNA template and 9 µl PCR grade nuclease free water (Promega Corporation, Madison, WI) were added to obtain a final reaction volume of 20 µl. The final concentration of the primers and probes was 0.5 µM in each PCR reaction mixture. The following cycling programme was used on the LightCycler®480 (Roche Diagnostics, Germany) for the amplification: pre-incubation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 10 sec, annealing at 52°C for 15 sec, extension at 72°C for 10 sec, followed by a final extension step at 72°C for 10 min. A single acquisition for fluorescence signals was done at the end of each extension step. Following the completion of the cycles, fluorescent signals were analysed and quantified using the LightCycler® 480. The samples were considered positive when the cycle threshold (C_T) value was 35 or less in the FAM (465 to 510) or HEX (533 to 580) channel.

Prior to the multiplex real-time PCR assay, a singleplex real-time PCR for each of the four *U. parvum* serotypes was evaluated. Amplification was done in a final volume of 20 µl consisting of 4 µl of the LightCycler® Taqman® Master reaction mix (Roche Diagnostics, Germany), 0.5 µl of the forward primer (20 µM) and 0.5 µl of the reverse primer (20 µM), 0.5 µl of the *U. parvum* Taqman probe (20 µM) (1, 3, 6 or 14), 5 µl of DNA template and 9.5 µl PCR grade nuclease free water (Promega Corporation, Madison, WI). The cycling and detection were the same as that of the multiplex PCR.

3.3.7 Statistical analysis

The sample size calculation was based on an assumption of 40% prevalence in the asymptomatic group. An increase of 20% (percentage points) in the group with symptoms to 60% prevalence was regarded clinically relevant. A sample size of 99 subjects per group would have 85% power to detect this difference when testing at the 0.05 level of significance using the continuity corrected chi-square test. The sensitivity and specificity of the PCR assay was compared with the "gold standard" culture method.

3.4 RESULTS

3.4.1 Culture for the detection of *Ureaplasma* species

Fourty percent (79/100) specimens showed a colour change from yellow to pink in the U9 broth (Figure 3.1). Of these 40% U9 positive specimens, 35% (28/79) were positive when subcultured on A2 medium (Appendix A: Table A1). Fifty-seven percent (16/28) positive specimens were from symptomatic men and 42% (12/28) positive specimens were from asymptomatic men. Thirty-four percent (20/59) negative specimens on A2 (34%) were identified as *M. hominis* or contaminated with yeast growing and 40% (31/79) of the U9 positive specimens did not grow on Shepard's A2 agar medium.

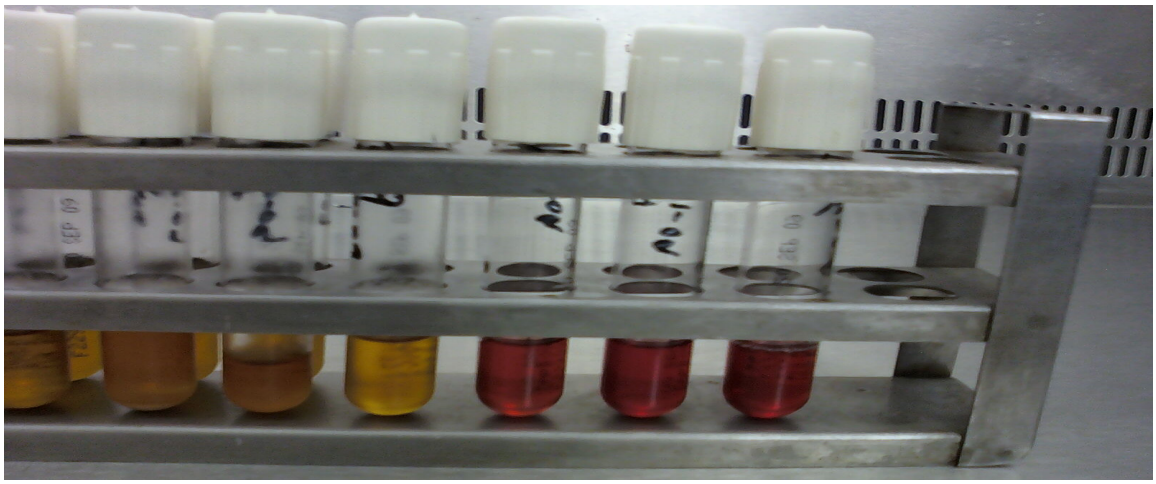

Figure 3.1: Shepard's U9 broth showing positive *Ureaplasma* species by hydrolyzing urea and pH indicator changing colour from yellow to red

3.4.2 Susceptibility testing results

Susceptibility testing using the Mycofast evolution 3 kit (ELITech Microbiology, France) showed that 9 isolate did not grow on the kit. All the other 14 isolates that grew on the kit were sensitive to doxycycline, pristinamycine, roxycycline and azithromycin. One isolate (from an asymptomatic male) was resistant to ciprofloxacin and josamycin but intermediately resistant to ofloxacin. One isolate (from a symptomatic male) was resistant to ciprofloxacin. None of the isolates was resistant to all antibiotics (Table 3.2). Five isolates were identified as *M. hominis* by the kit (two from asymptomatic and two from symptomatic men).

