

# **Epidemiology and Antibiotic Susceptibility Patterns of *Mycoplasma* sp. and *Ureaplasma urealyticum***

by  
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*Dissertation presented for the degree of Doctor of  
Philosophy in Medical Microbiology at the  
University of Stellenbosch*



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December 2010

## **Declaration**

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## ABSTRACT

**Overview:** Mycoplasmas and ureaplasmas are not routinely diagnosed and are under researched in South Africa. Prevalence, population shifts especially concerning genital flora and implications in infection or other conditions are unknown. Information pertaining to *Mycoplasma pneumoniae* in respiratory disease is similarly lacking. There is little information on antimicrobial susceptibilities and resistance development against Sexually Transmitted Infections (STI) syndromic management approaches.

**Aims:** a) Elucidate mycoplasmal and ureaplasma prevalence and contributing factors concerning cervical colonisation or preterm delivery in conjunction with HIV and *Chlamydia trachomatis* b) Investigate prevalence of *M. pneumoniae* in respiratory infections in conjunction with HIV, *Mycobacterium tuberculosis* and *Pneumocystis jiroveci*. c) Determine antimicrobial susceptibilities of mycoplasmas and ureaplasmas and analyse resistance genes. d) Assess the inter-generic transfer potential of resistance gene (*tetM*) between *Ureaplasma* spp. and *Neisseria gonorrhoeae*.

**Genital setting:** The prevalence of genital mycoplasmas, ureaplasmas and *Chlamydia* on women attending their first prenatal visit, in conjunction with preterm labour or HIV status was investigated. For preterm labour (2003), 199 women were monitored for preterm delivery (<37 weeks); for colonisation and HIV (2005), 219 women were screened. Microbial detection was performed on DNA extracted from endocervical swabs employing PCR techniques. Colonisation was seen to be highest in the 14–20 year group from 2003. In women aged ≥21 years, co-colonisation was 13% although there was a shift from co-colonisation with *Mycoplasma hominis* and *Ureaplasma* spp. in 2003 to other dual/triple combinations in 2005. Overall major trends from both collection periods were that the prevalence of *Ureaplasma* spp. tended to be higher in women ≥26 years, whilst prevalence of *C. trachomatis* and *M. hominis* were lower. No association was evident between colonisation with *M. hominis*, *U. urealyticum*, *Ureaplasma parvum* and labour outcome. HIV status



had no effect on the prevalence/co-colonisation of *M. hominis*, *Ureaplasma* spp. or *C. trachomatis*.

**Respiratory setting:** Studies were conducted to determine the prevalence of community acquired atypical pneumonias in adults (*M. pneumoniae* and *P. jiroveci*) and neonates (mycoplasmas, ureaplasmas and *Chlamydia trachomatis*) in order to improve treatment management programmes in the Port Elizabeth region. Sputum specimens from 102 adult patients presenting with pneumonia/symptoms of pneumonia admitted to hospitals were assessed by PCR. Details of patient's gender, age, HIV and *Mycobacterium tuberculosis* status were provided by the hospitals. Women were seen to be at high risk for community-acquired *P. jiroveci* colonisation. Overall, prevalence of *P. jiroveci* was 52.9% (54/102 patients). *P. jiroveci* was mainly associated with HIV (25/74) (*P. jiroveci* and HIV positive patients in patient sample for which clinical data and HIV status was available) and co-infection with *M. tuberculosis* was observed in 12 HIV cases and one HIV negative patient. No DHPS (20) or DHFR (17) resistance associated mutations were found in *P. jiroveci*. *M. pneumoniae* was detected in one patient. For prevalence studies (2007-2008) on atypical pneumonia in neonates, 69 endotracheal aspirates were obtained. PCR detection of *M. hominis*, *U. urealyticum* and *C. trachomatis* was performed and *U. parvum* detected in two specimens.

**Antibiotic susceptibilities and resistance genes:** The following investigations on clinical isolates of *U. parvum* and *U. urealyticum* were conducted (i) antibiotic susceptibility profiles, (ii) detection of drug target gene mutations, or gene acquisitions and (iii) inter-generic resistance gene transfer potential to *Neisseria gonorrhoeae*. Culture techniques applied to 132 endocervical specimens provided 66 *Ureaplasma* cultures (35 *U. parvum*, 9 *U. urealyticum*, 22 *U. parvum* + *U. urealyticum*). MIC determinations to ofloxacin, erythromycin, tetracycline, doxycycline, azithromycin and josamycin were performed. Thirty-seven ureaplasma cultures were fully susceptible to all antibiotics tested; 21 showed intermediate resistance to erythromycin, azithromycin and ofloxacin; while seven were resistant to tetracycline, three of which were also resistant to doxycycline and one also resistant to

azithromycin. Concerning ofloxacin resistance directed at quinolone resistance determining regions, a substitution of Ser83Leu in ParC was demonstrated in one intermediately-resistant *Ureaplasma* (MIC 4 µg/ml) while a triple substitution of Asp112Glu in GyrA along with Ala125Thr and Ala136Thr in ParC was found in six further intermediately-resistant strains. No mutations were found in strains with MICs 1 µg/ml. No mutations were detected in 23S rRNA operons, L4 or L22 proteins. *TetM* and *int*-Tn genes were found in seven tetracycline-resistant strains. On screening 59 tetracycline-susceptible and -intermediate strains, eleven whilst possessing an *int*-Tn gene lacked a large region of *tetM* and 48 only contained small regions of *tetM*. The *tetM* genes of the seven tetracycline-resistant strains were sequenced and comparisons performed against GenBank sequences of *Neisseria gonorrhoeae*, *Streptococcus pneumoniae* and *U. urealyticum*. For five strains *tetM* was seen to be highly mosaic in structure containing regions that were similar to those of the GenBank strains and others that were unique. In the *tetM* leader region, four hot spot recombination sites were identified that could certainly influence the formation of the mosaic structures, upstream insertion sequences/open reading frames and transposon regions that regulate expression. On characterising the *int*-Tn genes of the seven tetracycline-resistant strains, three types were present indicating transposons from different origins had integrated into ureaplasma genomes. Reciprocal tetracycline resistance gene transfer between ureaplasmas and *N. gonorrhoeae* were unsuccessful. However, low-level tetracycline resistance (MICs 4-8 µg/ml) was transferred to a *U. parvum* recipient from one *U. urealyticum* and three *U. parvum* donors that carried *tetM* with MICs 16-64 µg/ml. On *tetM* PCR analysis, *tetM* was not detected in the transformants.

**Conclusions:** The importance of genital mycoplasmas, ureaplasmas and *C. trachomatis* in long term aetiologies requires further investigations, certainly in relation with syndromic management regimens that fail to reduce colonisation rates. The high prevalence of *P. jiroveci*, the presence of *M. pneumoniae* in cases of pneumonia and detection of *U. parvum* in two cases of neonatal pneumonia investigated emphasises that in the absence of definitive diagnoses, it is crucial to monitor treatment responses carefully, especially

when first line antibiotic preferences are  $\beta$ -lactams, in order to ensure adequate and informed delivery of medical care. The finding of transposon and/or *tetM* regions in all ureaplasmas investigated with or without full expression of tetracycline resistance, in conjunction with *tetM* gene diversity, certainly places ureaplasmas strongly in the picture for intra- and inter-generic exchange of antibiotic resistance genes.

## OPSOMMING

**Oorsig:** Mikoplasma en ureaplasma word nie roetinegeweg gediagnoseer nie en in Suid Afrika is nog min navorsing daarvoor gedoen. Prevalensie, populasie verskuiwings, veral in genital flora, en die impliksies van infeksie en ander toestande is onbekend. Inligting rakende *Mycoplasma pneumoniae* in respiratoriese siekte is ook gebrekkig. Daar is min inligting beskikbaar rakende die antimikrobiale vatbaarheid en die ontwikkeling van weerstandigheid gesien teen die benadering tot sindromiese hantering van seksueel oordraagbare siektes.

**Doelwitte:** a) Om inligting te verskaf oor die prevalensie van mikoplasma en ureaplasma en bydraende faktore betreffende voortydige kraam tesame met MIV en *Chlamydia trachomatis*. b) Ondersoek van die prevalensie van *M. pneumoniae* in respiratoriese infeksies tesame met MIV, *Mycobacterium tuberculosis* en *Pneumocystis jiroveci*. c) Bepaling van die antimikrobiale vatbaarheid van mikoplasma en ureaplasma en analise van weerstandigheds gene. d) Bereken die inter-genetiese oordrag potensiaal van weerstandigheds gene (*tetM*) tussen *Ureaplasma* spp. en *Neisseria gonorrhoeae*.

**Genitale omgewing:** Die prevalensie van genitale mikoplasma, ureaplasma en *Chlamydia* in vroue tydens hul eerste prenatale besoek, tesame met vroegtydige kraam en MIV status is ondersoek. In voortydige kraam (2003), is 199 vroue gemonitor vir voortydige kraam (<37 weke); vir kolonisasie en MIV (2005), is 219 vroue getoets. Mikrobiale toetsing is gedoen deur DNS te win vanaf endoservikale deppers met PKR tegnieke. Kolonisasie was die hoogste in die ouderdomsgroep 14–20 jaar, in 2003. In vroue van ≥21 jaar was mede-kolonisasie 13% alhoewel daar 'n verskuiwing was van mede-kolonisasie met *Mycoplasma hominis* en *Ureaplasma* spp. in 2003 tot ander dubbel/trippel kombinasies in 2005. Die oorkoepelende tendens in al twee die tydperke van waarneming was dat die prevalensie van *Ureoplasma* spp. geneig was om hoër te wees in vroue ≥26 jaar, terwyl prevalensie van *C. trachomatis* en *M. hominis* laer was. Geen assosiasie kon getoon word tussen kolonisasie met

*M. hominis*, *U. urealyticum*, *Ureaplasma parvum* en uitkoms van kraam nie. MIV status het geen effek gehad op die prevalensie/mede-kolonisasie van *M. hominis*, *Ureaplasma* spp. of *C. Trachomatis* nie.

**Respiratories:** Studies is gedoen om die prevalensie van gemeenskaps verworwe atipiese pneumonie in volwassenes (*M. pneumoniae* en *P. jiroveci*) en neonate (mikoplasma, ureaplasma en *Chlamydia trachomatis*) te bepaal om behandeling en hantering programme in die Port Elizabeth area te verbeter. Sputum monsters van 102 volwasse pasiënte wat presenteer het met pneumonie of simptome van pneumonie en wat tot hospitale toegelaat was, is ontleed. Besonderhede van die pasiënte se geslag, ouderdom, MIV en *Mycobacterium tuberculosis* status is deur die hospitale verskaf. PKR is gedoen met inleiers gerig teen die volgende gene: *P. jiroveci* vir die aantoning van mitokondriale groot subeenheid RNS en vir die analise van mutasies vir ko-trimoksasool weerstandigheid dihydropteroaat sintetase (DHPS) en dihydrofolaat reduktase (DHFR); *M. pneumoniae* vir die aantoning van P1 adhesien en 16S rRNS. Vroue het 'n hoë risiko vir gemeenskapsverworwe *P. jiroveci* kolonisasie gehad. In die algemeen was die prevalensie van *P. jiroveci* 52.9% (54/102 pasiënte). *P. jiroveci* was hoofsaaklik geassosieer met MIV (25/74) (*P. jiroveci* en MIV positiewe pasiënte in die pasiënt monster waarvoor daar kliniese data en MIV status bekend was) en mede-infeksie met *M. tuberculosis* is gesien in 12 MIV gevalle en een MIV negatiewe pasiënt. Geen DHPS (20) of DHFR (17) weerstandigheds geassosieerde mutasies is gevind in *P. Jiroveci* nie. *M. pneumoniae* was aangetoon in een pasiënt. Vir prevalensie studies (2007-2008) op atipiese pneumonie in neonate is 69 endotracheale aspirate verkry. PKR toetsing vir *M. hominis*, *U. urealyticum* en *C. trachomatis* is gedoen met 'primers' soos voorheen gepubliseer. *Ureaplasma parvum* is aangetoon in twee neonate met PKR met negatiewe kultuur resultate.

**Antibiotika sensitiwiteite en weerstandigheds gene:** Die volgende toetse is gedoen op kliniese isolate van *U. parvum* en *U. urealyticum* (i) antibiotika sensitiwiteits profiele, (ii) aantoning van teiken geen mutasies, of geen aanwinste en (iii) potensiaal vir inter-generiese weerstandigheds geen

oordrag na *Neisseria gonorrhoeae*. Kultuur tegnieke toegepas op 132 endoservikale monsters het 66 *Ureaplasma* kulture gelewer (35 *U. parvum*, 9 *U. urealyticum*, 22 *U. parvum* + *U. urealyticum*). MIK bepaling vir ofloksasien, eritromisien, tetrasiklien, doksisisiklien, azitromisien en josamisien is gedoen. Sewe-en-dertig kulture was ten volle sensitief vir alle antibiotika wat getoets is; een-en-twintig het intermediêre weerstandigheid teenoor eritromisien, azitromisien en ofloksasien getoon, terwyl sewe weerstandig was vir tetrasiklien, drie daarvan was ook weerstandig vir doksisisiklien. Wat betref ofloksasien weerstandigheid gemik teen kwinoloon weerstandigheds bepalende gebiede, is vervanging van Ser83Leu in ParC gedemonstreer in een intermediêre weerstandige *Ureaplasma* (MIK 4 µg/ml) terwyl 'n trippel vervanging van Asp112Glu in GyrA saam met Ala125Thr en Ala136Thr in ParC gevind is in ses ander intermediêre weerstandige stamme. Geen mutasies is gevind in stamme met MIKs van MICs 1 µg/ml nie. Geeneen van die ureaplasma was weerstandig vir eritromisien/azitromisien nie en geen mutasies is gevind in 23S rRNA operons, L4 of L22 proteïene nie. *TetM* en *int-Tn* gene is gevind in sewe tetrasiklien weerstandige stamme. 58 Tetrasiklien sensitiewe en –intermediêre stamme is getoets, waarvan elf 'n *int-Tn* geen gekort het sowel as 'n groot deel van *tetM*, terwyl 48 slegs klein dele van *TetM* bevat het. Die *tetM* gene van die sewe tetrasiklien-weerstandige stamme se geenvolgorde is bepaal en vergelykings is getref teenoor die GenBank volgordes van *Neisseria gonorrhoeae*, *Streptococcus pneumoniae* en *U. urealyticum*. In vyf stamme is gevind dat die *tetM* geen hoogs mosaïek in struktuur was met areas wat ooreenstem met die in GenBank stamme, en ander areas wat uniek is. In die *tetM* leier area, is vier 'hot spot' herkombinasie areas geïdentifiseer wat sekerlik die vorming van die mosaïek strukture kon beïnvloed, asook transposon areas wat geenuitdrukking bepaal. Met karakterisering van die *int-Tn* gene van die sewe tetrasiklien-weerstandige stamme, was drie tipes teenwoordig waarin transposons vanaf verskillende oorsprong aangedui was, geïntegreerd met die ureaplasma genome. Resiprokale tetrasiklien weerstandigheds geen oordrag tussen ureaplasma en *n. gonorrhoea* was nie suksesvol nie. Lae-vlak tetrasiklien weerstandigheid (MIK's van 4 – 8 µg/ml) is wel suksesvol oorgedra na 'n *U. parvum* ontvanger vanaf een *U. urealyticum* en drie *U. parvum* ontvangers

wat *tetM* gedra het met MIKs van 16-64 µg/ml. Met die analise van *tetM* met PKR, kon *tetM* nie aangetoon word in die transformante nie.

**Gevolgtrekkings:** Die belang van genitale mykoplasma, ureaplasma en *C. trachomatis* in langtermyn etologie benodig verdere ondersoek, veral in die lig van die sindromiese behandeling regimes wat nie kolonisasie verminder nie. Die hoë prevalensie van *P. jiroveci*, die teenwoordigheid van *M. pneumoniae* in gevalle van pneumonie en die aantoning van *U. parvum* in twee gevalle van neonatale pneumonie benadruk dat, in die afwesigheid van 'n definitiewe diagnose, dit noodsaaklik is om respons tot behandeling sorgvuldig te monitor, veral indien die eerste lyn antibiotika keuse β-laktam antimikrobiale middels of kefalosporiene is, sodat behoorlike en ingeligde gesondheidsorg gelewer kan word. Die bevinding van transposon en/of *tetM* gebiede in alle ureaplasma wat ondersoek is met of sonder volle uitdrukking van tetrasiklien weerstandigheid, in samehang met *tetM* diversiteit, plaas verseker ureaplasma sterk in die prentjie vir intra- en inter-generiese uitruiling van antibiotika weerstandigheids gene.

## ACKNOWLEDGEMENTS

The author records her appreciation to:

Dr L.J. Chalkley, (promoter), Department of Medical Microbiology, University of Stellenbosch, without her, this thesis would not have been possible. The author is grateful for her constant availability, assistance with the preparation of the manuscripts that were published, for guidance, recommendations concerning all research experiments and incredible patience. The good advice and encouragement has been invaluable on both an academic and a personal level.

Prof E. Wasserman, (co-promoter), Department of Medical Microbiology, University of Stellenbosch, for financial assistance, her support and providing *N. gonorrhoeae* cultures for the resistance gene transfer component of the project.

Profs H.J. Odendaal and G.B. Theron, Department of Obstetrics and Gynaecology, University of Stellenbosch, Tygerberg hospital, for co-ordination of sample collection for the initial 2003 study and 2005-2006 series, respectively.

Staff and patients of the Obstetrics and Gynaecology Unit at Tygerberg hospital for their participation in this study.

G.A. van Zijl for obtaining patients' consent and co-ordinating samples and detailed records for the 2005-2006 series of samples.

N. du Plessis, Department of Medical Microbiology, University of Stellenbosch for excellent administrative assistance during the past 6 years.

Staff (especially Drs GS Ocana, F. Khan and B. Brown) and patients at Dora Nginza and Livingstone hospitals, Port Elizabeth, for participation and assistance with collection of the respiratory specimens.



M.C. Scheckle, R.B. Nicholas and SJ du Plessis, Department of Biochemistry and Microbiology, Nelson Mandela Metropolitan University, for their invaluable technical assistance with various components of the project.

Prof R.J. Naude, Department of Biochemistry and Microbiology, Nelson Mandela Metropolitan University, and Prof A. Leitch, Dean of the Faculty of Science for their support and encouragement during the course of this project.

Dr J. Pietersen, Nelson Mandela Metropolitan University, for assistance with statistical analysis.

M. de Jongh, University of Limpopo and F. Radebe, National Institute of Communicable Disease – National Health Laboratory Services, University of the Witwatersrand, for providing *N. gonorrhoeae* cultures for the resistance gene transfer component of the project.

Medical Research Council, National Research Foundation (NRF-Thuthuka), and Nelson Mandela Metropolitan University (NMMU) for funding to carry out this study.

Her family for their unequivocal support, encouragement and incredible patience throughout this study.

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## LIST OF ABBREVIATIONS

A	Adenine
A2058G	Adenine to guanine mutation at position 2058
A2149C	Adenine to cytosine mutation at position 2149
A2181T	Adenine to thymine mutation at position 2128
Ala	Alanine
ATCC	American Type Culture Collection
Asn	Asparagine
Asp	Aspartic acid
AZM	Azithromycin
BAL	Broncho-alveolar lavage
BLAST	Basic local alignment search tool
bp	Base pairs
C	Cytosine
CEM 101	Investigational ketolide
C2243N	Cytosine to any nucleotide at position 2243
CO <sub>2</sub>	Carbon dioxide
CFU/ml	Colony forming units per millilitre
CLSI	Clinical and laboratory standards institute
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
DOX	Doxycycline
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
E	Erythromycin
EF-G	Elongation factor G
EF-Tu	Elongation factor Tu
ELISA	Enzyme linked immunosorbent assay
Erm	Methyltransferase
g	grams
G2056U	Guanine to uracil at position 2056
G2057U	Guanine to uracil at position 2057
G+C	Guanine and cytosine
Gln	Glutamine
Glu	Glutamate
<i>gyrA</i>	Gene encoding GyrA which is a structural component of DNA gyrase

<i>gyrB</i>	Gene encoding GyrB which is a structural component of the enzyme DNA gyrase
h	Hours
HIV	Human immunodeficiency virus
I	Intermediate
<i>Int-Tn</i>	Gene encoding integrase which is part of the Tn 1545 transposon
IS1221	Insertion sequence 1221
IOM	International Organization of Mycoplasmology
JOS	Josamycin
kb	Kilobases
LB	Luria broth
L4	Protein component of ribosomes
L22	Protein component of ribosomes
Leu	Leucine
l	Litre
Lys	Lysine
<i>md1</i>	Genes that encode efflux pumps for drug resistance
<i>md2</i>	Genes that encode efflux pumps for drug resistance
MDR	Multidrug resistance efflux pumps
MIC	Minimum inhibitory concentration
MLSK	Macrolide-lincosamide-streptogramin-ketolide
M	Molar
min	minutes
ml	millilitre
mM	Millimolar
mRNA	Messenger rRNA
mtLSUrRNA	Mitochondrial large subunit ribosomal RNA
NALC	N-acetyl cysteine
NHLS	National Health Laboratory Services
NMMU	Nelson Mandela Metropolitan University
OFX	Ofloxacin
PBS	Phosphate buffered saline
PcP	<i>Pneumocystis pneumonia</i>
<i>parC</i>	A gene encoding ParC which is a structural component of the enzyme topoisomerase IV
<i>parE</i>	A gene encoding ParE which is a structural component of the enzyme topoisomerase IV
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
RNA	ribonucleic acid

rRNA	Ribosomal ribonucleic acid
S	Susceptible
Ser	Serine
SOC medium	Super optimal broth with catabolite repression
spp.	Species
SP4	Spiroplasma medium
STIs	Sexually transmitted infections
T	Thymine
TAE	Tris-acetate-EDTA
Taq	<i>Thermus aquaticus</i>
TB	Tuberculosis
TE	Tris-EDTA
TMP-SMX	Trimethoprim-sulphamethoxazole
TET	Tetracycline
<i>tetM</i>	A gene which encodes for a the TetM protein which confers resistance to tetracycline
Thr	Threonine
Tn1545	Transposon known to carry the <i>tetM</i> determinant
Tn916	Transposon which is known to harbour the <i>tetM</i> gene
U	uracil
µg/ml	Micrograms per millilitre
µl	Microlitre
µm	Micrometer
µM	Micromolar
Up	<i>Ureaplasma parvum</i>
<i>Ure-A, Ure-B,</i>	Urease genes
<i>Ure-C, Ure-D,</i>	
<i>Ure-E, Ure-F,</i>	
<i>Ure-G</i>	
Uu	<i>Ureaplasma urealyticum</i>
v	Volume
xg	Gravitational force
Zn	Ziehl-Neelsen
23S	Subunit of ribosome
%	Percent
°C	Degrees Celsius

# CHAPTER ONE

## INTRODUCTION

### 1.1 MYCOPLASMAS AND UREAPLASMAS IN HUMAN DISEASES

#### 1.1.1 Taxonomy

Mycoplasmas and ureaplasmas are pleomorphic, nonmotile microorganisms that lack cell walls, and are among the smallest independently living organisms. Sixteen *Mycoplasma* and *Ureaplasma* species that can be pathogenic/ opportunistic to humans in some settings have been identified, the most known species include: *Mycoplasma hominis*, *Mycoplasma pneumoniae*, *Mycoplasma genitalium*, *Mycoplasma fermentans*, *Mycoplasma penetrans*, *Ureaplasma parvum* and *U. urealyticum*. *M. amphoriforme* is a recently identified species and its pathogenic role in human disease is currently under investigation (Waites and Talkington, 2005).

Mycoplasmas and ureaplasmas were classified in a separate phylum of Gram-negative bacteria *Mollicutes*, however, analyses of rRNA revealed they were similar to Gram-positive bacteria. They are now categorised as low G+C Gram-positive bacteria in the Phylum *Firmicutes*; nevertheless they appear pink when stained with the Gram stain (Bauman, 2007). *U. parvum* and *U. urealyticum* were originally considered one species *U. urealyticum* with subdivision into two biovars. In 2002, after prolonged debate, it was agreed that the former *U. urealyticum* should be divided into two species namely *U. parvum* (biovar 1) and *U. urealyticum* (biovar 2) (Kong and Gilbert, 2004). *U. parvum* contains serovars 1, 3, 6 and 14 while *U. urealyticum* contains the serovars 2, 4, 5, 7, 8, 9, 10, 11, 12 and 13 (Schelonka and Waites, 2007). A urease gene cluster has been found that consists of seven genes, *ureA*, *ureB*, *ureC* being responsible for the structural units of urease and *ureD*, *ureE*, *ureF*, *ureG* being responsible for the accessory proteins. Although these urease genes are found within the various serovars, there are key differences in their

distributions between *U. parvum* and *U. urealyticum* that can be used to separate the two species (Mallard *et al.*, 2004).

### 1.1.2 *Mycoplasma* spp. and *Ureaplasma* spp in urogenital infections

The importance of genital mycoplasmas in prematurity, pregnancy loss and chorioamnionitis have been topics of great interest although not satisfactorily resolved as analyses have been complicated by different study designs and inappropriate sampling sites (Waites and Talkington, 2005). Studies from which ureaplasmas and *M. hominis* were isolated from the endometrium or placenta have shown a consistent association with spontaneous abortion, however, this has not been substantiated by studies limited to sampling the lower genital tract such as endocervical specimens (Taylor-Robinson *et al.*, 2003a).

*M. hominis* has been shown to be associated with bacterial vaginosis and linked to a) increased frequencies of *Chlamydia trachomatis* (Odendaal *et al.*, 2002) and b) *U. urealyticum* infections and genital tract HIV RNA levels (Sha *et al.*, 2005). Movement of *M. hominis* into the chorioamnion causes inflammation (chorioamnionitis) and movement into the amniotic cavity results in the colonisation of the foetus (Kim *et al.*, 2003). Evidence has also been found linking *M. hominis* with pelvic inflammatory disease (Taylor-Robinson, 2007). Cervical colonisation (sample size of 218) women with *M. hominis* and *U. urealyticum* was associated with lower gestational age at delivery, lower birth weight, and increased neonatal morbidity and mortality (Luton *et al.*, 1994). However, no correlation was found for co-existence of *M. hominis*/*U. urealyticum* colonisation with typical and atypical urinary tract infections or for sterile pyuria (Latthe *et al.*, 2008). *M. hominis* has also been shown to cause postpartum and postabortal fever however it has also been argued that it may actually be only a marker for bacterial vaginosis (Taylor-Robinson, 2007).

*M. genitalium* has been shown to cause acute and chronic urethritis in men (Jensen *et al.*, 1993; Deguchi and Maeda, 2002; Taylor-Robinson *et al.*,

2003b). In women it is strongly associated with cervicitis, endometritis and infertility (Cohen *et al.*, 2002; Deguchi and Maeda, 2002; Uuskula and Kohl, 2002). Recently, studies in Peruvian women with a sample group comprising 661 cases with spontaneous preterm birth and 667 controls, cervical *M. genitalium* detection was found to be independently associated with younger maternal age and preterm birth (Hitti *et al.*, 2010).

In contrast, *M. genitalium* was not seen to be significantly associated with non-gonococcal urethritis in South African men with genital ulcer disease (Ballard *et al.*, 2002). Further studies from South Africa have shown *M. genitalium* infection in male urethritis syndrome and genital ulcer syndrome (Black *et al.*, 2008), with a high prevalence of *M. genitalium* and other STIs observed in asymptomatic men (Lewis *et al.*, 2008).

*M. penetrans* has been isolated from the urogenital tracts of individuals with HIV infection (Hussain *et al.*, 1999). *M. fermentans* has been reported to be an opportunist in HIV infection (Ainsworth *et al.*, 2001; Waites and Talkington, 2005) and to be responsible for bacteraemia in a high percentage of patients with rheumatoid arthritis (Gil *et al.*, 2009).

Ureaplasmas are able to colonise the vagina and cervix and have been implicated as a cause of preterm labour possibly through the production of cytokines that can cause premature contractions. They are also associated with spontaneous abortion, choriamnionitis, foetal body weight fluctuations, and the introduction of other bacterial species into the womb followed by intense inflammation (Taylor-Robinson, 2007; Kim *et al.*, 2003). Vaginal carriage of *Ureaplasma* spp. is not reliably predictive of preterm labour, but there is an association when it is present in the amniotic fluid or placenta. In contrast it has been reported that women whose cervixes were culture positive for *Ureaplasma* spp. were more likely to develop pregnancy complications than women with a negative culture (Waites *et al.*, 2005). The presence of *U. urealyticum* in placental parenchyma before 28 weeks was found to higher risks of foetal and maternal inflammation and was associated with increased risk of preterm labour and delivery (Olomu *et al.*, 2009). A high

prevalence of *U. urealyticum* was observed in women with unexplained chronic urinary (voiding) symptoms (Baka *et al.*, 2009). *Ureaplasma* spp. can bind to sperm cells and by inhibiting free movement, infertility can result (Waites and Talkington, 2005). *M. genitalium* and *Ureaplasma* spp. were found to be widespread among infertile male patients in Tunisia (Gdoura *et al.*, 2008).

Extragenital infections caused by *Ureaplasma* spp. and *M. hominis* include neonatal pneumonia (Waites *et al.*, 2005; Biernat-Sudolska *et al.*, 2006), abscesses (Waites and Talkington, 2005), septic arthritis (Phuah *et al.*, 2007) and surgical wound infections in patients with malignancies (Krijnen *et al.*, 2006), organ transplant recipients (Hopkins *et al.*, 2002) and mediastinitis (Mattila *et al.*, 1999). There have been reports of *U. urealyticum* meningitis in an adult patient after a complicated kidney transplantation and organ rejection (Gessdorfer *et al.*, 2008) and detection of an erythromycin-resistant *U. urealyticum* meningitis in a premature infant (Chung *et al.*, 2007).

### **1.1.3 Neonatal diseases caused by genital mycoplasmas and ureaplasmas**

*U. urealyticum* and *U. parvum* can be transmitted vertically from the parent to offspring either *in utero* or perinatally (Biernat-Sudolska *et al.*, 2006; Pinna *et al.*, 2006). *U. urealyticum* and *U. parvum* infections of the newborn have been associated with bacteraemia, pneumonia, chronic lung disease and the central nervous system (Cultrera *et al.*, 2006; Waites *et al.*, 2005). There is strong evidence from clinical and experimental animal studies that ureaplasmas can invade the amnionic sac and induce an inflammatory response resulting in chorioamnionitis, preterm labour and neonatal lung injury (Waites *et al.*, 2009a).

Evidence of vertical ureaplasma transmission and neonatal infection comes from observations that ureaplasmas can be isolated from endotracheal secretions in up to 40% of newborn infants within 30 minutes to 24 hours after

birth and from maternal and umbilical cord blood at the time of delivery (Schelonka and Waites, 2007). Newborns with birth weights of less than 1000 g appear to be at a higher risk of infection when the mother is colonised, with the rate of vertical transmission approaching 90% for these infants. The presumed mechanisms of infection include foetal exposure to ascending ureaplasma intra-uterine infection, passage through an infected birth canal, and haematogenous dissemination through the placenta into umbilical vessels. These exposures can lead to colonisation of the skin, mucosal membranes, and respiratory tract, and sometimes to dissemination into the bloodstream and central nervous system (Schelonka and Waites, 2007).

Case studies have reported the presence of *M. hominis* in eye infections and brain abscesses in the neonate (Waites *et al.*, 2005). In addition, in neonates with respiratory distress, *Ureaplasma* spp. alone or in combination with *M. hominis* has been detected more frequently than *M. hominis* alone (Waites *et al.*, 2005; Waites and Talkington, 2005). Hence, it would be expected for such infections in neonates to be predominantly caused by *Ureaplasma* spp.

#### **1.1.4 *Mycoplasma* spp. and *Ureaplasma* spp. in respiratory disease**

*M. pneumoniae* is a common cause of acute upper and lower respiratory tract infections particularly in children, young adults and the elderly; and community-acquired pneumonia which often results in hospitalisation due to severity of the illness (Madani and Al-Ghamdi., 2001; Waites and Talkington, 2004). Prevalence of *M. pneumoniae* infections within healthy populations can vary from between 13.5-34% to 45% in adults with asthma (Biscardi *et al.*, 2004; Lauderdale *et al.*, 2005; Braun *et al.*, 2006; Shankar *et al.*, 2006). *M. pneumoniae* infection may contribute to asthma exacerbation (Varshney *et al.*, 2009).

*U. urealyticum* and *U. parvum* infections of the newborn have been associated with pneumonia, and chronic lung disease (Schelonka and Waites, 2007). Cultrera *et al.* (2006), has suggested that *U. parvum* may play a



greater role in the pathogenesis of respiratory distress syndrome than *U. urealyticum*.

As many as 25% of patients infected with *M. pneumoniae*, may experience extrapulmonary manifestations, such as neurological and central nervous system damage, in the form of encephalitis, motor neuron damage, dermatological disorders such as erythematous maculopapular and vesicular rashes, conjunctivitis, non-specific ear disorders and glomerulonephritis (Palumbo *et al.*, 2008; Kano *et al.*, 2007; Guleria *et al.*, 2005; Waites and Talkington, 2004; Sotgiu *et al.*, 2003; Said *et al.*, 1999). The release of pro-inflammatory cytokines, in association with *M. pneumoniae*, has been implicated as a possible mechanism aggravating chronic pulmonary diseases, such as asthma and, possibly, triggering rheumatoid arthritis or reactive arthritis (Bebear, 2008; Harjacek *et al.*, 2006; Waites and Talkington, 2004; Haier *et al.*, 1999).

## 1.2 TREATMENT OF MYCOPLASMAS AND UREAPLASMAS

As mycoplasmas and ureaplasmas lack cell walls, antibiotics that target wall synthesis are ineffective.