Table 3.2: The antibiotic susceptibility profile of *Ureaplasma* species isolates determined by the Mycofast evolution 3 kit (N=28)

Isolate Number	Symptomatic or asymptomatic men	Results	DO	PT	ROX	AZM	JM	CIP	OFX
8	Asymptomatic	+	S	S	S	S	S	S	S
11	Asymptomatic	+	S	S	S	S	R	R	I
12	Symptomatic	+	S	S	S	S	S	S	S
24	Asymptomatic	+	S	S	S	S	S	S	S
27	Asymptomatic	-							
29	Symptomatic	+	S	S	S	S	S	S	S
34	Asymptomatic	+	S	S	S	S	S	S	S
38	Symptomatic	+	S	S	S	S	S	R	S
43	Asymptomatic	+	S	S	S	S	S	S	S
46	Asymptomatic	-							
69	Asymptomatic	+	S	S	S	S	S	S	S
70	Asymptomatic	-							
85	Asymptomatic	+	S	S	S	S	S	S	S
87	Asymptomatic	+	S	S	S	S	S	S	R
102	Asymptomatic	+	S	S	S	S	S	S	S
118	Asymptomatic	-							
123	Asymptomatic	C							
126	Asymptomatic	C							
135	Symptomatic	C							
138	Symptomatic	-							
159	Symptomatic	C							
160	Symptomatic	+	S	S	S	S	S	S	S
161	Symptomatic	-							
167	Symptomatic	C							
173	Asymptomatic	+	S	S	S	S	S	S	S
184	Symptomatic	-							
185	Symptomatic	-							
194	Symptomatic	-							

C: Contamination, S: Sensitive, R: Resistant, +: Positive, -: Negative, DO: doxycycline, PT: pristinamycin, ROX: roxithromycin, AZM: azithromicin, JM: josamycin, CIP: ciprofloxacin, OFX: ofloxacin

3.4.3 Real-time PCR detection of *U. urealyticum*

The *Ureaplasma urealyticum* real-time PCR kit detected 16% (31/200) positive samples, 52% (16/31) were from symptomatic men (Appendix A: Table A1; Table 3.4) and 48% (15/31) from asymptomatic men (Appendix A: Table A1; Table 3.4). Positive results were called when the cycle threshold (C_T) in FAM channel was less or equal to 38. Fluorescence was measured during annealing in the FAM (530 nm) channel and the internal control was measured in the VIC/JOE (560 nm) channel (Figure 3.2).

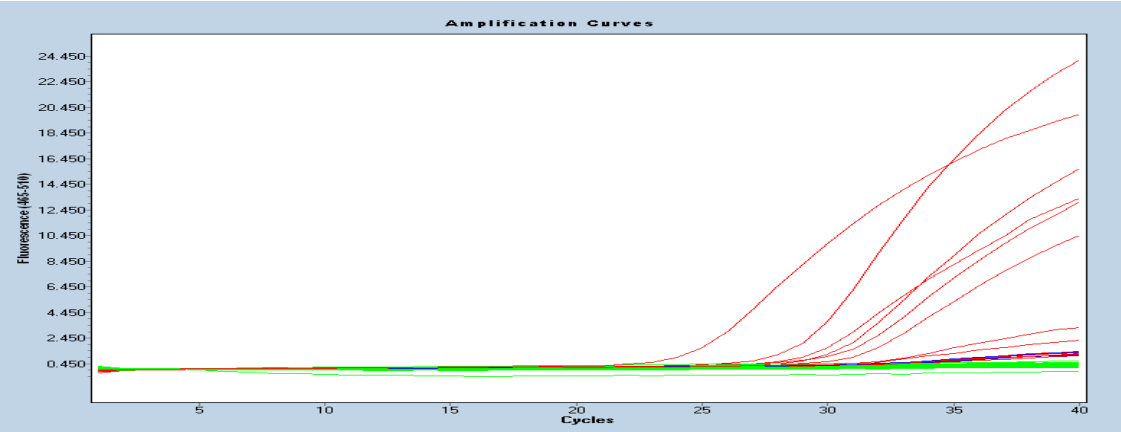

Figure 3.2: **Real-time PCR results for the detection of *Ureaplasma urealyticum* using the *U. urealyticum* real-time PCR kit (Shanghai ZJ Bio-Tech, China) on a LightCycler®480 (Roche Diagnostics, Germany).**

With respect to the proportion of positive *U. urealyticum* with the UU real time PCR kit, no significant difference (P=0.83) between symptomatic and asymptomatic patients was observed. The 95% confidence interval for the proportion of positive UU in the symptomatic group was (0.083; 0237), whereas for the asymptomatic group it was (0.075; 0225).

3.4.4 Multiplex TaqMan real time PCR for the detection of *U. parvum*

Results of the multiplex real-time PCR assay showed that 15% (29/200) men were colonized by *U. parvum*, 11% (11/100) from symptomatic men and 18% (18/100) from asymptomatic men (Table 3.3). The proportion of positive *U. parvum* detected from symptomatic and asymptomatic patients did not differ significantly (p=01598). The 95% confidence interval for the proportion of positive *U. urealyticum* in the symptomatic group was (0.044; 0.176) and for the asymptomatic group it was (0.100; 0.260).

Table 3.3: Relationship between clinical symptomatology and *Ureaplasma* species (N=200)

Male participant N=200	***Ureaplasma* species**	
	U. urealyticum	***U. parvum***
Symptomatic	8% (16/200)	6% (11/200)
Asymptomatic	8% (15/200)	9% (18/200)

Serotype 6 was distributed with the same frequency (9%) amongst men with symptoms and those without symptoms (Table 3.4). Serotype 14 was more (6%) distributed in asymptomatic men than in symptomatic men whereas serotypes 1 and 3 (6% and 4%) were more associated with symptomatic men than asymptomatic men. The distribution of serotypes did not differ significantly between the symptomatic and asymptomatic group (p= 0.309; Fisher's exact test) (Figure 3.4).