Macrolide, lincosamide and streptogramin antibiotics (MLSKs) are commonly used for the treatment of human mycoplasma and ureaplasma infections. Ketolides (a derivative of erythromycin), tetracyclines and fluoroquinolones are also used. Mycoplasmas present different phenotypes of intrinsic resistance to macrolides. *M. pneumoniae* a respiratory mycoplasma is susceptible to all MLSKs. In contrast, *M. hominis* a genital species is naturally resistant to 14- and 15- membered macrolides and ketolides but is susceptible to josamycin a 16-membered macrolide and lincosamides. Intrinsic resistance to 14- membered macrolides has been observed in other species, like *M. fermentans* (Bebear and Robertson, 1996; Bebear and Bebear, 2002; Pereyre

*et al.*, 2002). *Ureaplasma* spp. are intrinsically-resistant to the lincosamides (eg. clindamycin) (Bebear and Bebear, 2002).

Macrolides are most commonly used for the treatment of *Ureaplasma* spp. infections in pregnant women and neonates (Bebear and Kempf, 2005; Pereye *et al.*, 2007a). The MLSK group, are also used for the treatment of respiratory tract infections or when tetracyclines or fluoroquinolones are contra-indicated, for instance in newborns, children or during pregnancy. For *Ureaplasma* spp. and *M. hominis* respiratory tract infections in neonates, erythromycin and josamycin respectively are normally considered first line treatment (Waites and Talkington, 2005). Recently, an investigational ketolide, CEM-101, was reported to be a potent compound, inhibiting growth of clinical isolates of *Mycoplasma* and *Ureaplasma* spp. at  $\leq 0.5$   $\mu\text{g/ml}$  (Waites *et al.*, 2009b).

Tetracyclines are used for the treatment of urogenital infections like non-gonococcal urethritis and pelvic inflammatory disease in which mycoplasmas/ureaplasmas may be implicated. They are also indicated in respiratory tract infections due to *M. pneumoniae* in adults.

Fluoroquinolones used against both *Mycoplasma* spp. and *Ureaplasma* spp. include ciprofloxacin, ofloxacin, gatifloxacin (Waites and Talkington, 2004; Blasi, 2004) and newer agents like sparfloxacin, gemifloxacin, garenoxacin and moxifloxacin (Bebear *et al.*, 2008). However, these antimicrobials cannot be given during pregnancy or to children due to juvenile cartilage damage (Bebear and Kempf, 2005).

### 1.3 DETECTION OF MYCOPLASMAS AND UREAPLASMAS

#### 1.3.1 Conventional culture

Diagnosis is hampered in pathology laboratories because mycoplasmas and ureaplasmas are not readily/reliably visualised by staining methods and grow poorly or not at all on conventional media. Due to the lack of a cell wall, mycoplasmas are very susceptible to adverse environmental conditions including drying, osmotic change, toxic metabolites, and extreme environmental fluctuations such as temperature. Quality controlled culture media specifically designed to support mycoplasmal growth is expensive. In addition, there is no single medium formulation that will adequately support the growth of all species. Media components include animal serum (foetal calf, horse serum), peptones, yeast extract, metabolic substrates (glucose, cysteine, arginine, and urea) and a pH indicator (phenol red). Atmospheric conditions for broths are aerobic, while agar requires the addition of 5 – 10% CO<sub>2</sub>, with an incubation temperature of 35 - 37°C (Duffy and Waites, 2008).

For genital mycoplasmas and ureaplasmas A8/ A7 agar is used, with 10B/ U9 broth for *Ureaplasma* spp. and arginine broth for *M. hominis* recommended. Antibiotics such as penicillin, cefoperazone, ampicillin or nystatin can be incorporated into media to inhibit bacterial and fungal contamination or overgrowth.

*M. hominis* produces 200 – 300 µm colonies on A8 agar and causes colour change in arginine broth. Colonies of *Ureaplasma* spp. growing on A8 agar have a brownish appearance due to urease activity in the presence of the CaCl<sub>2</sub> indicator contained in the agar (Waites *et al.*, 2001). *U. urealyticum* hydrolyses urea and releases ammonia, which results in an alkaline shift and the medium changes from yellow to pink. Broth cultures for *Ureaplasma* spp. should be examined for such colour changes twice a day for up to 7 days because on depletion of urea, ureaplasmas undergo a steep death phase (Pinna *et al.*, 2006). Therefore ureaplasmas need to be sub-cultured

timeously to a medium such as A8 or A7 agar, which will support further growth.

On the basis of colony morphology, *M. pneumoniae* cannot be differentiated from other *Mycoplasmas* (Daxboek *et al.*, 2003). It has been recommended that culture should also be combined with additional diagnostic techniques (Waites and Talkington, 2004). *Spiroplasma* medium (SP4), which was developed 20 years ago, for the cultivation of *Spiroplasma*s, was found to enhance the isolation of *M. pneumoniae* from clinical specimens. The presence of *M. pneumoniae* is indicated, in broth cultures by a distinct colour change of the medium, from red to yellow, as a result of glucose utilisation and on agar by distinct “fried-egg”, granulated colony formation. Culture of *M. pneumoniae* from respiratory specimens is slow, insensitive, expensive, and requires specialised growth media, with incubation periods of up to several weeks (Waites and Talkington, 2004; Daxboek *et al.*, 2003).

### 1.3.2 Commercial kits

The development of commercially prepared media and diagnostic kits offer simpler alternatives for the detection of *Mycoplasma* spp. and *Ureaplasma* spp. in urogenital and neonatal respiratory samples. Commercially prepared media or kits require laboratories to perform internal quality control and that product limitations should be noted (Waites *et al.*, 2001; Duffy and Waites, 2008). Commercially prepared culture media currently available are arginine broth (*M. hominis*) and U9 broth (*Ureaplasma* spp.) [Bio-Rad], while diagnostic test kits for identification and antibiotic susceptibility include: MycoDuo (Bio-Rad); MycoIST2 (bioMerieux), Mycoview (Ivagen) and Pneumofast kit for *M. pneumoniae*.

In the Mycoplasma Duo kit (Bio-Rad), identification is indicated by a colour change (from yellow to pink) of U9 and arginine broths based on the metabolism of urea and arginine. Results are read within 24 – 48 hours. This method allows for culture, identification and differential titration of *Ureaplasma*

spp. and *M. hominis*. Cheah *et al.* (2005), showed that the overall agreement between the polymerase chain reaction (PCR) and Mycoplasma Duo Kit for detection of *Ureaplasma* spp. in endotracheal aspirates from neonates was 96%.

MycolST2 (bioMerieux) allows for the identification, culture and antibiotic susceptibility testing of *Ureaplasma* spp. and *M. hominis*. Antibiotic susceptibility testing includes nine antibiotics at two different concentrations and uses similar antibiotics as those in the SIR kit with the inclusion of ciprofloxacin and clarythromycin. There have been contrasting reports concerning antibiotic resistance profiles when using the MycolST2 kit (Smayevsky *et al.*, 1995; Kenny and Cartwright, 2001). In addition, false positive results can be obtained as other bacteria (e.g. *Proteus*, *Klebsiella*) are capable of hydrolysing urea (Biernat-Sudolska *et al.*, 2006).

The Mycoview (Ivagen) test kit enables the identification and the differential titration of *U. urealyticum* and *M. hominis* from urogenital specimens. The test is based on natural resistance and specific metabolic properties: *U. urealyticum* is resistant to lincomycin and hydrolyses urea; *M. hominis* is resistant to erythromycin and hydrolyses arginine. Growth of the two species is visualised by a change in media colour from yellow – orange to red or pink.

The Pneumofast kit uses an SP4-type agar plate for isolation and morphological identification, while the Pneumofast assay is used for the enumeration and identification of *M. pneumoniae* within a liquid medium, and in addition provides susceptibility testing of four antibiotics (doxycycline, minocycline, ciprofloxacin and erythromycin). Identification is also based on the typical colony morphology of *M. pneumoniae*, namely its granular, fried-egg shape with a diameter of 10 to 150 µm, and its characteristic colour change in Pneumofast broth from red to orange to yellow (with no signs of turbidity) as a result of glucose utilisation (Madani and Al-Ghamdi, 2001). When comparing culture techniques with PCR, the sensitivity of culture is extremely low, with a maximum analytical sensitivity of 60% being obtained only in experienced, well-trained laboratories (Ieven *et al.*, 1996).

Serology is also an important diagnostic tool in routine laboratory practice. (Daxboek *et al.*, 2003; Tjhie *et al.*, 1994). Numerous commercially designed serological tests are available, including the complement fixation test, the microparticle agglutination assay and various enzyme linked immunosorbent assay tests (Daxboek *et al.*, 2003; Johnston and Martin, 2005; Loens *et al.*, 2003). A major disadvantage associated with serological assays, is that sensitivity is dependent on the precise timing of collection and therefore accurate diagnosis relies on the collection of serum samples at two specific points during the illness (McDonough *et al.*, 2005).

### **1.3.3 Molecular detection**

Although isolation by culture is considered the gold standard for detection, it has been shown to be inefficient for recovery of *Ureaplasma* spp. and *M. hominis* (Biernat-Sudolska *et al.*, 2006; Pinna *et al.*, 2006). Nelson *et al.* (1998), found that PCR was more sensitive than culture in specimens analysed soon after collection. If neonates acquire ureaplasmas during birth, the organism would be at a low concentration at day zero and increase over time. Thus rapid PCR assays in which early diagnoses are important can be of tremendous benefit in designing antibiotic treatment protocols (Nelson *et al.*, 1998). Real time PCR assays have been shown to be extremely useful for the simultaneous detection and biovar discrimination of ureaplasmas in clinical specimens (Yi *et al.*, 2005). Cao *et al.* (2007), have developed two Real-Time Taqman PCR assays for quantitative detection of *U. parvum* and *U. urealyticum*. The detection rate of real-time Taqman PCR was seen to be higher than that of culture methods and conventional PCR.

Sequences of the 16S rRNA gene and the 16S rRNA – 23S rRNA intergenic spacer regions, genes for urease, and the 5' ends of multiple-banded antigen genes have all been used in PCR-based assays to differentiate *U. parvum* from *U. urealyticum* (Blanchard, 1990; Cordova *et al.*, 2000; Robertson *et al.*, 1993). Many of the primer targets used in a study carried out by Kong *et al.* (2000), were based on previous observations that heterogeneity of the

intergenic spacer regions was greater than that within the genes. Primers based on these regions would therefore be more discriminatory for identification and sub-typing of *Ureaplasma* spp. and include primers: UP5A – U5A (16S rRNA – 23S rRNA gene spacer regions); UP2 – UPA2 and U52 – UUA2 (UreA – UreB and UreB – UreC gene spacer regions, respectively) (Kong *et al.*, 2000). Primers UU4 and UU5 have been used to target the urease gene for the identification of both *U. parvum* and *U. urealyticum* in cases of simultaneous infection (Cultrera *et al.*, 2006). Various primers which target different areas in the 16S rRNA gene have been designed to differentiate between species of mycoplasmas (Cordova *et al.*, 2000).

PCR is an appealing tool for the diagnosis of *M. pneumoniae*. However, false positive results, due to primer specificity or carry over contamination from previous PCR reactions, can occur (Murdoch, 2003; Ursi *et al.*, 2003), while the presence of inhibitory substances in the clinical sample could produce false negative results. Most studies have used lower respiratory tract samples, such as sputum and bronchoalveolar lavage (BAL). A major limitation of lower respiratory tract samples is that they are difficult to collect and require invasive techniques (Murdoch, 2003). Reznikov *et al.* (1995) and Ramirez *et al.* (1996), found that upper respiratory tract samples such as throat and nasopharyngeal swabs were appropriate and the preferred sample type with convenient collection and fewer PCR inhibitors, while Menendez *et al.* (1999), concluded that PCR of throat swab samples had a lower sensitivity than serology. In contrast, Welti *et al.* (2003) and Murdoch (2003), suggested that nasopharyngeal secretions, sputum, endotracheal aspiration, BAL samples, pleural fluid and lung tissue were acceptable specimens for PCR, but the highest sensitivity and specificity was obtained with sputa and BAL.

Different target sequences, within specific genes, have been used for *M. pneumoniae* PCR detection and include: P1 adhesin gene [Ramirez *et al.* (1996)] and 16S rRNA [Tjhie *et al.* (1994)]. The P1 adhesin gene is an appropriate target for PCR, because of its repetitive nature within the genome (Himmelreich *et al.*, 1997). Although similar P1 adhesin genes have been

found in other *Mycoplasma* species, some of the highly conserved regions are unique to *M. pneumoniae* enabling PCR primers directed at these regions to be species specific (Su *et al.*, 1987). Various PCR assays have been used for the detection of *M. pneumoniae*, such as real time PCR (Daxboek *et al.*, 2003), capillary PCR (Honda *et al.*, 2000), multiplex PCR (Loens *et al.*, 2003), loop-mediated isothermal amplification (Saito *et al.*, 2005), nucleic acid sequence-based amplification and enzyme-linked gel assay (Loens *et al.*, 2003 and Obyn *et al.*, 1996).

#### **1.4 ANTIMICROBIAL SUSCEPTIBILITY TESTING**

There are no internationally recommended guidelines for susceptibility testing of human mycoplasmas and ureaplasmas as there are for other prokaryotes under the Clinical and Laboratory Standards Institute (previously known as the National Committee for Clinical and Laboratory Standards).

Agar disk diffusion is not acceptable for susceptibility testing of ureaplasmas because of the small size of colonies and prolonged growth periods. The Etest agar gradient diffusion technique has been adapted to determine fluoroquinolone, tetracycline and macrolide susceptibilities of *Ureaplasma* spp. but Etests are not widely used due to restrictions associated with agar plate culture of ureaplasmas (Dosa *et al.*, 1999; Waites *et al.*, 1999; Ngan *et al.*, 2004).

The Mycoplasma Chemotherapy Working Team of the International Organisation for Mycoplasmaology (IOM) is working on a guideline susceptibility testing document. Standard microbroth dilution methods using microtitre plates are currently being used for susceptibility testing of mycoplasmas and ureaplasmas (Duffy *et al.*, 2000; Duffy and Waites, 2008).



Commercially produced broth media [U9 (*Ureaplasma* spp.) and arginine (*M. hominis*)] and susceptibility testing kits are available. Susceptibility testing kits include: SIR *Mycoplasma* kit (Bio-Rad) and the *Mycoplasma* IST 2 kit (bioMérieux). Both kits comprise microwells containing different concentrations of an antibiotic. Antibiotics used for resistance profiling in the SIR kit include: doxycycline (4 µg/ml and 8 µg/ml), tetracycline (4 µg/ml and 8 µg/ml), azithromycin (2 µg/ml and 4 µg/ml), josamycin (2 µg/ml and 8 µg/ml), erythromycin (1 µg/ml and 4 µg/ml), ofloxacin (1 µg/ml and 4 µg/ml), clindamycin (2 µg/ml) and pristinamycin (8 µg/ml). If mycoplasmas/ureaplasmas inoculated into the antibiotic containing wells are susceptible to an antibiotic present, growth will be inhibited and the broth will remain yellow. *M. hominis* is intrinsically-resistant to erythromycin and azithromycin, *Ureaplasma* spp. are intrinsically-resistant to clindamycin while josamycin is active against both *M. hominis* and ureaplasmas. Tetracycline and doxycycline are also assessed as there have been reports of low-level resistance, although frequency/rate of resistance tends to vary according to country and levels of antibiotic exposure. Ofloxacin is reported to be active against *M. hominis* and ureaplasmas but resistance frequencies to date are not well known (SIR kit manual; Bio-Rad). The SIR kit has been validated by laboratories in France and used in preliminary antibiotic resistance screening tests (Bebear *et al.*, 2003; Degrange *et al.*, 2008).

The *Mycoplasma* IST 2 kit is similar to the SIR kit, however, MycoIST2 uses different antibiotics with clarithromycin and ciprofloxacin at concentrations 1 and 4 µg/ml, and 1 and 2 µg/ml respectively, while azithromycin concentrations are 0.12 and 4 µg/ml. There have been contrasting reports concerning antibiotic resistance profiles when using the MycoIST2 kit (Smayevsky *et al.*, 1995; Kenny and Cartwright, 2001) and false positive results have been obtained (Biernat-Sudolska *et al.*, 2006).

Antibiotic susceptibility testing for *M. pneumoniae* is not routinely performed as resistant isolates are considered rare and results are only available after a prolonged period due to the fastidious nature of *M. pneumoniae* (Bebear, 2008).

Minimum inhibitory concentration (MIC) ranges for various antimicrobials against *Mycoplasma* spp. and *Ureaplasma* spp. have been reported (Table 1.1) (Bebear and Kempf, 2005).

**Table 1.1:** MIC ranges ( $\mu\text{g/ml}$ ) for various antimicrobials against *Mycoplasma* spp. and *Ureaplasma* spp. (Bebear and Kempf, 2005).

Antimicrobial	<i>M. pneumoniae</i>	<i>M. genitalium</i>	<i>M. hominis</i>	<i>M. fermentans</i>	<i>Ureaplasma</i> spp.
<i>Tetracyclines<sup>b</sup>, glycylicyclines</i>					
Tetracycline	0.63-0.25	0.06-0.12	0.2-2	0.1-1	0.05-2
Doxycycline	0.02-0.5	$\leq 0.01$ -0.3	0.03-2	0.05-1	0.02-1
Minocycline	0.06-0.5	$\leq 0.01$ -0.2	0.03-1	ND	0.06-1
Tigecycline	0.06-0.25	ND	0.125-0.5	ND	1-16
<i>MLSK group</i>					
Erythromycin	$\leq 0.004$ -0.06	$\leq 0.01$	32->1,000	0.5->64	0.02-4
Roxithromycin	$\leq 0.01$ -0.03	$\leq 0.01$	>16->64	32-64	0.06-4
Clarithromycin	$\leq 0.004$ -0.125	$\leq 0.01$ -0.06	16->256	1-64	$\leq 0.004$ -2
Azithromycin	$\leq 0.004$ -0.01	$\leq 0.01$ -0.03	4->64	0.05-8	0.06-0.5
Josamycin	$\leq 0.01$ -0.02	0.01-0.02	0.05-2	0.12-0.5	0.03-4
Spiramycin	$\leq 0.01$ -0.25	0.12-1	32->64	2-4	4-32
Midecamycin	$\leq 0.015$	ND	0.25	0.06	ND
Clindamycin	$\leq 0.008$ -2	0.2-1	$\leq 0.008$ -2	0.01-0.25	0.2-64
Lincomycin	4-8	1-8	0.2-4	0.12	8-256
Pristinamycin	0.02-0.05	ND	0.1-0.5	ND	0.1-1
Quinupristin/ Dalfopristin	0.008-0.12	0.05	0.03-2	0.12-0.5	0.03-0.5
Telithromycin	0.0002-0.06	$\leq 0.015$	2-32	0.06-0.25	$\leq 0.015$ -0.25
Cethromycin	$\leq 0.001$ -0.016	ND	$\leq 0.008$ -0.031	$\leq 0.008$	$\leq 0.008$ -0.031
<i>Fluoroquinolones<sup>b</sup></i>					
Pefloxacin	2	ND	1-4	ND	1-8
Norfloxacin	ND	ND	4-16	ND	4-16
Ciprofloxacin	0.5-2	2	0.5-4	0.02-0.25	0.1-4
Ofloxacin #	0.05-2	1-2	0.5-4	0.02-0.25	0.2-4
Sparfloxacin	$\leq 0.008$ -0.5	0.05-0.1	$\leq 0.008$ -0.1	$\leq 0.01$ -0.05	0.003-1
Levofloxacin	0.5-1	0.5-1	$\leq 0.008$ -0.5	0.05	0.12-2
Trovafoxacin	$\leq 0.008$ -0.5	0.03-0.06	$\leq 0.008$ -0.06	$\leq 0.008$ -0.03	$\leq 0.008$ -0.5
Gatifloxacin	0.06-1	0.12	0.06-0.25	0.12-0.25	0.25-2
Moxifloxacin	0.06-0.3	0.03	0.06	$\leq 0.015$ -0.06	0.12-0.5
Gemifloxacin	$\leq 0.008$ -0.12	0.05	0.0025-0.01	0.001-0.01	$\leq 0.008$ -0.25
Garenoxacin	0.015-0.12	0.06-0.12	0.008-0.25	$\leq 0.015$	0.06-0.25
<i>Chloramphenicol</i>	2-10	0.5-25	4-25	0.5-10	0.4-8
<i>Aminoglycosides</i>					
Gentamicin	4	ND	2-16	0.25->500	0.1-13
<i>New agents</i>					
Linezolid	64-256	ND	2-8	ND	>64
Evernimicin	2-16	ND	1-16	ND	8-16

<sup>b</sup>: Susceptible strains

<sup>#</sup>: Bebear *et al.* (2008) reported ofloxacin MIC range to be 0.5-8  $\mu\text{g/ml}$  for *Ureaplasma* spp;

## 1.5 ANTIMICROBIAL DRUG RESISTANCE

Resistance to an antibiotic can originate in different ways: (i) resistance due to natural genetic makeup, resulting in all bacteria belonging to that species being resistant (intrinsic resistance); (ii) resistance developed by mutation(s) in an antimicrobial target region and (iii) resistant gene/gene regions acquired from another microorganism, in cases ii and iii resistance is limited to a particular isolate/clone. Genital mycoplasmas and ureaplasmas are intrinsically resistant to certain members of the MLSK-group as described in section 1.2. Target alterations associated with quinolone and macrolide resistance development, and transposon located *tetM* genes have been described in mycoplasmas and ureaplasmas (Bebear and Kempf, 2005).

Fluoroquinolones act by inhibiting DNA replicating enzymes, gyrase and topoisomerase IV. Mutations in the quinolone resistance determining regions (QRDR's) of genes encoding gyrase subunits (*gyrA* and *gyrB*), and topoisomerase IV genes (*parC* and *parE*) have been correlated with quinolone MICs to determine which mutations/amino acid substitutions contribute to resistance development in ureaplasmas (Bebear *et al.*, 1997; Bebear *et al.*, 1999; Bebear *et al.*, 2003).

Until 2006, mutations resulting in amino acid substitutions at Ser83Leu and Asp87Lys in ParC and a triple substitution of Asp112Glu in GyrA protein along with Ala125Thr and Ala136Thr in ParC had all been suggested to be associated with fluoroquinolone resistance development in *Ureaplasma* spp. (Bebear *et al.*, 2000; Zhang *et al.*, 2002; Bebear *et al.*, 2003). However, recently Beeton *et al.* (2009a), attributed the triple mutations to species-specific polymorphisms found in all *Ureaplasma* serovars and therefore not considered to be associated with a resistance phenotype.

*GyrA*, *gyrB*, *parC* and *parE* mutations in 13 fluoroquinolone-resistant clinical isolates of *U. parvum* from two research groups were compared by Duffy *et al.* (2006), the most frequently observed amino acid replacements were Asp112Glu in GyrA and Ser83Leu in ParC which was seen in tandem for

twelve isolates. An American isolate exhibiting an ofloxacin MIC of 128 µg/ml was found to contain the Ser83Leu substitution in ParC but the only GyrA mutation noted was Gln103Lys (Duffy *et al.*, 2006). Beeton *et al.* (2009a), on conducting a similar comparative study to Duffy *et al.* (2006), that now incorporated reports from six groups and comprised 32 resistant isolates, drew attention only to the Ser83Leu substitution in ParC which was found in 19/32 isolates. On adding a study isolate with a ciprofloxacin MIC 8 µg/ml which had a single ParC substitution Asp82Asn Beeton *et al.* (2009a), concluded that only mutations within an 8-amino acid region of the most common ParC substitution Ser83Leu should be considered to be associated with quinolone resistance.

The active efflux of fluoroquinolones is mediated by endogenous multidrug resistance (MDR) efflux pumps, increased expression of which can develop a MDR resistance phenotype. Raheison *et al.* (2002), reported an active efflux system, in ethidium bromide selected strains of *M. hominis* that exhibited a MDR resistance phenotype with increased MICs to ciprofloxacin. Two genes (*md1* and *md2*) coding for putative multidrug resistance ABC transporters were identified and that the resistant strains exhibited higher expression levels of proteins involved in the membrane pump. To-date resistance to fluoroquinolones has been reported among human genital *Mycoplasma* and *Ureaplasma* species (Bebear and Kempf, 2005; Duffy *et al.*, 2006; Xie and Zhang, 2006). Characterisation of *in vitro*-selected mutants of *U. parvum* that were resistant to macrolides revealed no significant difference in MICs, with or without reserpine indicating the absence of a putative efflux mechanism (Pereye *et al.*, 2007a).

The methyltransferase enzyme (Erm family) or enzymes modifying macrolides have not been identified in mycoplasmas/ureaplasmas (Bebear and Kempf, 2005). Strains exhibiting low, but elevated levels (i.e. greater than reported susceptibility MICs yet lower than resistant MICs) of macrolide resistance have been found not to contain mutations in the 23S rRNA encoding operon or L22 and L4 proteins. Erythromycin and clarithromycin resistance in *Ureaplasma* spp. has recently been found to include novel point mutations in

the L4 protein, attributed to a six base pair deletion corresponding to two deleted amino acids arginine and glutamine at residue numbers 66 and 67 (Beeton *et al.*, 2009b). It has also been suggested that transition mutations in 23S rRNA could account for macrolide resistance development in *U. urealyticum* (Pereyre *et al.*, 2007a; Dongya *et al.*, 2008). Macrolide and ketolide resistance is thought to be predominantly centred in domain V of 23S rRNA, involving specific G2056U, G2057U and A2058G transition mutations (Pereyre *et al.*, 2007a). A 23S rRNA operon investigation on roxithromycin and azithromycin resistance found a C2243N transition mutation appeared to facilitate resistance to both macrolides, with A2149C and A2181T mutations associated with roxithromycin resistance only (Dongya *et al.*, 2008). Pereyre *et al.* (2007a and 2007b), have also proposed that several mutations on both rRNA subunits and ribosome protein components are prerequisites for full expression of high-level macrolide resistance. The increasing prevalence of macrolide-resistant strains of *M. pneumoniae* has become a significant clinical issue in paediatric patients (Matsubara *et al.*, 2009) and has been associated with prolonged severe respiratory infection in children (Li *et al.*, 2009).

Concerning acquisition of resistance genes/gene regions from other bacteria, only cryptic plasmids have been found in *Mollicutes* of which none have been described in species that colonise/infect humans (Bebear and Kempf, 2005). The extent to which tetracycline resistance occurs in genital mycoplasmas/ureaplasmas varies geographically and according to prior antimicrobial exposure. The *tetM* gene has been found in the Tn916-Tn1545 family of conjugative transposons in both *Streptococcus* spp. (Su *et al.*, 1992) and in *M. hominis* (Bebear and Kempf, 2005). The mechanism by which the TetM protein functions is to cover a ribosomal site that would otherwise enable tetracycline to bind and prevent translation. The *tetM* gene has DNA regions homologous to those of elongation factors EF-G and EF-Tu and thus the TetM protein protects the ribosome and allows for the continuation of translation to occur (Bebear and Kempf, 2005).

Tetracycline resistance in clinical isolates of *Ureaplasma* spp. and *M. hominis* have been reported in Singapore (Ngan *et al.*, 2004), France (Degrange *et al.*,

2008) and United Kingdom (Beeton *et al.*, 2009b). Tetracycline-resistant strains that are concurrently resistant to doxycycline have been described (Blanchard *et al.*, 1992 and Beeton *et al.*, 2009b). Such strains could influence the prevalence of mycoplasmas/ureaplasmas in countries that include doxycycline in sexually transmitted infection syndromic management regimens.

## **1.6 POTENTIAL FOR INTERGENERIC TRANSFER OF ANTIBIOTIC RESISTANCE GENES**

As natural exchange of chromosomal DNA between cells of mycoplasmas has only been proposed by Teachman *et al.*, (2002), it is thought that mycoplasmas/ ureaplasmas probably do not acquire DNA by conventional transformation processes. The lack of a cell wall in mycoplasmas/ ureaplasmas would be expected to facilitate the introduction of exogenous DNA into the cells that may well involve transient fusion of the cell membranes at the zone of contact (Razin *et al.*, 1998). The very small genomes of mycoplasmas/ureaplasmas lack a substantial part of standard recombination mechanisms available in larger bacterial genomes, however, they do contain large numbers of repeat DNA sequences considered vital for adaptation and chronic colonisation of their hosts (Rocha *et al.*, 2005).

Although mycoplasmal phages are known to exist, transduction has not been described. Several mycoplasmas have been shown to acquire the conjugative transposon *Tn916* by mating with an enterococcal donor, but conjugal transfer of any genetic element including *Tn916* from a mycoplasmal donor has not been described. Circumstantial evidence suggests that horizontal gene exchange has occurred between species of *Mycoplasma* with the *IS1221* insertion sequence having been found in *M. hyorhinis*, *M. hyopneumoniae* and *M. flocculare* (Teachman *et al.*, 2002).

The possibility of exchange of DNA between mycoplasmas and other pathogenic infectious agents within the urogenital tract, such as *Neisseria gonorrhoeae* may contribute to gene transfer, which may promote antibiotic resistance in such pathogens.



## 1.7 SCOPE AND OBJECTIVES

The molecular basis of *Mycoplasma* and *Ureaplasma* pathogenicity remains largely elusive. Due to conflicting reports from around the world concerning the pathogenic nature of mycoplasmas and ureaplasmas, it is important to clarify the situation as it pertains to South Africa where there are high incidences of sexually transmitted diseases and HIV. Resistance to quinolones, tetracyclines and macrolides have been reported in mycoplasmas and ureaplasmas, however, ongoing surveillance of antibiotic resistance development and potential consequences is lacking in South Africa.

### Objectives:

1. To validate both conventional culture and molecular methods used for the detection of *Mycoplasma* spp. and *Ureaplasma* spp. from clinical specimens.
2. To investigate the prevalence of genital mycoplasmas, ureaplasmas and *Chlamydia* on women attending their first prenatal visit, in conjunction with preterm labour or HIV status.
3. To determine the prevalence of community-acquired atypical pneumonias in adults (*M. pneumoniae* and *P. jiroveci*) and neonates (mycoplasmas, ureaplasmas and *C. trachomatis*).
4. To determine antibiotic susceptibility profiles of clinical isolates of *U. parvum* and *U. urealyticum*, and detect target gene mutations/acquisitions responsible for resistance development.
5. To investigate, as tetracycline resistance genes (*tetM*) are transposon located, inter-generic resistance gene transfer potential to *N. gonorrhoeae*.

## CHAPTER TWO

### MATERIALS AND METHODS

#### 2.1 GENITAL SETTING: PATIENTS AND ENDOCERVICAL SPECIMENS

Consenting pregnant women participated in these studies for which ethics approval was obtained from the Committee for Human Research, Stellenbosch University, [Reference numbers: 2001/C067 (2003 study) and N04/02/038 (2005 study)].

Extended mycoplasma and ureaplasma prevalence studies (2003 and 2005); 2003: Specimens were collected from the first 199 women attending the Bishop Lavis Midwife Obstetric Unit in 2003 (Odendaal *et al.*, 2006). Preterm delivery was defined for women delivering before 37 weeks gestation. The HIV status of the women was not obtained.

2005: For the *Mycoplasma* spp., *Ureaplasma* spp., *Chlamydia trachomatis* and HIV, co-infection/co-colonisation study; 219 women attending the Obstetrics Unit, Tygerberg Hospital were screened in 2005. The HIV Abbott Determine® HIV-1/2 rapid test was used and positive tests at the Obstetrics Unit were confirmed by ELISA performed at the Virology Laboratory at the Faculty of Health Sciences. The HIV results were recorded anonymously to laboratory reference numbers.

For the ureaplasma antibiotic resistance study, 191 women attending the Obstetrics Unit, Tygerberg Hospital were screened in 2006.

All specimens were taken by nurses, independently to those for diagnostic investigations. Specimens were endocervical swabs (Dacron) that were transferred onto Microbank beads (Davies Diagnostics) by swabs being expressed into broth in Microbank tubes and stored at -80°C within 30 min of specimen collection.

## **2.2 RESPIRATORY SETTING: PATIENTS AND SPECIMENS**

Approval to conduct the study was obtained from the Human Ethics Committee, Nelson Mandela Metropolitan University (Reference number: H06SB005).

### **2.2.1 Adult pneumonia**

Consenting adult patients (102) with pneumonia, or symptoms of pneumonia, admitted to the general ward of Dora Nginza (101 specimens collected) and Livingstone Hospitals (one specimen collected) during the period 2006 – 2008, participated in the study. X-Rays, HIV status, sputum and blood samples for culture requested by doctors, were forwarded to the National Health Laboratory Services (NHLS), Port Elizabeth. National recommendations for diagnosis of *M. tuberculosis* were conducted: two sputa samples were sent for Ziehl-Neelsen-microscopy, if one was positive for acid fast bacilli, then X-rays were performed; if the two samples were negative, then a third sputum sample was taken. HIV status was determined employing ELISA tests performed at NHLS, Port Elizabeth. HIV status and routine microbiological data recorded in patients' files was extracted by Dr GS Ocana. Sputum samples independently collected for study purposes were immediately stored at -80°C. Adult samples were collected whenever doctors suspected that adult patients might have pneumocystis pneumonia.

### **2.2.2 Neonatal pneumonia**

Following parent consent, neonates (age < 1 month) with respiratory problems admitted to Dora Nginza Hospital Neonatal Intensive Care Unit (NICU), were included in the study, duration 2007 to 2008. Sixty-nine endotracheal aspirate specimens were collected by the neonatal ICU nurse and immediately stored at -80°C. Neonate samples were collected whenever paediatricians at the NICU suspected that neonates had atypical pneumonia.

## 2.3 CULTURE: CONVENTIONAL MEDIA

American Type Culture Collection (ATCC) strains that were purchased and used for media quality control were: *M. hominis* (ATCC 14027), *U. urealyticum* (ATCC 27618), *U. parvum* (ATCC 27815) and *M. penetrans* (ATCC 55252) and *M. pneumoniae* (ATCC 29343).