3.4.5 Sensitivity and specificity of the two real-time PCR assay for the detection of ureaplasmas

A two by two table was used for the calculation of the sensitivity and specificity of the real-time PCR assays. Results of the UU real-time PCR kit showed that the sensitivity and specificity against culture was 38% and 96% respectively (Appendix C: Table 1). The sensitivity and specificity of the multiplex real-time PCR assay against the culture method (gold standard) was 68% and 95%, respectively (Appendix C: Table C2). Four men were colonized by both species, two from symptomatic and two from asymptomatic.

Of the 28 positive culture specimens, 71% (20/28) were positive by both PCR assay, 50% (10/20) from symptomatic men and another 50% (10/20) from symptomatic men (Table 3.4). Fifty-five percent (11/20) *Ureaplasma* positive specimens were positive by the multiplex PCR assay, 25% (5/20) with the UU PCR assay while 10% (4/20) were positive with both multiplex and UU PCR assays (Table 3.4). Serotype 6 was the most frequently detected serotype followed by serotypes 1, 14 and 3. Twenty-nine specimens were positive with PCR assays but 23 faild to grow on culture and 6 were contaminated.

Table 3.4: Comparison of culture and positive PCR assay results (N=52)

Specimen numbers	Symptomatic or Asymptomatic men	Culture results	UU PCR	Multiplex PCR serotypes
3	Asymptomatic	C	+	-
11	Asymptomatic	+	+	-
12	Symptomatic	+	-	3 and 6
14	Symptomatic	-	-	1
24	Asymptomatic	+	-	6
27	Asymptomatic	+	+	-
29	Symptomatic	+	+	-
44	Asymptomatic	+	+	6
47	Asymptomatic	+	-	6
49	Asymptomatic	-	+	-
57	Symptomatic	-	+	-
59	Asymptomatic	C	+	6
63	Asymptomatic	C	+	-
66	Asymptomatic	+	-	1 and 6
70	Asymptomatic	+	-	14
73	Asymptomatic	-	+	-
84	Asymptomatic	-	-	14
85	Asymptomatic	+	-	14
91	Asymptomatic	-	+	-
93	Asymptomatic	-	+	-
97	Asymptomatic	-	+	-
103	Asymptomatic	-	+	-
106	Symptomatic	-	+	-
107	Symptomatic	-	+	-
109	Symptomatic	-	+	-
111	Symptomatic	-	+	-
116	Symptomatic	-	+	-
119	Symptomatic	-	+	-
120	Asymptomatic	+	-	6
121	Asymptomatic	+	-	6
126	Asymptomatic	+	+	6 and 14
132	Asymptomatic	-	+	-
135	Symptomatic	+	+	-
138	Symptomatic	+	-	1 and 6
148	Asymptomatic	-	-	6

Table 3.4: Comparison culture and positive PCR assay results (Cont)

Specimen numbers	Symptomatic or Asymptomatic men	Culture results	UU PCR	Multiplex PCR serotypes
149	Asymptomatic	C	-	6
156	Asymptomatic	-	-	14
157	Asymptomatic	-	-	1
159	Symptomatic	+	+	1
160	Symptomatic	+	+	6
161	Symptomatic	+	+	-
165	Symptomatic	-	+	-
167	Symptomatic	+	+	-
168	Symptomatic	C	+	-
172	Asymptomatic	C	+	-
173	Asymptomatic	+	-	1
184	Symptomatic	+	-	1 and 6
185	Symptomatic	+	-	3 and 6
187	Symptomatic	-	+	-
188	Symptomatic	-	-	3, 6 and 14
189	Symptomatic	-	-	1, 3, 6 and 14
194	Symptomatic	+	-	1

+: positive, -: negative, C: contaminated

3.5 DISCUSSION

This study undertook to detect genital *Ureaplasma* species and to characterize *Ureaplasma parvum* in men with and without urogenital symptoms. Ureaplasmas are considered as normal flora of the urogenital tract in healthy men but can cause non-gonococcal urethritis and is implicated to affect fertility (Pinna *et al*., 2006). It is suggested that not all the serotypes of *Ureaplasma* species cause disease since different serotypes are frequently detected in both symptomatic and asymptomatic individuals; therefore, the pathogenic role of *Ureaplasma* species is difficult to prove (De Francesco *et al*., 2009).

Susceptibility testing was performed on the 28 A2 culture isolates (A2 medium) in this study using antibiotics that are commonly used for the management of *Ureaplasma* infections. Nine of the 28 isolates did not grow on the Mycofast evolution 3 kit. This could be because the kit is validated to be used on direct specimens and not on isolates or it might be because the A2 agar plate had mixed isolates of ureaplasmas and other bacteria that was picked and used to inoculate the Mycofast evolution 3 kit (ELITech Microbiology, France). Five of the 28 isolates

were contaminated, all the wells changed colour from yellow to red,. Fourteen isolates were excluded from the analysis, five were contaminated while nine failed to grow. All the isolates 100% (14/14) were susceptible to doxycyclin, pristinamycin, roxycyclin and azithromycin. One isolate from a asymptomatic male was resistant to ciprofloxacin and jentamicin but intermediately resistant to ofloxacin, while another isolate from a symptomatic male was resistant to ciprofloxacin. These findings were in agreement with the results of other studies (Kanakas *et al*., 1999; Cakan *et al*., 2003, Beeton *et al*., 2009), which found that resistance of *Ureaplasma* spp to ciprofloxacin and ofloxacin ranged between 39% to 58% using E-test and broth dilution tests. None of the isolates were resistant to all antibiotics tested, which correlated with the study of De Francesco *et al*. (2009) where not one of 158 isolates included in the study were resistant to all antibiotics tested.