Efforts were made to isolate and culture *Mycoplasma* sp. and *Ureaplasma* sp. from both endocervical swabs and sputa. Approximately 100 µl of endocervical swab specimen (and two microbank beads) were inoculated into media as specified by the ATCC for each of the micro-organisms as described below. Broths/cultures were incubated at 37°C, ATCC controls for 3 days and clinical samples to 10 days under aerobic conditions. A8 plates both commercially available (bioMerieux) and made onsite at NMMU according to the method described by Duffy and Waites, (2008), (IOM Workshop, China) were also used for the culture of *M. hominis*, *U. parvum* and *U. urealyticum* with incubation at 37°C in a candle extinction jar for 7 - 10 days. The media used for each species is described in more detail below.

### 2.3.1 *M. hominis*

#243 ATCC broth medium: Basal medium [17.5 g heart infusion broth, 700 ml distilled water] was autoclaved followed by aseptic addition of filter sterilised solutions [200 ml horse serum (heat inactivated), 100 ml yeast extract solution and 1000 U/ml benzyl penicillin].

### 2.3.2 *U. urealyticum*

#1331 ATCC broth medium: Basal medium [14.7 g PPLO broth, 700 ml distilled water] pH 6 adjustment with HCl was autoclaved followed by aseptic addition of filter sterilised solutions [200 ml horse serum, 100 ml yeast extract solution, 0.1 g L-cysteine HCL, 0.5 g urea, 5 ml Isovitalex enrichment, 10 mg phenol red and 1000 U/ml benzyl penicillin].

### 2.3.3 *U. parvum*

#2616 ATCC broth medium: Basal medium [3.5 g PPLO broth, 10 g pancreatic digest of casein (tryptone), 5 g pancreatic digest of gelatine (peptone), 500 ml distilled water] was autoclaved followed by aseptic addition of filter sterilised solutions [50 ml 10X CMRL-1066 medium, 35 ml yeast extract solution, 20 ml yeastolate [10% (w/v)], 170 ml foetal bovine serum, 20 ml of 0.1% phenol red solution, 1 g urea, 205 ml distilled water].

### 2.3.4 *M. penetrans*

#988 ATCC (*Spiroplasma*) broth medium: Basal medium [5.5 g PPLO broth, 5 g tryptone, 2.5 g glucose, 307.5 ml distilled water] was autoclaved followed by aseptic addition of filter sterilised solutions [25 ml 10X CMRL-1066 medium, 17.5 ml yeast extract solution, 50 ml 2% (w/v) yeastolate, 85 ml foetal bovine serum, 10 ml 0.1% (w/v) phenol red solution and 1000 U/ml benzyl penicillin].

### 2.3.5 *M. pneumoniae*

#988 ATCC (*Spiroplasma*) broth medium (Section 2.3.4).

Culture: Approximately 100 µl of the sputa specimens were inoculated into #988 ATCC (*Spiroplasma*) broth. Upon a colour change from red to yellow, sub-cultures were made directly onto #988 ATCC *Spiroplasma* agar (medium composition as broth with the addition of 10 g/l of agar) (Matsuoka *et al.*, 2004) and Modified New York City agar plates (whole blood component replaced with horse serum) manufactured by NHLS Port Elizabeth (Granato *et al.*, 1983). Inoculated broth tubes were incubated at 37°C for 10 – 30 days under aerobic conditions, while agar plates were incubated at 37°C in a candle extinction jar for 7 - 30 days.

## 2.4 CULTURE: COMMERCIAL KITS

For detection of *M. hominis* and *Ureaplasma sp.*, the Mycoview (Ivagen), Mycoplasma IST2 (bioMerieux) and Mycoplasma Duo kits (Bio-Rad) were used and for *M. pneumoniae* the Pneumofast kit (International Microbio, Signes, France, Progen-SA).

### 2.4.1 MycoView kit (Ivagen)

The MycoView test kit was used for the identification and differential titration of *U. urealyticum* and *M. hominis* and antibiotic susceptibility profiles to nine antimicrobial agents. The principle of the test is based on specific metabolic properties and natural resistance: *U. urealyticum* (hydrolysis of urea and resistance to lincomycin); *M. hominis* (hydrolysis of arginine and resistance to erythromycin). Growth was indicated by a change in colour of pH indicator from yellow-orange to red or pink. The MycoView kit comprises the following: transport broth (2 ml), culture medium (freeze dried medium), MycoView strip (2 strips of 12 test-wells, sealed in an aluminium pouch, with desiccant), incubation rack for 4 strips, with a lid and paraffin. The format of the MycoView strip is shown in Fig. 2.1.

Approximately 100 µl of endocervical swab specimen with two microbank beads were inoculated into a vial of transport broth. A vial of culture medium was regenerated with one inoculated transport broth. The regenerated medium which contained the inoculum was gently mixed 4 to 5 times with a pipette before inoculating the MycoView strip. Twelve wells of the strip were inoculated with 100 µl of culture followed by one drop of paraffin to each well. The wells were sealed with film, placed in the rack with the lid closed and incubated at 37°C for 48-72 h. All wells were expected to be limpid as a turbid appearance was indicative of bacterial or yeast contamination. Urogenital mycoplasmal/ureaplasma growth was confirmed by a change in colour of the medium from yellow to red/pink.

Well n°	Abb.	Description of the test
1	C	Growth control
2	U.u	Identification of U.u species from a $\geq 10^4$ CCU/ml specimen
3	M.h	Identification of M.h species from a $\geq 10^4$ CCU/ml specimen
4	L	Resistance test to Lincomycin (8 µg/ml); it is also a detection test of U.u at low level ( $10^3$ CCU/ml)
5	E	Resistance test to Erythromycin (4 µg/ml); it is also a detection test of M.h, even at low level ( $10^3$ CCU/ml)
6	ROX	Resistance test to Roxithromycin (4 µg/ml)
7	AZM	Resistance test to Azithromycin (4 µg/ml)
8	JM	Resistance test to Josamycin (4 µg/ml)
9	MNO	Resistance test to Minocycline (8 µg/ml)
10	DO	Resistance test to Doxycycline (8 µg/ml)
11	OFX	Resistance test to Ofloxacin (4 µg/ml)
12	NOR	Resistance test to Norfloxacin (2 µg/ml)

**Figure 2.1:** MycoView strip format (Ivagen kit manual).

### 2.4.2 Mycoplasma IST2 (bioMerieux)

The Mycoplasma IST2 kit combines a selective culture broth with a strip containing 22 tests. A positive test is indicated by a change of broth colour from yellow to red as a result of the phenol red indicator in the broth (urea for *U. urealyticum* and arginine for *M. hominis*). The combination of three antibiotics and one antifungal agent provides selectivity, ensuring that any contaminating flora present in the specimen does not affect the test. The kit comprises the following: reagent R1 (3 ml broth); reagent R2 (1 ml lyophilised urea-arginine broth), mineral oil and strips that contained 22 tests. Strips are divided into 3 sections: identification (cupules no. 1-3); indicative enumeration (cupules no. 4 and 5); susceptibility tests (cupules no. 6-22) (Fig. 2.2). Approximately 100 µl of endocervical swab specimen (and two microbank beads) were inoculated into R1 solution which was combined with a vial of R2 and vortexed until the lyophilised pellet was completely dissolved. Of the combined R1/ R2 solution 55 µl was dispensed into each of the 22 test cupules on the strip followed by addition of 2 drops of mineral oil to each cupule and lid placed over the strip. Strips were incubated at 37°C for 24-48 h.

Cupules	Tests	Substrates
No. 1	0 (Control)	Phenol red (0.05 g/l)
No. 2	Uu	Phenol red (0.05 g/l) Lincomycin
No. 3	Mh	Erythromycin

 (A)
  

Cupules	Tests	Substrates
No. 4	Uu ≥ 10 <sup>4</sup>	Phenol red (0.05 g/l) Lincomycin Inhibition agent
No. 5	Mh ≥ 10 <sup>4</sup>	Erythromycin Inhibition agent

 (B)
  

Cupules	Antibiotics and Abbreviations	Concentrations mg/l	
No. 6 & 7	Doxycycline	DOT	4 8
No. 8 & 9	Josamycin	JOS	2 8
No. 10 & 11	Ofloxacin	OFL	1 4
No. 12 & 13	Erythromycin	ERY	1 4
No. 14 & 15	Tetracycline	TET	4 8
No. 16 & 17	Ciprofloxacin	CIP	1 2
No. 18 & 19	Azithromycin	AZI	0.12 4
No. 20 & 21	Clarithromycin	CLA	1 4
No. 22	Pristinamycin	PRI	2

 (C)

**Figure 2.2:** Composition of MycolIST2 strips. (A) Identification (cupules no. 1-3). (B) Indicative enumeration (cupules no. 4 and 5). (C) Susceptibility tests (cupules no. 6-22)

### 2.4.3 Mycoplasma Duo kits (Bio-Rad)

The Mycoplasma Duo kit was used for the identification of *U. urealyticum* and *M. hominis* based on hydrolysis of urea and arginine respectively where a change in colour from yellow to red was a positive test. The Kit comprises twenty microplates: Each microplate has 6 microwells which contains dehydrated substrates for identification, *Mycoplasma* growth factors, with agents to inhibit the growth of concomitant polymorphic flora (Fig. 2.3); 2 ml vials of suspension medium, 15 ml diluent, plastic micropipettes, adhesive sheets. Approximately 100  $\mu$ l of endocervical swab specimen and two microbank beads were inoculated into suspension medium. Diluent (200  $\mu$ l) was transferred to each of the three wells of the lower row of the microplate: U $\geq$ 10<sup>4</sup>, D, and H $\geq$ 10<sup>4</sup>. A micropipette was then used to transfer 200  $\mu$ l of specimen containing suspension medium into each of the 3 wells of the upper row of the microplate: U, X, H and 25  $\mu$ l was added to well D. Using a different pipette contents of well D were mixed and 25  $\mu$ l transferred to well U $\geq$ 10<sup>4</sup> and 25  $\mu$ l transferred H $\geq$ 10<sup>4</sup>. The microplate was covered with an adhesive sheet and incubated at 37°C for 24 - 48 h.



**Figure 2.3:** Presentation of Mycoplasma Duo microplate. U and U $\geq$ 10<sup>4</sup>: identification and titration of UU (containing urea). H and H $\geq$ 10<sup>4</sup>: identification and titration of MH (containing arginine). D: dilution well. X: selective mycoplasma enrichment: preparation of standardised inoculum for antibiotic susceptibility testing.



#### **2.4.4 Pneumofast Kit (International Microbio, Signes, France, Progen-SA)**

The Pneumofast kit was used for isolation, identification, enumeration and antibiotic resistance testing of *M. pneumoniae* from clinical specimens. The kit comprises: *M. pneumoniae* base (MP base), *M. pneumoniae* supplement (MP supplement), *M. pneumoniae* agar (MP agar) and Pneumofast trays. MP supplement was regenerated by the addition of 1.5 ml of MP base to the MP supplement vial. MP agar vials were liquefied in a boiling water bath and allowed to cool to 55°C, and 1 ml of the regenerated MP supplement was introduced into the agar base and poured into a 55 mm petri dish and allowed to solidify. The remaining MP base was then added to the remaining MP supplement forming the Pneumofast broth. Two hundred microlitres of each sputa sample and the different dilutions of the ATCC culture, ATCC 29343 (7, 11 and 12.5 times dilutions) were added to Pneumofast broth, and 100 µl of the mixture was added to each well of the Pneumofast tray, which was then covered with two drops of paraffin oil. Each tray was sealed within a sterile petri dish and incubated anaerobically at 37°C. Three drops of each of the biological samples were placed randomly onto an agar plate. The agar plates were incubated at 37°C within a candle extinction jar. The Pneumofast trays and agar plates were monitored daily for approximately 12 to 30 days. Samples resistant to ampicillin (40 µg/ml), sulfa-trimethoprim (4 µg/ml), and lincomycin (1 µg/ml) but were inhibited by erythromycin (8 µg/ml) were positively identified as *M. pneumoniae*.

#### **2.5 CULTURE: ARGININE AND U9 BROTHS (BIO-RAD)**

Commercially made media; U9 and arginine broths were also used to culture *M. hominis* and *Ureaplasma* spp. Sterile distilled water (2 ml) was added to each ampoule to reconstitute the lyophilised broths (U9 or arginine). The reconstituted broths were transferred to sterile eppendorfs and used immediately. To six wells of a microtitre plate row, 180 µl of U9 broth or arginine broth was transferred. Endocervical specimens (20 µl) were then

transferred to the first well of each broth row (U9 and arginine). The contents of the first well were mixed with a pipette and 20 µl from the first well was transferred into the second well and serial 10<sup>-1</sup> dilutions conducted. The microtiter plates were then sealed with aluminium sealing tape and incubated at 37°C for 24 to 96 h. A colour change in the wells from yellow to red-pink indicated the presence of *M. hominis* (arginine broth) and *U. urealyticum* (U9 broth).

## **2.6 DNA EXTRACTION: PCR DETECTION OF *MYCOPLASMA* SPP. AND *UREAPLASMA* SPP.**

### **2.6.1 Endocervical specimens**

DNA was extracted from 200 µl of endocervical swab specimens (microbank bead broth) employing the High Pure PCR Template Preparation Kit (Roche) as for the isolation of nucleic acids from whole blood, buffy coat, or cultured cells as described by the manufacturer. For ATCC control cultures and cultures used in Chapter six (detection of antibiotic resistance genes), 200 µl of broth culture was centrifuged at 3000 *xg* for 5 min, and the resulting pellet re-suspended in 200 µl PBS with DNA extracted using the Roche High Pure PCR Template as for the isolation of nucleic acids from bacteria.

### **2.6.2 Sputa specimens**

#### **2.6.2.1 *P. jiroveci***

For *P. jiroveci* approximately 200 µl of sputa was treated with an equal volume of 0.1 M 1,4-dithiothreitol, (Roche), vortexed and incubated at 37°C for 30 min, until liquefied. The suspension was centrifuged at 3000 *xg* for 15 min and the pellet re-suspended in 500 µl sterile deionised water. *P. jiroveci* control DNA that had been extracted from paraffin embedded lung histological sections confirmed as PcP positive by methanamine silver staining and PCR mitochondrial large subunit ribosomal RNA (mtLSUrRNA), dihydropteroate synthase (DHPS), dihydrofolate reductase (DHFR) genes, were kindly supplied by FJL Robberts *et al.* (2007).

### **2.6.2.2 *M. pneumoniae***

Approximately 200 µl, of the sputa specimens, were liquefied with an equal volume of 3% N-acetyl cysteine, (NALC, Merck) and incubated at 37°C for 15 to 30 min (Ursi *et al.*, 2003; Qasem *et al.*, 2002) followed by an additional 10 min at 65°C, then 15 min incubation at 100°C to ensure that any tubercle bacilli or other respiratory pathogens present had been inactivated prior to DNA extraction (Pitcher *et al.*, 2006). DNA was extracted from 200 µl of each pre-treated respiratory specimen using the Roche High Pure PCR Template Preparation Kit as for the isolation of nucleic acids from whole blood, buffy coat, or cultured cells as described by the manufacturer. For ATCC control cultures, 200 µl of broth culture sample was centrifuged at 3000 *xg* for 5 min, and the resulting pellet re-suspended in 200 µl PBS with DNA extracted using the Roche High Pure PCR Template as for the isolation of nucleic acids from bacteria.

### **2.6.3 Endotracheal aspirates (neonates)**

The endotracheal aspirate tube was flushed with sterile PBS and 200 µl of the endotracheal aspirate suspension liquefied with an equal amount of 3% (w/v) N-acetyl Cysteine (NALC, Merck) and treated as described above for *M. pneumoniae* in section 2.7.2.2.

## **2.7 PCR DETECTION**

### **2.7.1 Mycoplasma detection kit (Takara)**

PCR detection of *M. hominis*, *M. fermentans* and *Ureaplasma* spp. was performed using a *Mycoplasma* PCR detection kit (Takara Bio Inc., Japan). Primers (F1 and R1): amplification of 16S-23S rRNA spacer region, products; 370 bp fragment *M. hominis*, 482 bp fragment *Ureaplasma* spp. and 491 bp fragment *M. fermentans*. Designed nested primers F2 and R2: target highly conserved spacer region and 23S gene, products; 148 bp fragment *M. hominis*, 154 bp fragment *Ureaplasma* spp. and 195 bp fragment *M. fermentans*. Amplification was performed in a total volume of 50 µl. The reaction mixture for the first PCR comprised: 5 µl 10x PCR buffer, 4 µl

deoxynucleotide triphosphate mixture [2.5 mM of each dNTP (Takara)], 0.5 µl of each primer (20 pmol/µl), 1.25 units of *Taq* DNA polymerase (Takara Taq™) and 3 µl of template DNA. The reaction mixture for the second PCR reaction comprised: 5 µl 10x PCR buffer, 4 µl deoxynucleotide triphosphate mixture [2.5 mM of each dNTP (Takara)], 0.5 µl of each primer (20 pmol/µl), 1.25 units of *Taq* DNA polymerase (Takara Taq™) and 1 µl of the first PCR reaction product. Amplification was performed in an iCycler PCR machine (Bio-Rad). Amplification: First reaction, 1 cycle at 94°C for 30 sec; 35 cycles denaturation 94°C for 30 sec, annealing 55°C for 2 min and elongation 72°C for 1 min, followed by a final elongation step at 72°C for 5 min. Second reaction, 30 cycles: denaturation 94°C for 30 sec, annealing 55°C for 2 min and elongation 72°C for 1 min, followed by a final elongation step at 72°C for 5 min. The amplification products of the first PCR reaction were separated on 3% (w/v) agarose gels for 1 h at 100 V in Tris-acetate EDTA buffer (40 mM Tris base, 5 mM sodium acetate, 1 mM EDTA, pH 8) and amplified products of the second PCR reaction were separated on 4% (w/v) agarose gels for 75 min at 100 V. Ethidium bromide stained DNA products were visualised by UV transillumination and images captured using an Alpha Imager™3400 gel system (Alpha Innotech). A 100 bp DNA ladder (Promega) was included in each gel run.