U9 broth positive specimens were cultured on solid agar for confirmation of *Ureaplasma* species. Thirty-one out of the seventy-nine (39%) specimens did not grow on the A2 agar medium and this could be attributed to a number of reasons, which include the possibility that the bacteria lost viability before being subcultured, the high pH levels in the media or because the U9 broth was contaminated with other urogenital organisms that have the urease enzyme, eg *Proteus* species. Only 35% (28/79) of the U9 positive specimens grew on A2 agar medium. The A2 agar medium was prone to contamination (34%); therefore, it was difficult to observe the bacteria of interest especially when the plate was contaminated with yeasts. Differentiation between *Ureaplasma* spp was not possible because the A2 agar medium only detects up to the genus level. Overall, the culture method was difficult to perform, because *Ureaplasma* species are fragile and susceptible to adverse conditions and take up to seven days to grow. Although culture is considered the "gold standard", the clinical sensitivity of the culture methods remains unclear (Mallard *et al*., 2005; Dhawan *et al*., 2006).

The *U. urealyticum* real-time PCR kit (Shanghai ZJ Bio-Tech, China) was used for the detection of *Ureaplasma urealyticum*. *Ureaplasma urealyticum* was detected with the same frequency 8% (16/200) and 8% (15/200) in men with and without urogenital symptoms. These results correlated with the studies of Polvsen *et al*. (2001) and Gupta *et al*. (2008) where the distribution of *U. urealyticum* amongst symptomatic and asymptomatic groups were found to be the same. However, these results were in contrast with the findings of other studies (Polvsen *et al*., 2002; Deguchi *et al*., 2004; Schlicht *et al*., 2004; Ondondo *et al*., 2010) where it was reported that the distribution *U. urealyticum* was significantly higher in symptomatic men than

asymptomatic men. In a study done by Sturm and colleagues (2004) in Durban, KwaZulu Natal, South Africa, 335 men with symptoms and 100 men without symptoms were tested for different bacteria, including *Neisseria gonorrhoeae* as an established pathogen and *U. urealyticum* as a potential pathogen. *Neisseria gonorrhoeae* was tested by culture and *U. urealyticum* by a PCR assay. Among the symptomatic men, *N. gonorrhoeae* was detected in 52% (175/335) of cases and *U. urealyticum* in 36% (121/335) of cases. In asymptomatic men *N. gonorrhoeae* was not detected, while *U. urealyticum* was detected in 30% (30/100) men. *Ureaplasma urealyticum* was distributed with the same frequency amongst symptomatic and asymptomatic men in the current study; therefore, it can be concluded that the presence of this species was evidence of colonization rather than infection.

Ureaplasma parvum was detected with the real-time PCR assay (Cao *et al*., 2007a) more frequently from asymptomatic men 9% (18/200) compared to 6% (11/200) symptomatic men but there was no significant difference in the distribution of *U. parvum* amongst the two groups. In a study by Yoshida *et al*. (2002) in Japan the detection rate of *U. parvum* was much higher amongst asymptomatic men 21.4% (9/42) than from symptomatic men 1.8% (1/55). Ondondo and colleagues (2010) did a study in the USA, which recruited 119 heterosexual men with genital symptoms and 117 men without symptoms and detected *U. parvum* more in asymptomatic men 31% (36/117) compared to 14% (17/119) in symptomatic men.

No statistical significant association was observed between the distribution of *U. parvum* serotypes and clinical symptoms. Serotype 1 and 3 were detected more from symptomatic men than asymptomatic men (6% vs 4% and 4% vs 1% respectively), whereas serotype 6 was evenly distributed in men with symptoms and those without symptoms. Serotype 14 was more distributed in asymptomatic men in this study (6% vs 3%). The distribution of serotypes in this study differed from that reported by De Francesco *et al*. (2009) in females, where serotype 1 was equally distributed in symptomatic and asymptomatic women and serotype 6 was most prominent in the asymptomatic group. Furthermore, De Francesco *et al*. (2009) noted that serotype 14 was associated with the symptomatic group; however, in the present study, it was associated with the asymptomatic group.

The culture method missed 27 *Ureaplasma* spp that were detected by the real-time PCR assay, 11 from symptomatic men and 16 from asymptomatic men. This might be because the participants were already on treatment and therefore the organisms were no longer viable but

the DNA could still be detected or that the organisms might have died during transportation of the specimen to the laboratory. Another explanation might be the increased sensitivity of the real-time PCR assay compared with conventional culture for fastidious bacteria, such as *Ureaplasma*. Xiao and colleagues (2010) compared culture with a multiplex real-time PCR assay and detected 15% more *Ureaplasma* spp that were tested negative by the culture method. Several studies comparing culture methods with PCR assays for the detection of *Ureaplasma* spp in clinical specimens have shown that PCR is better than culture in terms of sensitivity and time (Abele-Horn *et al*., 2000; Heggie *et al*., 2001; Horner *et al*, 2001; Yoon *et al*., 2003). The six specimens that were positive with culture but negative with PCR could be due to culture results being false positive or a DNA extraction that was not successful.

The two real-time PCR assays used in this study to target both the urogenital species of *Ureaplasma* had a sensitivity of 36% (UU kit) and 61% (UP multiplex PCR) whereas the specificity of the two real-time PCR used in this study was 88% (UU kit) and 94% (multiplex PCR). The sensitivity of the real-time PCR assays in this study was lower compared to the study by Xiao and colleagues (2010), which was 97%. However, the specificity of the real-time PCR was higher (88% and 94%) than that reported by Xiao *et al*. (2010), which was 79%. This might be due to the strain variation of *Ureaplasma* species in different regions. It has been proven that the sensitivity of PCR assays in male specimens ranges between 64% and 100% compared with the sensitivity of PCR in female specimens that ranges between 94% and 100% (Teng *et al*., 1994; Polvsen *et al*., 1998; Govender *et al*., 2010). A possible reason for the low PCR sensitivity in male genital specimens compared to female genital specimens is that male specimens are prone to PCR inhibitors and have low numbers of bacteria present in the specimens (Teng *et al*., 1994; Polvsen *et al*., 1998). Further studies are required to improve the sensitivity of real-time PCR on male genital specimens.

The limitations of this present study are that demographic data, such as age, sexual history and socio-economic status were not obtained. This information would have been more advantageous in the comparison of the different serotypes amongst the symptomatic and asymptomatic men. Another limitation is that *U. urealyticum* serotypes were not characterized in this study; this would have helped in the attempt to relate *Ureaplasma* spp serotypes to clinical manifestations. The sample size may have been too small and larger numbers are required if a difference are to be shown.

3.6 CONCLUSIONS

Standardized culture and susceptibility methods are required as current methods are difficult and time consuming. This can aid in the management of patients and will facilitate the comparison of results between different laboratories.

The *Ureaplasma* real-time PCR kit (Shanghai ZJ Bio-Tech, China) for the detection of *U. urealyticum* proved to be rapid and easy to perform. Another advantage of this kit is that it can perform both qualitative and quantitative detection of the bacteria even though quantification of the bacteria was not performed in this study. However, this kit only detects *U. urealyticum* and thus does not differentiate between the two urogenital species of *Ureaplasma*. The kit cannot be used in routine diagnostic laboratories in developing countries because it is very expensive. There is an urgent need for easy to perform and inexpensive real-time PCR assays for the diagnosis of *Ureaplasma* spp in developing countries, which have a high burden of sexually transmitted diseases.

Further studies to improve multiplex-real-time PCR assays are required using South African specimens. This may lead to the improvement of the sensitivity of the real-time PCR assays. Implementation of these assays in South African diagnostic laboratories may decrease the turnaround time from two to five days to one day, since these assays are easy to perform and a large number of specimens can be tested at the same time.

In this study, there was no significant difference of *Ureaplasma* spp in men with symptoms and those without symptoms. This study concludes that the *Ureaplasma* spp are more colonizers rather than pathogens.

3.7 ACKNOWLEDGMENTS

The authors wish to thank the Department of Medical Microbiology, University of Pretoria and National Health Laboratory Service (NHLS) for the financial support of this study. Miss NE Maningi would like to thank the University of Pretoria, the National Research Foundation and the Medical Research Council for the financial support received during her MSc study.

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CHAPTER 4

CONCLUDING REMARKS

Ureaplasma species colonize the mucosal surfaces of the urogenital tract and respiratory tract in humans and can cause many diseases especially in immunocompromised individuals (Waites *et al*., 2009). It has been found that not all serotypes of the two species are associated with the disease syndrome; however, available data are conflicting (Teng *et al*., 1994; Zheng *et al*., 1992; De Francesco *et al*., 2009). Genital *Ureaplasma* species have been associated with non-gonococcal urethritis in men (Takashi *et al*., 2007). In this study, it was shown to be a colonizer because there were no association of *Ureaplasma* species with nongonococcal urethritis.

Tetracycline and macrolides are the most effective drugs for the treatment of *Ureaplasma* species, but the emergence of resistance have been recorded in some studies (Kechagia, 2008; Beeton *et al*., 2009; De Francesco *et al*., 2009). Ofloxacin and ciprofloxacin have been proven to be less effective for the treatment of *Ureaplasma* species and a number of strains are intermediately resistant to these drugs (De Francesco *et al*., 2009). Antimicrobial susceptibility testing of ureaplasmas and mycoplasmas in diagnostic laboratories in South Africa relies on broth dilution kits. The kits are based on natural resistance and specific metabolic properties; with *U. urealyticum* resistant to lincosamide and with the ability to hydrolyze urea, while *M. hominis* is resistant to erythromycin and with the ability to hydrolyze arginine. Growth of the two species is visualized by a change in the medium's colour from yellow to orange to red or pink (Govender *et al*., 2010). When using the kits, results take two to five days to be available and lack standardization (Govender *et al*., 2010).

Prior to 1994, detection of *Ureaplasma* species in clinical specimens relied on specific culture techniques (Blanchard *et al*., 1993). Although culture is considered the "gold standard", culture has been found to have many limitations: cultivating the bacteria in laboratory media is time consuming (two to five days), lacks sensitivity, requires repeated microscopic observations, is costly (requires special media and expertise), is labour-intensive, requires special handling of specimens to maintain viability of the bacteria and is at risk of contamination with other organisms (Blanchard *et al*., 1993; Stellrecht *et al*., 2004; Mallard *et*

al., 2005; Dhawan *et al*., 2006; Petrikkos *et al*., 2006). Another limitation of the culture method is that it cannot accurately differentiate between the species (Mallard *et al*., 2005).