### **2.7.2 Specific primers and conditions**

All PCR reactions included positive and negative controls. Control DNA was extracted from: *M. hominis* (ATCC 14027), *M. penetrans* (ATCC 55252), *M. genitalium* (ATCC 33530), *U. parvum* (ATCC 27815) (for prevalence and antibiotic resistance), *U. urealyticum* (ATCC 27618) (for prevalence and antibiotic resistance), *C. trachomatis* (ATCC VR-887), *M. pneumoniae* (ATCC 29343), and for *P. jiroveci* DNA was extracted from confirmed histological specimens (Section 2.7.2.1). All PCRs from which fragments of expected size were produced, were repeated for confirmation.

**2.7.2.1 *M. hominis*, *M. penetrans* *M. genitalium*, *U. parvum*,  
*U. urealyticum*, *C. trachomatis*, *P. jiroveci***

Specific PCR primers and conditions employed for the detection of *M. hominis*, *M. penetrans*, *M. genitalium*, *U. parvum*, *U. urealyticum*, *C. trachomatis*, *P. jiroveci* are given in Table 2.1.

Table 2.1: PCR primers employed in the detection of *Mycoplasma* spp., *Ureaplasma* spp., *C. trachomatis* and *P. jiroveci*.

Organism	Primer (F) (R)	Sequence (5' - 3')	Size of amplified product (bp)	Target gene	Reference
<i>M. hominis</i>	HOM+ UNI-	TGAAAGGCGCTGTAAGGCGC TAATCCTGTTTGCTCCCCAC	589	16S rRNA	Dussurget <i>et al.</i> (1994) Cordova <i>et al.</i> (2000)
<i>M. penetrans</i>	MYCPENETP MYCPENETN	CATGCAAGTCGGACGAAGCA AGCATTTCCTCTTCTTACAA	410	16s rRNA	Grau <i>et al.</i> (1994) Cordova <i>et al.</i> (2000)
<i>M. genitalium</i>	MgPa-1 MgPa-3	AGTTGATGAAACCTTAACCCCTTGG CCGTTGAGGGGTTTTCCATTTTTGC	281	Adhesin gene	Jensen <i>et al.</i> (1991) Cordova <i>et al.</i> (2000)
<i>U. parvum</i>	UPS2c UPA2c	CAGGATCATCAAGTCAATTTAG AACATAATGTTCCCCTTTTTATC	420	UreA - UreB gene spacer region	Biernat-Sudolska <i>et al.</i> (2006) Kong <i>et al.</i> (2000)
<i>U. urealyticum</i>	UUS2c UUA2c	CAGGATCATCAAATCAATTCAC CATAATGTTCCCCTTCGTCTA	420	UreB - UreC gene spacer region	Biernat-Sudolska <i>et al.</i> (2006) Kong <i>et al.</i> (2000)
<i>C. trachomatis</i>	T1 T2	GGACAAATCGTATCTCGG GAAACCAACTCTACGCTG	517	cryptic plasmid	Class <i>et al.</i> (1991)
<i>M. pneumoniae</i>	F1 R1	CCGCGAAGAGCAATGAAAACTCC TCGAGGCGGATCATTGGGGAGGT	375	P1 adhesin	Ramirez <i>et al.</i> (1996)
	16SrRNA F 16SrRNA R	AAGGACCTGCAAGGGTTCGT CTCTAGCCATTACCTGCTAA	277	16SrRNA	Nadal <i>et al.</i> (2001)
<i>P. jiroveci</i> (Nested PCR)	pAZ102-E pAZ102-H	GATGGCTGTTTCCAAGCCCA GTGTACGTTGCAAAGTACTC	346	mtLSUrRNA	Wakefield <i>et al.</i> (1996)
	pAZ102-X pAZ102-Y	GTGAAATACAAATCGGACTAGG TCACTTAATATTAATTGGGGAGC	260		
	F1 B45	CCTGGTATTAACCAGTTTTGCC CAATTTAATAAATTTCTTTCCAAATAGCATC	300	DHPS	Lane <i>et al.</i> (1997)
	AHUM BN	GCGCCTACACATATTATGGCCATTTTAAATC GGAACCTTCAACTTGGCAACCAC			
	FR280 FR1038	GCAGAAAGTAGGTACATTATTACGAGA AACCAGTTACCTAATCAAATATATTGC	798	DHFR	Ma <i>et al.</i> (1999)
	FR242 FR1018	GTTTGAATAGATTATGTTTCATGGTGTACG GCTTCAAACCTTGTGTAACGCG			

For *U. parvum* and *U. urealyticum* reaction mix (25 µl) comprised: 1X GoTaq Green master mix (Promega), 0.5 µM of each primer and 2 µl of template DNA. Amplification comprised: an initial step of 95°C for 5 min, followed by 40 cycles: 92°C for 1.5 min, 55°C for 2 min, 72°C for 1.5 min and final extension of 72°C for 8 min (Biernat-Sudolska *et al.*, 2006; Kong *et al.*, 2000). For *M. hominis*, *M. genitalium* and *M. penetrans*: reaction mix (25 µl) comprised 1X GoTaq Green master mix (Promega), 0.5 µM of each primer specific to the relevant microorganism and 2 µl of template DNA. Amplification comprised: an initial step of 95°C for 5 min, followed by 30 cycles: 95°C for 30 sec, 58°C for 1.5 min, 72°C for 1.5 min and final extension of 72°C for 8 min (Cordova *et al.*, 2000). For *C. trachomatis*: reaction mix (25 µl) comprised: 1X GoTaq Green master mix (Promega), 0.5 µM of each primer and 2 µl of template DNA. Amplification consisted of an initial step of 94°C for 2 min, followed by 40 cycles: 94°C for 1 min, 42°C for 2 min, 72°C for 3 min and final extension of 72°C for 7 min (Class *et al.*, 1991).

### **2.7.2.2 *M. pneumoniae* and *P. jiroveci***

Detection of *M. pneumoniae* employed primers F1 and R1, which are specific for the P1 adhesin gene (Table 2.1) (Ramirez *et al.*, 1996 and Buck *et al.*, 1992). The PCR reaction mixture comprised: 3 µl DNA template, 1.5 mM MgCl<sub>2</sub>, 0.5 µM of each primer, 0.2 mM of each dNTP, 0.625 units of the enzyme GoTaq® Flexi DNA polymerase and 5X Green GoTaq® Flexi buffer (Promega). Amplification of the P1 adhesin gene again included a three-step initial phase, comprising 94°C for 5 min, followed by 50°C for 5 min (addition of Taq polymerase) and 72°C for 5 min. Amplification was conducted for 35 cycles: 94°C for 30 sec, 65°C for 30 sec and 72°C for 30 sec followed by a final extension phase of 10 min at 72°C. Primers 16S rRNA F and 16S rRNA R were used in the single 16S rRNA PCR reaction. The PCR reaction mixture comprised: 3 µl DNA template, 2 mM MgCl<sub>2</sub>, 1.25 µM of each primer, 0.2 mM of each dNTP, 0.625 units of GoTaq® Flexi DNA polymerase and 5X Green GoTaq® Flexi buffer (Promega). A three-step initial phase of: 94°C for 5 min, followed by 50°C for 5 min (addition of Taq polymerase) and 72°C for 5 min was used. Amplification was conducted for 40 cycles: 94°C for 1 min, 60°C for 1 min and 72°C for 1 min followed by a final extension phase of 5 min at 72°C.

The PCR reaction mixture for the mtLSUrRNA method for detection of *P. jiroveci* comprised: 3  $\mu$ l DNA template, 3 mM MgCl<sub>2</sub>, 1  $\mu$ M of each primer, 0.4 mM of each dNTP and 0.625 units of the enzyme GoTaq® Flexi DNA polymerase and 5X Green GoTaq® Flexi buffer (Promega). PCR conditions used included a three-step initial phase, of 94°C for 2 min, 50°C for 5 min, followed by the addition of Taq polymerase and 72°C for 5 min. Amplification primers pAZ102-E and pAZ102-H comprised: 40 cycles at 94°C for 1.5 min, 55°C for 1.5 min and 72°C for 2 min followed by a final extension phase of 10 min at 72°C. From the first PCR reaction, 1  $\mu$ l was used for the second PCR reaction. Primers pAZ102-X and pAZ102-Y were used in the second PCR reaction: initial step, of 94°C for 5 min, followed by 10 cycles at 94°C for 1.5 min, 55°C for 1.5 min and 72°C for 2 min, and 30 cycles at 94°C for 1.5 min, 63°C for 1.5 min and 72°C for 2 min with a final extension phase of 10 min at 72°C.

The F1 and B45 primers were used in the first reaction of the DHPS nested PCR (Table 2.1). The PCR reaction mixture comprised: 3  $\mu$ l DNA template, 2mM MgCl<sub>2</sub>, 0.4  $\mu$ M of each primer, 0.2 mM of each dNTP, 0.625 units of GoTaq® Flexi DNA polymerase and 5X Green GoTaq® Flexi buffer (Promega). DHPS amplification included a three-step initial phase: 94°C for 5 min, followed by 52°C for 5 min (addition of Taq polymerase) and 72°C for 5 min. Amplification included 35 cycles of: 92°C for 30 sec, 52°C for 30 sec and 72°C for 1 min followed by a final extension phase of 5 min at 72°C. For the second round of amplification primers AHUM and BN and 1 $\mu$ l of the first round product were used. PCR conditions were the same as the first PCR, except that an initial step of 94°C for 5 min and annealing at 55°C were included.

The DHFR PCR mixture contained 2  $\mu$ l DNA template, 2mM MgCl<sub>2</sub>, 0.25  $\mu$ M of each primer, 0.2 mM of each dNTP, 0.625 units of GoTaq® Flexi DNA polymerase and 5X Green GoTaq® Flexi buffer (Promega). Amplification included a three-step initial phase of 94°C for 5 min, followed by 52°C for 5 min (addition of Taq polymerase) and 72°C for 5 min. Cycling parameters comprised 35 cycles of: 94°C for 1 min, 60°C for 2 min and 72°C for 3 min, with a final extension phase of 10 min at 72°C. For the second round of amplification 1 $\mu$ l of the first round product was used, and the PCR conditions remained the same except for an initial step of 94°C for 5 min.



## 2.8 AGAROSE GEL ELECTROPHORESIS

PCR products were separated in 2% (w/v) agarose gels for 45 min at 100 V using Tris-acetate EDTA buffer (40 mM Tris base, 5 mM sodium acetate, 1 mM EDTA, pH 8). Ethidium bromide (Promega) stained DNA products were visualised by UV transillumination and images captured using an Alpha Imager™3400 gel system (Alpha Innotech). Positive and negative controls and a 100 bp DNA ladder (Promega) were included in each gel run to determine approximate sizes of the PCR products.

## 2.9 SEQUENCING PROCEDURES AND COMPARISONS

PCR products (for prevalence and antibiotic resistance chapters) were prepared for sequencing using Wizard SV gel and PCR clean-up system (Promega) according to the manufacturer's instructions. For clean-up of a PCR product an equal volume of membrane binding solution (4.5 M guanidine isothiocyanate and 0.5 M potassium acetate, pH 5) was added to the PCR reaction mixture. The binding of the DNA, washing and elution steps were followed according to the suppliers instructions. Purified DNA samples were sequenced at the Central Analytical Facility, University of Stellenbosch. Sequence analyses were performed using Chromas 1.45, Bioedit 7.0.5 and Geneious 3.8.5, and sequences compared to regions of relevant target genes from micro-organisms deposited in GenBank.

### 2.9.1 Prevalence studies

For identification and detection of *Mycoplasma* spp. and *Ureaplasma* spp. PCR products were compared to known sequences on GenBank. *M. hominis*: GenBank accession number M96660 (Blanchard *et al.*, 1993). *U. parvum*: GenBank accession number AF085729 (Biernat-Sudolska *et al.*, 2006; Kong *et al.*, 2000; Kong *et al.*, 1999). *U. urealyticum*: (GenBank accession number AF085729). *P. jiroveci*: GenBank accession number: mtLSUrRNA M58605 (Sinclair *et al.*, 1991). *M. pneumoniae*: GenBank accession number: M18639 (Su *et al.*, 1987).

### **2.9.2 Antibiotic resistance genes**

Predicted PCR products from ATCC control cultures and sequences obtained from clinical strains were compared to corresponding regions obtained through BLAST search for QRDR regions of *U. parvum* (GenBank accession number: CP000942.1), *U. urealyticum* (GenBank accession number: AAYN02000002.1), *Streptococcus pneumoniae tetM* (GenBank accession number: X90939.1) and *N. gonorrhoea tetM* (GenBank accession number: L12241).

## **2.10 STATISTICAL ANALYSIS**

Statistical analysis was performed employing the program Statistica (version 8.0) and Pearson chi square and p- values determined. Sensitivity and specificity calculations: Sensitivity = [(no. True positives) / (no. true positives + no. false negatives)] x 100 = %; Specificity = [(no. True negatives) / (no. true negatives + no. false positives)] x 100 = %.

## **2.11 ANTIBIOTIC SUSCEPTIBILITY TESTS**

### **2.11.1 SIR Mycoplasma kit: antibiogram for urogenital Mycoplasma (Bio-Rad)**

Antimicrobial screening was performed according to instructions supplied in the product insert. A 24 h subculture from U9 broth stock (previously grown as described in section 2.5) was used. The expected titre obtained was  $10^6 - 10^7$  colour changing units/ml according to the published recommendations and the SIR kit instructions. Thus, 20  $\mu$ l of a 24 h culture in U9 broth medium was diluted with 1.8 ml of U9 broth (1/100 dilution). A standard inoculum of 100  $\mu$ l (1/100 dilution of the pre-culture, in U9 broth) was aliquoted into each well of the SIR microplate. The microplate was covered with sealing film and incubated at 37°C for 48 h.

### **2.11.2. Microbroth Dilution**

Minimum inhibitory concentrations (MIC) of antibiotics were determined by microbroth dilution tests as described by Bebear and Robertson (1996) and Waites *et al.* (2001). Antibiotic free broth was used for microbroth dilution tests for *U. urealyticum* ATCC #1331 broth (Section 2.3.2) and for *U. parvum* ATCC #2616 broth (Section 2.3.3). MICs to ofloxacin (Sigma), erythromycin (Sigma), azithromycin (Sigma) and tetracycline (Sigma) antibiotics were performed. All antibiotics were prepared at stock concentrations of 1 mg/ml in relevant diluents (either NaOH or ethanol) as specified by the supplier. For the MIC protocol, 100 µl of appropriate ATCC broth was added to wells 2 – 8. To wells 1 and 2, 100µl antibiotic at 64 µg/ml was added. Doubling dilutions were then performed across wells 2 to 8. The inoculum 100 µl per well was derived from combining 50 µl from the last positive growth well from resuscitation in the appropriate ATCC broth with 1 ml ATCC broth. Controls comprised: well 9: positive control (100 µl inoculum + 100 µl ATCC broth), well 10: antibiotic control (100 µl antibiotic containing media + 100 µl ATCC broth); well 11: negative control (200 µl ATCC broth).

## **2.12 DETECTION OF TARGET GENE MUTATIONS OR GENE ACQUISITIONS IN ANTIBIOTIC-RESISTANT UREAPLASMAS**

### **2.12.1. DNA Extraction and plasmid screening**

*U. parvum* and *U. urealyticum* DNA was extracted from 200 µl of U9 broth culture and *N. gonorrhoeae* DNA from 10 colonies grown on chocolate agar that were suspended in 200 µl of sterile water, using a High Pure PCR Template preparation kit (Roche) as for the isolation of nucleic acids from bacteria as described by the manufacturer. For *N. gonorrhoeae*, cells were additionally treated with 5 µl of 10 mg/ml lysozyme in 10 mM tris-HCl buffer at pH 8 for 15 min at 37°C prior to addition of binding buffer.

All clinical strains of *Ureaplasma* spp. which were resistant to tetracycline were screened for plasmids using the Pure Yield plasmid miniprep isolation kit (Promega). A tetracycline-resistant *N. gonorrhoeae* strain (A11) that carried a

*tetM* gene was used as a positive control and was kindly provided by Mari de Jongh, University of Limpopo.

### 2.12.2. PCR amplification of genes associated with antibiotic resistance

For the detection of resistant gene target alterations/acquisitions and transposon regions, PCR conditions and specific primers employed are given in Tables 2.2 – 2.6. In addition, tetracycline -susceptible and -intermediate resistant clinical stains of *U. parvum* or *U. urealyticum* were screened for *tetM* and *int*-Tn genes. PCR products were separated on agarose gels as described in section 2.8 and sequencing performed with comparisons as described in section 2.9.

**Table 2.2:** Primers and PCR conditions employed for the amplification of QRDR of gyrase and topoisomerase IV genes in *U. urealyticum* and *U. parvum* (Bebear *et al.*, 2000).

Gene	Primer	Sequence	Size (bp)
<i>gyrA</i>	gyrA-1	5'-TTGCTGCTTTTCGAAAACGG-3'	320
	gyrA-2	5'-CTGATGGTAAAACACTTGG-3'	
<i>gyrB</i>	gyrB-3	5'-CCTGGTAAATTAGCTGACTG-3'	300
	gyrB-4	5'-TTCGAATATGACTGCCATC-3'	
<i>parC</i>	parC-5	5'-ACGCAATGAGTGAATTAGG-3'	300
	parC-6	5'-CACTATCATCAAAGTTTGGAC-3'	
<i>parE</i>	parE-7	5'-ATGGGCGGAAAATTAACGC-3'	300
	parE-8	5'-CTTGGATGTGACTACCATCG-3'	
<b>PCR conditions</b> (Bebear <i>et al.</i> , 1997)		Amplification in final volume of 25 µl. Denaturation: 5-10 min @ 92°C 40 cycles:       1 min @ 92°C 1 min @ 57°C 2 min @ 72°C Final extension: 10 min @ 72°C	

**Table 2.3:** Primers and PCR conditions employed for the amplification of *int*-Tn gene for encoding the protein required for movement of Tn1545-like transposon of *Ureaplasma* spp. (de Barbeyrac *et al.*, 1996).

Gene	Primer	Sequence	Size (bp)
<i>int</i> -Tn	Int 1	5'-TGACTCTGCCAGCTTTAC-3'	579
	Int 2	5'-CCATAGGAACTTGACGTTGG-3'	
<b>PCR conditions</b> (de Barbeyrac <i>et al.</i> , 1996)		Amplification in final volume of 25 µl. Denaturation: 5 min @ 95°C 35 cycles: 1 min @ 95°C 1 min @ 60°C 1 min @ 72°C Final extension: 10 min @ 72°C	

**Table 2.4:** Primers and PCR conditions employed for the amplification of 397 bp regions of *tetM* genes (Blanchard *et al.*, 1992).

Gene	Primer	Sequence	Size (bp)
<i>tetM</i>	tetMF	5'-TTATCAACGGTTTATCAGG -3'	397
	tetMR	5'-CGTATATATGCAAGACG -3'	
<b>PCR conditions</b> (Blanchard <i>et al.</i> , 1992)		Amplification in final volume of 25 µl. Denaturation: 5 min @ 95°C 40 cycles: 25 sec @ 95°C 1 min @ 50°C 1 min @ 72°C Final extension: 10 min @ 72°C	

**Table 2.5:** Primers and PCR conditions employed for the amplification of 1.7 kb regions of *tetM* genes (Beeton *et al.*, 2009a).

Gene	Primer	Sequence	Size (kb)
<i>tetM</i>	tetMF	5'-TTATCAACGGTTTATCAGG-3'	1.7
	tetMR 2123	5'-GCATTTTCGGACAATAGAGGGG-3'	
<b>PCR conditions</b> (Beeton <i>et al.</i> , 2009a)		Amplification in final volume of 25 µl. Denaturation: 5 min @ 95°C 35 cycles: 1 min @ 95°C 1 min @ 54°C 1 min @ 72°C Final extension: 7 min @ 72°C	

**Table 2.6:** Primers and PCR conditions employed for the amplification of two operons (OP1 and OP2) of 23S rRNA, L4 and L22 proteins of *Ureaplasma* spp. (Beeton *et al.*, 2009a).

Gene	Primer	Sequence	Size (bp)
23S domain V	OP1 MH23S-11	5'-TAACTATAACGGTCCTAAGG -3'	1339
	UP23S-OP1	5'-ACCACCATTCAATGTTTGAC -3'	
23S domain V	OP2 MH23S-11	5'-TAACTATAACGGTCCTAAGG -3'	1424
	UP23S-OP2R2	5'-CGTATACTTTGCCATAGTGTTGCC -3'	
L4	UPL4-U	5'-TCTATTGATGGTAACTTCGC -3'	392
	UPL4-R	5'-GTTGAAGGTGTTTCTAAATCGC -3'	
L22	UPL22-U	5'-TTCGCACCGTAAAGCTTCTC -3'	458
	UPL22-R	5'-GTTCTGGATCAACGTTTTTCG -3'	
<b>PCR conditions</b> (Beeton <i>et al.</i> , 2009a)		Amplification in final volume of 25 µl. Denaturation: 5-10 min @ 92°C 40 cycles: 1 min @ 92°C 1 min @ 56°C (OP1 and OP2) and 60°C (L4 and L22) 2 min @ 72°C Final extension: 10 min @ 72°C	

**Table 2.7:** Primers designed and PCR conditions employed for the amplification of 1.3 kb regions of *tetM* genes (from base 2886-4222; Accession no.: X90939).

Gene	Primer	Sequence	Size (kb)
<i>tetM</i>	TMF1	5'-GCG TCT TGC ATA TAT ACG TCT TTA T-3'	1.3
	TMR1	5'-TCT GCA TTT CGG ACA ATA GAG GGG G-3'	
<b>PCR conditions</b>		Amplification in final volume of 25 µl. Denaturation: 5 min @ 95°C 35 cycles: 1 min @ 95°C 1 min @ 54°C 1 min @ 72°C Final extension: 7 min @ 72°C	

### **2.12.3 *tetM* sequence comparisons of *Ureaplasma* spp. strains from this study with *S. pneumoniae*, *N. gonorrhoeae* and *Ureaplasma* spp. GenBanked sequences**

*Ureaplasma* spp. *tetM* sequence comparisons were performed against the *tetM* sequence of *N. gonorrhoeae* strain 6418 (GenBank accession number: L12241) [USA, 1993, plasmid pOZ100] (Gascoyne-Binzi *et al.*, 1993). The genes aligned were: *S. pneumoniae*: X90939: [Italy, 1995, Transposon Tn5251 (Provvedi *et al.*, 1996); *U. urealyticum* SV9-Seattle: U08812 [1988, Transposon Tn916 doxycycline-susceptible (Sanchez-Pescador *et al.*, 1988).

## **2.13. ANTIBIOTIC RESISTANCE GENE TRANSFER**

*N. gonorrhoeae* and ureaplasma transformation experiments were performed employing media, conditions and recipient/donor concentrations of cells/DNA from which transformants have been obtained in other studies (Morse *et al.* 1996; Roberts and Kenny, 1987; Chalkley and Koornof, 1990; Potgieter and Chalkley, 1991; Voelker and Dybvig, 1996; Teachman *et al.*, 2002). Extended co-incubation strategies, with recipient growth from exponential to early stationary phase, were based on studies for *S. pneumoniae* (Chalkley and Koornhof, 1991). Clinical strains of *N. gonorrhoeae* were kindly provided by Mari de Jongh, University of Limpopo, Frans Radebe, NICD-NHLS, University of the Witwatersrand and Prof E Wasserman, NHLS, University of Stellenbosch.

### **2.13.1 Co-incubation of a *N. gonorrhoeae* recipient strain with tetracycline-resistant *Ureaplasma* donor DNA**

Antibiotic susceptible *N. gonorrhoeae* recipients were cultured on chocolate agar plates in a 5-10% CO<sub>2</sub> atmosphere at 37°C for 36 h. Twenty-colonies of *N. gonorrhoeae* were inoculated into 2.5 ml of a modified semi defined broth [3.5g Columbia broth base, (Laboratories CONDA); 0.5g Glucose, (Merck); 0.2493g Peptone special (vegetable), (Sigma); made up to 80 ml with sterile

distilled water and autoclaved. Sterile distilled water (14 ml), and the following filter-sterilized components were added aseptically: 4 ml Yeast extract, (Merck) (25% w/v); 2 ml bovine serum albumin (Roche) (0.5 g/l); (Takei *et al.*, 2005; Chalkley and Koornhof., 1991). The cell suspension was mixed and then divided into two 1.25 ml aliquots in microcentrifuge tubes. A third tube containing 1.25 ml of broth was included as a broth medium sterility control. The three tubes were incubated at 37°C in a heating block for 3 h. At this time, 10 µl of culture from the two tubes containing the recipient (*N. gonorrhoeae*) was removed and serial hundred-fold dilutions were prepared for plate counts on chocolate agar. Transformation experiment: to tube 1 containing the recipient culture, 10 µl of DNA extracted from a tetracycline-resistant *Ureaplasma* (which had been tested for sterility) was added; Culture control: to tube 2 containing recipient culture 10 µl of sterile water was added; Broth control: to tube 3 containing broth 10 µl of sterile water was added. All tubes were then incubated for 2 h after which 10 µl of culture from the tubes 1 and 2 was removed and serial hundred-fold dilutions were prepared for plate counts. Fresh media 1.2 ml was then added to all tubes and incubation continued for an additional 1 h. After this period, 10 µl of culture from the tubes 1 and 2 was removed and serial hundred-fold dilutions were prepared for plate counts. The culture from tube 1 was examined for tetracycline-resistant transformants by the Etests (AB Biodisk, Solna, Sweden) method 100 µl of culture using chocolate agar plates followed by incubation in a 5-10% CO<sub>2</sub> atmosphere at 37°C for 36 h. To test for any spontaneous tetracycline-resistant mutations/contaminants an Etest was performed on 100 µl of culture from tube 2. To confirm the broth was not contaminated, 50 µl of medium from tube 3 was spread across a chocolate agar plate with incubation in a 5-10% CO<sub>2</sub> atmosphere at 37°C for 36 h. In the event confirmatory testing was required, to the remaining culture in tubes 1 and 2 sterile glycerol was added [final concentration 10% (v/v)] and tubes stored at -80°C.



### **2.13.2 Co-incubation of *N. gonorrhoeae* recipients with tetracycline resistant *U. parvum* and *U. urealyticum* donors (culture+culture)**

Two susceptible *N. gonorrhoeae* strains were resuscitated on chocolate agar for 36 h at 37°C. Thirty colonies were inoculated into 3.75 ml of modified semi defined medium (Section 2.14.1) in Schott bottles. The suspension was mixed and divided into 3x 1.25 ml aliquots in microfuge tubes that were incubated for 3 h at 37°C on a heating block. Dilution plate counts were performed after the 3 h incubation period to establish cell numbers of *N. gonorrhoeae*. Thereafter, 900 µl of *N. gonorrhoeae* recipient culture was added to 100 µl tetracycline-resistant *U. parvum* or *U. urealyticum* (donor cultures) and cells left to settle/contact at 37°C for a further 3 h. After this period, 100 µl of 10% (v/v) glycerol was added to the culture which was then carefully mixed and stored overnight at -80°C. On removal from 24 h storage, tubes were incubated at 37°C for 30 min and then centrifuged for 15 min at 7 000 xg. The supernatant was removed, and pellet resuspended in 500 µl fresh modified semi defined medium containing 8 µg/ml tetracycline. The resuspended pellet was then incubated for 4 h at 37°C. To recover transformants, 100 µl of medium was spread across chocolate agar plates (2x 50 µl) and 20 µl streak plated on a chocolate agar with incubation in a candle extinction jar at 37°C for 48 h.

### **2.13.3 Co-incubation of *U. parvum* and *U. urealyticum* recipients with tetracycline-resistant *U. parvum* and *U. urealyticum* donors (culture+culture or culture+DNA)**

Four susceptible *Ureaplasma* recipients were used (2 *U. parvum* and 2 *U. urealyticum*) with grown in their specific media, for *U. urealyticum* ATCC #1331 broth and for *U. parvum* ATCC #2616 broth (Sections 2.3.2 and 2.3.3) for 20 h when cell concentrations were equivalent to approximately 10<sup>7</sup> cells/ml. Donor cells/DNA comprised: exponential phase cultures of one *U. urealyticum* and three *U. parvum* tetracycline-resistant strains; *N. gonorrhoeae* plasmid DNA containing *tetM*; DNA extracted from the one *U. urealyticum* and three *U. parvum* strains used in culture+culture experiments. For culture to culture transfer and DNA to culture transfer, 1/10 dilutions of donors were

used and for plasmid donor 1/15 dilution used. After addition of donor cells/DNA to a recipient incubation was conducted for 6 h at 37°C. Broth from tubes showing positive growth was used to determine tetracycline MICs by microbroth dilution (Section 2.12.2). Depending on MIC results, possible tetracycline-resistant transformant/recombinant cells were species identified and DNA extracted for PCR detection of *tetM* as described in section 2.13.2.

#### **2.13.4 Electroporation: *N. gonorrhoeae* recipient**

For *N. gonorrhoeae* electroporation, cell preparation and pulse conditions were based on procedures that have been found to be appropriate for *N. gonorrhoeae* and *E. coli* (Biorad Gene Pulser user guide; Genco *et al.* 2002; Sambrook *et al.* 1989; Shigekawa and Dower, 1988). Thirty colonies from a 36 h *N. gonorrhoeae* culture grown on chocolate agar plates were inoculated into 3.75 ml semi defined medium in Schott bottles and incubated for 4 h at 37°C. Cells were harvested by centrifugation at 5 000 xg for 10 min. The pellet was resuspended in 400 µl ice cold sterile distilled water, and centrifuged at 2500 xg for 15 min. The supernatant was removed and a further 400 µl ice cold sterile distilled water added and cells centrifuged at 2500 xg for 15 min. The pellet was then resuspended in 400 µl ice cold sterile 10% (v/v) glycerol, followed by centrifugation at 2500 xg for 15 min. This step was repeated. Cells were then resuspended in 250 µl ice cold sterile 10% (v/v) glycerol and aliquoted (5x50 µl) before storage at -80°C. Prior to electroporation an aliquot of cells was thawed, 5 µl of donor DNA added and cells transferred to chilled electroporation cuvettes (0.1 cm). Electroporation (Biorad Gene Pulser) conditions: 1.8kV, capacitance = 25 µFD, 200 ohms; left set on 125 µFD. After electroporation, the contents of the cuvette were transferred to a culture tube followed by the addition of 400 µl of semi defined media. After incubation at 37°C for 3 h, to enable recovery and expression of incorporated DNA, the culture was spread on chocolate agar plates for E-test detection of recombinant cells.

## **2.14 ELECTROPORATION AND TRANSFORMATION PROTOCOLS WITH *ESCHERICHIA COLI***

### **2.14.1 Electroporation: *E. coli* strain DH5 $\alpha$ recipient**

For *E. coli* DH5 $\alpha$  electroporation, cell preparation and pulse conditions were based on standard procedures described for *E. coli* (Biorad Gene Pulser user guide; Sambrook *et al.* 1989). *E. coli* DH5 $\alpha$  bacteria were grown on LB agar plates [10 g/l tryptone, 5 g/l yeast extract, 10g/l NaCl; pH adjusted to 7.5, 20 g agar, distilled water added to final volume of 1 l and autoclaved] for 24 h. Twenty colonies were inoculated into 4 ml LB broth [10 g/l tryptone, 5 g/l yeast extract, 10g/l NaCl; pH adjusted to 7.5, distilled water added to final volume of 1 l and autoclaved] in falcon tubes. Cells were incubated for 3 h at 37°C with shaking (225 rpm) and harvested by centrifugation and washing with ice cold sterile distilled water and 10% (v/v) glycerol as described for *N. gonorrhoeae* (section 2.13.4). Cells were then resuspended in 250  $\mu$ l ice cold sterile 10% (v/v) glycerol and aliquoted (5x50  $\mu$ l) before storage at -80°C. Prior to electroporation aliquots of cells (*E. coli* DH5 $\alpha$ ) were thawed, 5  $\mu$ l of donor DNA (whole cell from ureaplasmas); positive control: (pUC18 DNA) added and cells transferred to chilled electroporation cuvettes (0.1 cm). Electroporation (BioRad Gene Pulser) conditions: 1.8kV, capacitance = 25  $\mu$ FD, 200 ohms; left set on 125  $\mu$ FD. After electroporation, the contents of the cuvette were transferred to a culture tube followed by the addition of 400  $\mu$ l of LB media with incubation at 37°C for 3 h, to enable recovery and expression of incorporated DNA. The cells were spread on LB agar plates with ampicillin (100  $\mu$ g/ml) for detection of transformants with pUC18 control DNA or LB agar plates with tetracycline (8  $\mu$ g/ml) to detect any transformants containing *tetM* from *Ureaplasma* spp. strains. Plates were incubated at 37°C for 16-20 h.

### **2.14.2 Electroporation: *E. coli* DH5 $\alpha$ recipient (transformed with pUC18)**

The *E. coli* DH5 $\alpha$ -pUC18 recipient was grown on LB agar plates containing ampicillin for 24 h and processed as described above (2.14.1) to determine whether transfer of donor DNA (from *Ureaplasma* spp.) to *E. coli* DH5 $\alpha$  with a plasmid carrying an antibiotic resistance gene would occur.