Polymerase chain reaction-based assays have been developed for the diagnosis of *Ureaplasma* species and its serotypes. The assays proved to be equal to if not superior to the traditional selective culture techniques (Blanchard *et al*., 1993; Teng *et al*., 1994a; Sturm *et al*., 2004; Dhawan *et al*., 2006, Coa *et al*., 2007a; Xiao *et al*., 2010). Amplification with PCR assays take place even in the presence of a low number of the organisms and viability of the bacteria is not a prerequisite (Colaizy *et al*., 2003). Species identification by the PCR assays is possible within 24 hours and the *Ureaplasma* species can be identified up to serotype level (Waites *et al*., 2005). Molecular methods are sensitive, specific and easy to perform and require minimal preparation of clinical specimens (Waites *et al*., 2005).

Molecular assays, such as real-time PCR assays targeting the *mba* and *ure* genes have been developed for the identification of *Ureaplasma* species and detection of mutations that lead to different serotypes and resistance of the strains to the drugs (Bébéar and Kempf, 2005; Beeton *et al*., 2009; Xiao *et al*., 2010). Real-time PCR assays are becoming an important alternative tool compared to conventional PCR assays and culture for the initial quantification, detection and characterization of *Ureaplasma* serotypes in clinical specimens (Cao *et al*., 2007b; Xiao *et al*., 2010). Multiple studies including this present study have shown that real-time PCR assays can detect and characterize *Ureaplasma* species in less than 24 h compared to two to five days with culture methods (Cao *et al*., 2007a; Cao *et al*., 2007b; Xiao *et al*., 2010).

All PCR assays reported so far, have failed to separate all 14 serotypes because of limited sequence variation in the genes targeted; however, Xiao *et al*. (2010) has successfully separated all 14 ureaplasma serotypes targeting the *mba* gene, which has many variations on the different serotypes. This novel PCR assay has overcome many limitations that hampered serologically based typing methods.

Organisms sharing the same niche are more likely to exchange their genetic material through horizontal gene transfer. Even though this condition is rare in bacteria with minimal genomes Xiao *et al*. (2011) have reported horizontal gene transfer in *Ureaplasma* serotypes. This has raised questions about the relevance of *Ureaplasma* serotyping to assess pathogenecity on the

serotype level because the genes encoding the phenotypic epitopes on which the serotypes are based maybe changed, combined or lost after horizontal gene transfer (Xiao *et al*., 2011).

This is the first serotyping study conducted in South Africa; therefore, more studies are needed to confirm the findings of this study. In this study, serotypes 6 of the *U. parvum* species was detected with the same frequency in symptomatic and asymptomatic men. In the men where both *Ureaplasma* species were present, serotype 6 was associated with *U. urealyticum*. This is in contrast with other studies where serotype 3 was reported to be mostly associated with *U. urealyticum* in symptomatic patients. Serotypes 3 and 6 can adhere to the mucosal membrane, which can lead to inflammation (Moss *et al*., 2008). In some male participants more than one serotype was detected. The combination of serotypes 3 and 6 was the most common followed by the combination of serotypes 6 and 14. Serotype 14 was detected more frequently in asymptomatic than symptomatic men. These results are in contrast to the study by De Francesco *et al*. (2009) where serotype 14 was detected more frequently in symptomatic than in asymptomatic participants. Although serotype 1 was more frequently detected in symptomatic men than in asymptomatic men in this study, De Francesco *et al*. (2009) reported equal distribution of serotype 1 in symptomatic and asymptomatic participants.

Culture methods are used in diagnostic laboratories in developing countries, such as South Africa because culture methods are cheaper compared to available molecular methods. Implementation of PCR assays will help in the rapid identification of the bacteria and will allow the rapid administration of treatment to patients (Colaizy *et al*., 2003). This can prevent transmission of *Ureaplasma* species between couples and to babies through the birth canal (Colaizy *et al*., 2003). The exposures of the neonate to *Ureaplasma* spp can lead to colonization of the skin, mucosal membranes, respiratory tract and sometimes to dissemination into the bloodstream and the central nervous system (Schelonka and Waites, 2007; Viscardi, 2010). Therefore, in this case it is very important to develop cheaper and quicker *Ureaplasma* diagnostic methods to prevent the transmission of ureaplasmas to partners and eventually to the babies. Follow-up diagnosis should be obtained in treated infants to check for the clearance of the bacteria from the body.

4.1 FUTURE RESEARCH

Future research should concentrate on the quantitative detection of both genital species of *Ureaplasma* in men with and without urogenital symptoms. Quantification might assess whether the bacterial load of *Ureaplasma* spp is associated with pathogenecity of the bacteria in the urogenital tract.

More serotyping studies are needed to assess horizontal gene transfer amongst the *Ureaplasma* serotypes, since there are indications that serotype markers and some genes/fragment throughout the genome were exchanged among different serotypes especially since the *mba* gene was thought to be serotype specific. This could help in finding the mechanisms involved in horizontal gene transfer in ureaplasmas.

The standardization of the real-time PCR assays used by different investigators may be of great benefit to obtain results that are comparable across different population and regions. Furthermore, research should also concentrate on developing easier, quicker, cheaper and more reliable diagnostic methods for the detection and characterization of *Ureaplasma* species so that these assays can be used for routine diagnosis in developing countries where the incidence of STIs is high. More research on this topic remains to be done in South Africa, since there is geographical variation in the detection of genital *Ureaplasma* species. Evidence is needed to confirm or to disprove the main conclusion from this study that the *Ureaplasma* spp are more frequently harmless colonizers rather than pathogens.

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Appendix A

Table A1: The distribution of *Ureaplasma* species in asymptomatic men and symptomatic men (N=200)

Patient numbers	Asymptomatic or symptomatic men	U9 colour change	A2 growth	UU PCR	Multiplex PCR
1	Asymptomatic	-	-	-	-
2	Asymptomatic	+	-	-	-
3	Asymptomatic	+	C	+	-
4	Asymptomatic	-	-	-	-
5	Asymptomatic	-	-	-	-
6	Asymptomatic	-	-	-	-
7	Asymptomatic	+	-	-	-
8	Asymptomatic	+	+	-	-
9	Asymptomatic	-	-	-	-
10	Asymptomatic	-	-	-	-
11	Asymptomatic	+	+	+	-
12	Symptomatic	+	+	-	+
13	Symptomatic	-	-	-	-
14	Symptomatic	-	-	-	+
15	Symptomatic	-	-	-	-
16	Symptomatic	-	-	-	-
17	Symptomatic	+	-	-	-
18	Symptomatic	-	-	-	-
19	Symptomatic	-	-	-	-
20	Symptomatic	+	C	-	-
21	Symptomatic	-	-	-	-
22	Symptomatic	-	-	-	-
23	Asymptomatic	-	-	-	-
24	Asymptomatic	+	+	-	+
25	Asymptomatic	+	C	-	-
26	Asymptomatic	-	-	-	-
27	Asymptomatic	+	+	+	-
28	Asymptomatic	-	-	-	-
29	Symptomatic	+	+	+	-
30	Symptomatic	+	-	-	-
31	Symptomatic	-	-	-	-
32	Symptomatic	-	-	-	-
33	Asymptomatic	-	-	-	-
34	Asymptomatic	+	+	-	-
35	Asymptomatic	-	-	-	-
36	Symptomatic	-	-	-	-
37	Symptomatic	+	-	-	-
38	Symptomatic	+	+	-	-
39	Symptomatic	-	-	-	-
40	Symptomatic	-	-	-	-
41	Symptomatic	-	-	-	-
42	Asymptomatic	+	c	-	-
43	Asymptomatic	+	-	-	-
44	Asymptomatic	+	+	+	+
45	Asymptomatic	-	-	-	-
46	Asymptomatic	-	-	-	-
47	Asymptomatic	+	+	-	+
48	Asymptomatic	+	-	-	-
49	Asymptomatic	-	-	+	-
50	Asymptomatic	+	c	-	-

+: positive, -: negative and c: contaminated

Appendix A

Table A1: The distribution of *Ureaplasma* species in asymptomatic men and symptomatic men (N=200) (cont)

Patient numbers	Asymptomatic or symptomatic men	U9 colour change	A2 growth	UU PCR	Multiplex PCR
51	Symptomatic	-	-	-	-
52	Symptomatic	-	-	-	-
53	Symptomatic	-	-	-	-
54	Symptomatic	-	-	-	-
55	Symptomatic	+	-	-	-
56	Symptomatic	-	-	-	-
57	Symptomatic	-	-	+	-
58	Symptomatic	-	-	-	-
59	Asymptomatic	+	c	+	+
60	Asymptomatic	-	-	-	-
61	Asymptomatic	-	-	-	-
62	Asymptomatic	+	c	-	-
63	Asymptomatic	+	c	+	-
64	Asymptomatic	+	-	-	-
65	Asymptomatic	-	-	-	-
66	Asymptomatic	+	+	-	+
66	Symptomatic	+	-	-	-
67	Symptomatic	-	-	-	-
68	Symptomatic	+	-	-	-
70	Asymptomatic	+	+	-	+
71	Asymptomatic	-	-	-	-
72	Asymptomatic	+	-	-	-
73	Asymptomatic	-	-	+	-
74	Asymptomatic	-	-	-	-
75	Asymptomatic	+	c	-	-
76	Asymptomatic	-	-	-	-
77	Symptomatic	-	-	-	-
78	Symptomatic	+	-	-	-
79	Symptomatic	+	-	-	-
80	Symptomatic	+	-	-	-
81	Symptomatic	+	-	-	-
82	Symptomatic	-	-	-	-
83	Symptomatic	+	-	-	-
84	Asymptomatic	+	-	-	+
85	Asymptomatic	+	+	-	+
86	Asymptomatic	-	-	-	-
87	Asymptomatic	+	+	-	-
88	Symptomatic	+	-	-	-
89	Symptomatic	-	-	-	-
90	Asymptomatic	-	-	-	-
91	Asymptomatic	-	-	+	-
92	Asymptomatic	-	-	-	-
93	Asymptomatic	-	-	+	-
94	Asymptomatic	-	-	-	-
95	Asymptomatic	-	-	-	-
96	Asymptomatic	-	-	-	-
97	Asymptomatic	-	-	+	-
98	Asymptomatic	-	-	-	-
99	Asymptomatic	-	-	-	-
100	Asymptomatic	-	-	-	-
101	Asymptomatic	-	-	-	-
102	Asymptomatic	-	-	-	-

+: positive, -: negative, c: contaminated

Appendix A

Table A1: The distribution of *Ureaplasma* species in asymptomatic en and symptomatic men (N=200) (cont)

Patient number	Asymptomatic or symptomatic men	U9 colour change	A2 growth	UU PCR	Multiplex PCR
103	Asymptomatic	-	-	+	-
104	Symptomatic	-	-	-	-
105	Symptomatic	-	-	-	-
106	Symptomatic	-	-	+	-
107	Symptomatic	-	-	+	-
108	Symptomatic	-	-	-	-
109	Symptomatic	-	-	+	-
110	Symptomatic	-	-	-	-
111	Symptomatic	-	-	+	-
112	Symptomatic	-	-	-	-
113	Symptomatic	-	-	-	-
114	Symptomatic	-	-	-	-
115	Symptomatic	-	-	-	-
116	Symptomatic	-	-	+	-
117	Symptomatic	-	-	-	-
118	Symptomatic	-	-	-	-
119	Symptomatic	-	-	+	-
120	Asymptomatic	+	+	-	+
121	Asymptomatic	+	+	-	+
122	Asymptomatic	-	-	-	-
123	Asymptomatic	+	+	-	-
124	Asymptomatic	+	-	-	-
125	Asymptomatic	-	-	-	-
126	Asymptomatic	+	+	+	+
127	Asymptomatic	+	-	-	+
128	Asymptomatic	-	-	-	-
129	Asymptomatic	-	-	-	-
130	Asymptomatic	-	.-	-	-
131	Asymptomatic	-	-	-	-
132	Asymptomatic	-	-	+	-
133	Asymptomatic	-	-	-	-
134	Asymptomatic	-	-	-	-
135	Symptomatic	+	+	+	-
136	Symptomatic	-	-	-	-
137	Symptomatic	-	-	-	-
138	Symptomatic	+	+	-	+
139	Symptomatic	-	-	-	-
140	Symptomatic	-	-	-	-
141	Symptomatic	+	-	-	-
142	Asymptomatic	+	-	-	-
143	Asymptomatic	-	-	-	-
144	Asymptomatic	-	-	-	-
145	Asymptomatic	-	-	-	-
146	Asymptomatic	-	-	-	-
147	Asymptomatic	+	c	-	-
148	Asymptomatic	+	-	-	+
149	Asymptomatic	+	c	-	+
150	Asymptomatic	+	c	-	-
151	Asymptomatic	+	c	+	-
152	Asymptomatic	-	-	-	-
153	Asymptomatic	-	-	-	-
154	Asymptomatic	-	-	-	-

+: positive, -: negative and c: contaminated

Appendix A

Table A1: The distribution of *Ureaplasma* species in asymptomatic en and symptomatic men (N=200) (cont)

Patient number	Asymptomatic or symptomatic men	U9 colour change	A2 growth	UU PCR	Multiplex PCR
155	Asymptomatic	+	-	-	-
156	Asymptomatic	-	-	-	+
157	Asymptomatic	+	-	-	+
158	Asymptomatic	-	-	-	-
159	Symptomatic	+	+	+	+
160	Symptomatic	+	+	+	+
161	Symptomatic	+	+	+	-
162	Symptomatic	+	c	-	-
163	Symptomatic	+	c	-	-
164	Symptomatic	+	c	-	-
165	Symptomatic	-	-	+	-
166	Symptomatic	-	-	-	-
167	Symptomatic	+	+	+	-
168	Symptomatic	+	c	+	-
169	Symptomatic	-	-	-	-
170	Symptomatic	-	-	-	-
171	Symptomatic	-	-	-	-
172	Asymptomatic	+	c	-	+
173	Asymptomatic	+	+	-	+
174	Asymptomatic	-	-	-	-
175	Asymptomatic	-	-	-	-
176	Asymptomatic	+	-	-	-
177	Asymptomatic	-	-	-	-
178	Symptomatic	+	c	-	-
179	Symptomatic	+	-	-	-
180	Symptomatic	-	-	-	-
181	Symptomatic	+	-	-	-
182	Symptomatic	-	-	-	-
183	Symptomatic	-	-	-	-
184	Symptomatic	+	+	-	+
185	Symptomatic	+	+	-	+
186	Symptomatic	+	c	-	+
187	Symptomatic	-	-	+	-
188	Symptomatic	+	-	-	+
189	Symptomatic	+	-	-	+
190	Symptomatic	-	-	-	-
191	Symptomatic	-	-	-	-
192	Symptomatic	-	-	-	-
193	Symptomatic	-	-	-	-
194	Symptomatic	+	+	-	+
195	Symptomatic	-	-	-	-
196	Symptomatic	-	-	-	-
197	Symptomatic	-	-	-	-
198	Symptomatic	-	-	-	-
199	Symptomatic	-	-	-	-
200	Symptomatic	-	-	-	-

+: positive, -: negative and c: contaminated

Appendix B

Table B1: *Ureaplasma parvum* serotype distribution in urine specimens (N=29)

Specimens number	Serotype 1	Serotype 3	Serotype 6	Serotype 14
12	-	+	+	-
14	+	-	-	-
24	-	-	+	-
44	-	-	+	-
47	-	-	+	-
59	-	-	+	-
66	+	-	+	-
70	-	-	-	+
84	-	-	-	+
85	-	-	-	+
120	-	-	+	-
121	-	-	+	-
126	-	-	+	+
127	-	-	-	+
138	+	-	+	-
148	-	-	+	-
149	-	-	+	-
156	-	-	-	+
157	+	-	-	-
159	+	-	-	-
160	-	-	+	-
172	-	+	+	-
173	+	-	+	-
184	+	-	+	-
185	-	+	+	-
186	-	-	+	+
188	-	+	+	+
189	+	+	+	+
194	+	-	-	-

+: positive and -: negative

Appendix C

Table C1: Sensitivity and specificity of the UU real-time PCR for the detection of *Ureaplasma* spp against the culture method (N=166)

	Culture			
PCR		+	-	Total
	+	10	6	16
	-	16	134	150
	Total	26	140	166

+: positive and -: negative

Sensitivity: 38%

Specificity: 96%

Table C2: Sensitivity and specificity of the multiplex real-time PCR for the detection of *Ureaplasma* spp against the culture method (N=175)

	Culture			
PCR		+	-	Total
	+	17	7	24
	-	8	143	151
	Total	25	150	175

+: positive and -: negative

Sensitivity: 68%

Specificity: 95%