### 2.14.3 Transformation with *E. coli* JM109 competent cells

*E. coli* JM109 commercially available competent cells (Promega) were used for transformation experiments for transfer of whole cell DNA from *Ureaplasma* spp. Control DNA (pGEM-3Z vector) was used as a positive control to determine whether transformation did occur. Sterile culture tubes (Falcon) were chilled on ice, one per transformation. Frozen competent cells were placed on ice for 5 min until thawed and 100 µl transferred to each chilled tube. DNA (10 µl) from each of the seven tetracycline-resistant *Ureaplasma* spp. was added to tubes containing 100 µl competent *E. coli* cells. For the positive control, 1 µl of pGEM-3Z vector DNA (Promega) was added to 100 µl competent cells. Tubes containing competent *E. coli* cells and DNA (i.e. either from *Ureaplasma* spp strains or control pGEM-3Z vector DNA) was placed on ice for 10 min followed by heat shock (42°C water bath) for 45-50 seconds and immediately placed on ice for 2 min. This was followed by addition of 900 µl of cold (4 °C) SOC medium [2g tryptone, 0.5g yeast extract, 1ml 1M NaCl, 0.25 ml KCl and 97 ml double distilled water and autoclaved, followed by addition of filter sterilised 1 ml Mg<sup>2+</sup> stock (1M MgCl<sub>2</sub>, 1M MgSO<sub>4</sub>) and 1 ml 2M glucose, made up to 100 ml with sterile double distilled water and complete medium filter sterilised] to each transformation reaction that was then incubated at 37°C for 60 min with shaking (225 rpm). Cells (100 µl of undiluted and 1:10 dilution) were plated on LB agar plates containing either ampicillin (100 µg/ml) for pGEM-3Z vector control DNA or tetracycline (8 µg/ml) to detect any transformants with *tetM* DNA from the ureaplasmas. Plates were incubated at 37°C for 16-20 h.

## CHAPTER THREE

### VALIDATION OF METHODS

#### 3.1 INTRODUCTION

Conventional diagnostic microbiology can be seriously limited in cases where causative microorganisms are considered “difficult” to culture; these include genital *Mycoplasma* and *Ureaplasma* spp. and *M. pneumoniae*.

Routine investigations on mycoplasmas and ureaplasmas are compromised not only by time factor to isolation but also by the complexity and expense of culture media that requires rigorous quality control measures. Commercial culture kit assays have been developed that are based on selective media and enzymatic colour changes and include: Mycoview (Ivagen), Mycoplasma IST2 (bioMerieux), and Mycoplasma Duo (Bio-Rad) (Cheah *et al.*, 2005; Smayevsky *et al.*, 1995; Kenny and Cartwright, 2001; Biernat-Sudolska *et al.*, 2006; Evans *et al.*, 2007). These assays designed for the detection of *M. hominis*, *U. parvum* and *U. urealyticum* have not been used widely or have limited availability (Evans *et al.*, 2007). The Pneumofast kit a commercial culture detection kit for *M. pneumoniae* has been recommended for identification and antibiotic susceptibility determinations (Madani and Al-Ghamdi, 2001).

The introduction of molecular biological tools in diagnostic microbiology has made for a more informed understanding of infection, transmission and survival of pathogenic microorganisms that are either non-culturable or difficult to culture. Polymerase chain reaction techniques such as conventional and real-time, multiplex PCR (Waites and Talkington, 2005); and the commercial Takara Mycoplasma PCR detection kit have been fine tuned, incorporating high copy

number/specific gene amplifications for rapid and accurate detection and identification.

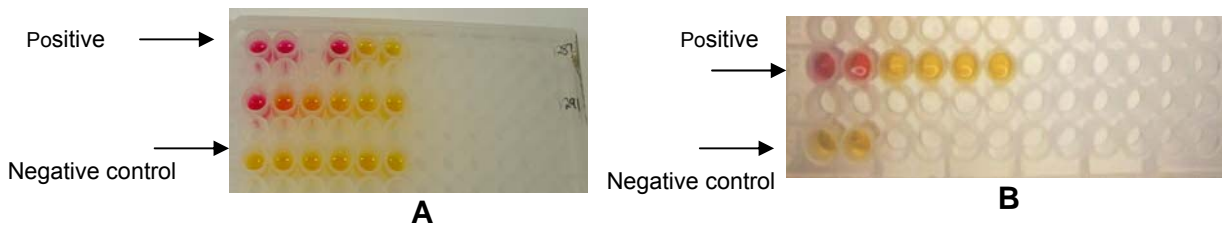
Evaluation of detection methods and specific confirmatory tests/verifications that were performed in the identification of *Mycoplasma* spp. and *Ureaplasma* spp. are described in this chapter so as not to detract from results presented on prevalence and antibiotic susceptibilities in Chapters 4 - 6.

## **3.2 RESULTS**

### **3.2.1 Comparison of conventional culture methods, commercial detection kits and PCR (Takara PCR detection kit and publication recommended primers)**

Specimens that showed growth in #243 ATCC and arginine (Bio-Rad) broths were after DNA extraction from broth culture, confirmed by Takara-PCR to be *M. hominis*. No subsequent growth of *M. hominis* on solid media was achieved. All specimens for which a positive culture in U9 broth (Bio-Rad) [Fig. 3.1(A)] was obtained were PCR positive for *U. urealyticum* and/or *U. parvum* using publication recommended primers (Table 2.1). Positive culture results from #1331 ATCC and #2616 ATCC broth media [Fig. 3.1(B)], were also PCR positive for *U. urealyticum* or *U. parvum* respectively. On subculture to solid media only a sparse growth of *Ureaplasma* spp. was observed.

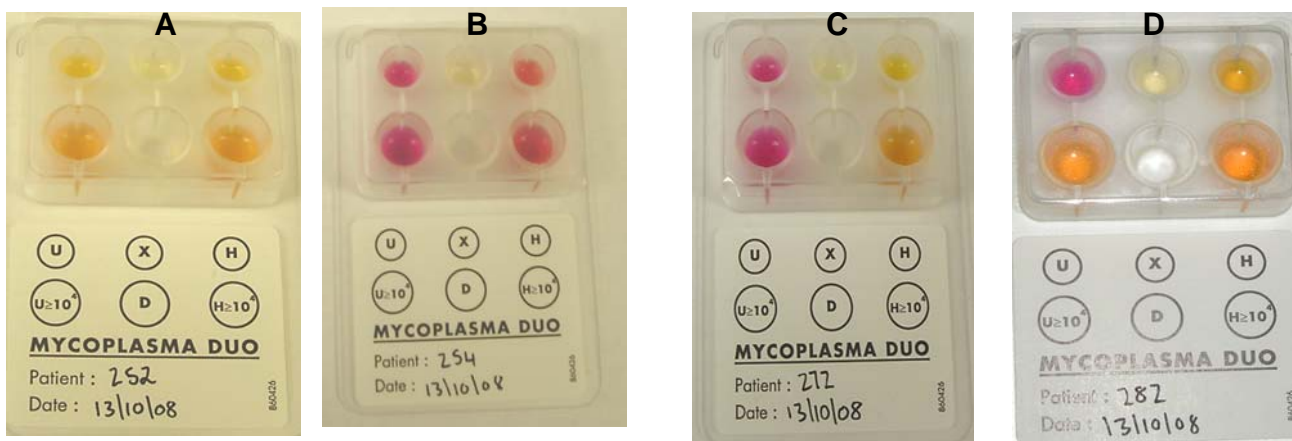
Initially, the first 14 specimens were screened by all four kits: Mycoview, Myco IST2 (Fig. 3.2), Mycoplasma Duo (Fig. 3.3) and Takara-PCR.



**Figure 3.1:** A: U9 Broth microwell culture. B: #1331 ATCC broth culture. Red wells indicate positive *Ureaplasma* spp. and yellow wells represent negative culture.



**Figure 3.2:** MycolIST2 test strip. A positive *U. urealyticum* is indicated by a pink colour change while a negative result is yellow.



**Figure 3.3:** MycoDuo kit. A: Negative result for *Ureaplasma* spp. and *M. hominis*; B: Positive *Ureaplasma* spp. and *M. hominis*; C: Positive *Ureaplasma* spp. – high titre; D: Positive *Ureaplasma* spp. – low titre.

On comparing the Takara kit with Mycoview and Mycoplasma Duo kits, Takara provided 100% sensitivity and specificity (Table 3.1). When Takara PCR was compared with specific broth media, sensitivity of Takara was only 27% for *Ureaplasma* spp. (Table 3.2). However, on comparison with specific primers (Table 3.3), sensitivity was 100% for *Ureaplasma* spp., showing that broth cultures were producing false positive results. Concerning *Ureaplasma* spp., Takara kit did not differentiate between *U. urealyticum* and *U. parvum*. Performance of Takara with PCR based on publication recommended primers (after new classification of ureaplasmas into *U. urealyticum* and *U. parvum*) provided excellent comparisons for *M. hominis* with good sensitivity and reasonable specificity for ureaplasmas (Table 3.3).

**Table 3.1:** Comparison of detection kits for the identification of *M. hominis* and *Ureaplasma* spp. on 14 specimens.

Test kit	<i>M. hominis</i>	<i>Ureaplasma</i> <i>spp</i>	<i>M. hominis</i> + <i>Ureaplasma</i> <i>spp.</i>	Negative	Sensitivity (%)	Specificity (%)
Takara-PCR	5	1	1	7	100	100
Mycoview	2	5	1	6	100	60
Myco IST2	1	2	0	11	33	87
Mycoplasma Duo	1	6	1	6	100	55



**Table 3.2:** Comparison of culture with Takara-PCR on 20 specimens.

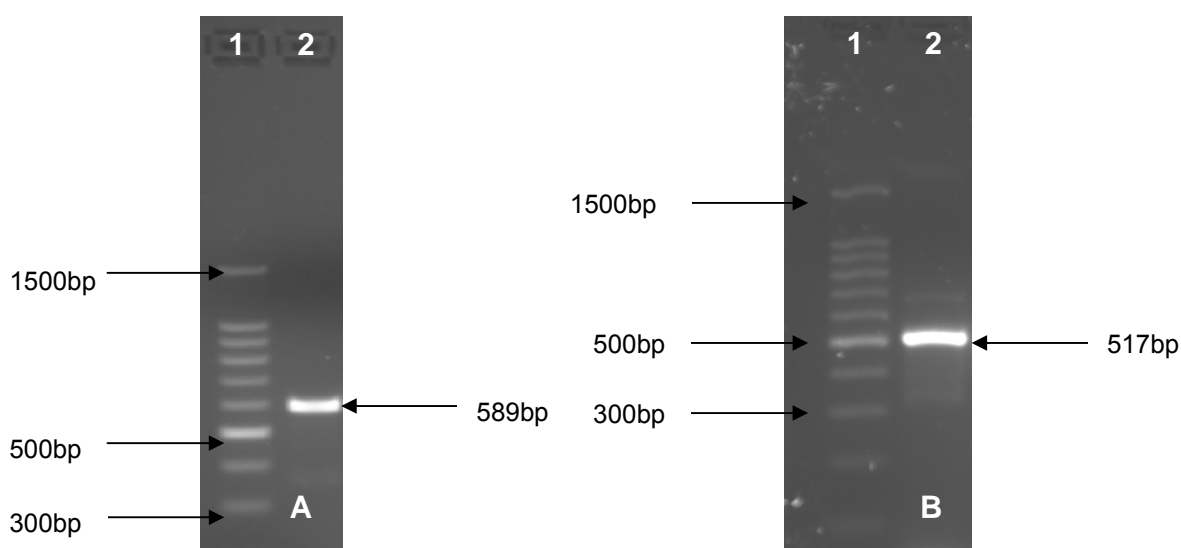
Test kit	<i>M. hominis</i>	<i>U. urealyticum</i> / <i>U. parvum</i>	<i>M. hominis</i> + <i>Ureaplasma</i> spp.	Negative	Sensitivity (%)	Specificity (%)
#243 ATCC broth (Mh)	8	N/A		12	73	100
Arginine broth (Mh)	5	N/A	1	14	50	100
Takara-PCR	10	2	1	9 <i>M. hominis</i> 17 <i>Ureaplasma</i> spp.	100 (Mh) 27 (U)	100 (Mh) 90 (U)
U9 broth (Up +Uu)	N/A	11	0	9	92	100
#1331 ATCC broth (Uu)	N/A	4	0	16	100	100
#2616 ATCC broth (Up)	N/A	8	0	12	100	100

**Table 3.3:** Comparison of MycoIST2 with Takara-PCR and publication recommended PCR primers on 48 specimens.

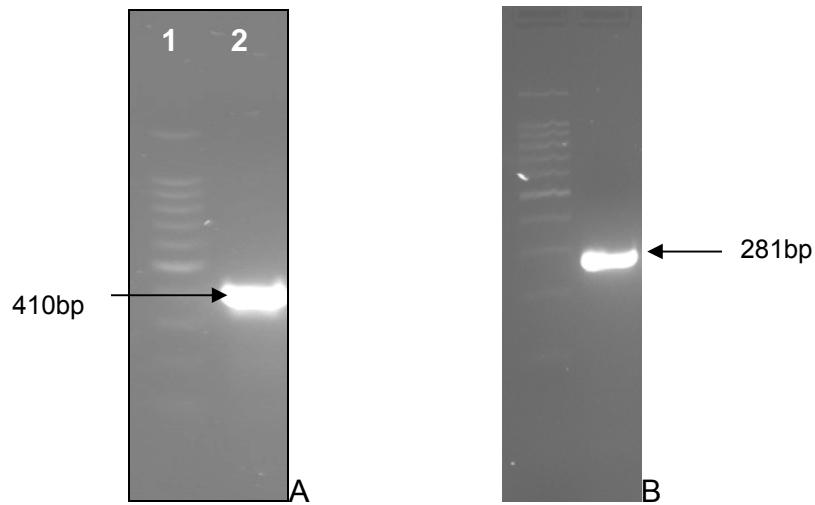
Test kit or PCR primers	<i>M. hominis</i>	<i>U. urealyticum</i>	<i>M. hominis</i> + <i>Ureaplasma</i> spp.	Negative	Sensitivity (%)	Specificity (%)
Myco IST2	5	11	2	41 <i>M. hominis</i> 35 <i>Ureaplasma</i> spp.	26 (Mh) 100 (U)	100 (Mh) 78 (U)
Takara-PCR	27	8	6	15 <i>M. hominis</i> 34 <i>Ureaplasma</i> spp.	100 (Mh) 100 (U)	100 (Mh) 76 (U)
HOM <sup>+</sup> + UN1 <sup>-</sup> (Mh)	6	N/A	2	40	24	100
UUS2c + UUA2c (Uu)	N/A	3	0	45	100	100

### 3.2.2 PCR Detection: Genital mycoplasmas, ureaplasmas and *C. trachomatis*

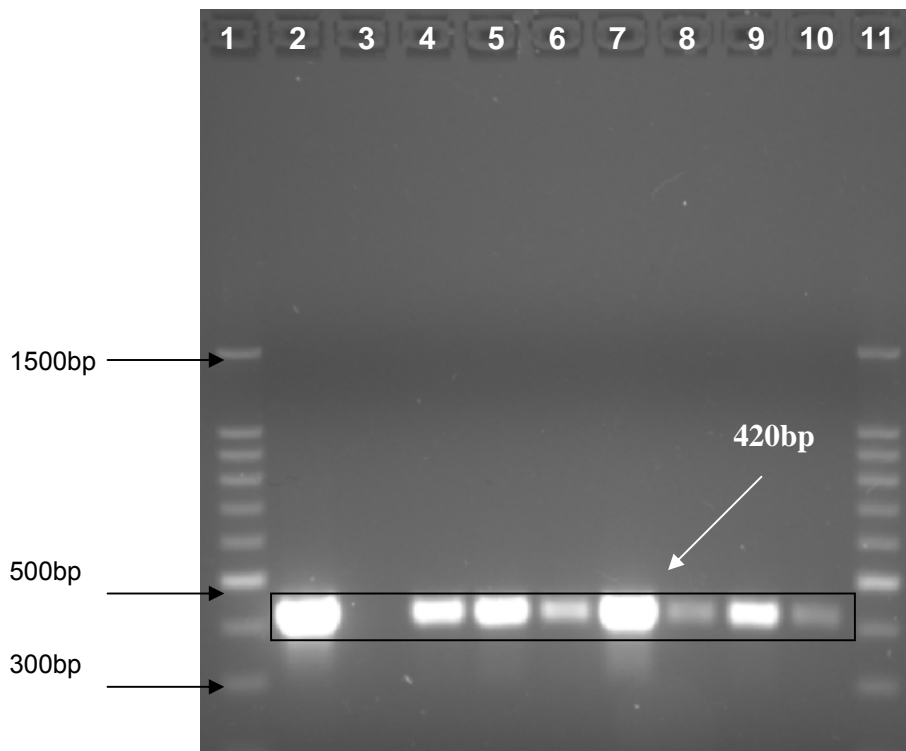
Control ATCC cultures were constantly employed to confirm PCR reactions and conditions for genital mycoplasmas, ureaplasmas and *C. trachomatis*; and that fragments of expected sizes were produced especially when no PCR products were evident from specimens (Figs. 3.4-3.9). In contrast to low detection rates employing HOM<sup>+</sup> and UNI<sup>-</sup> primers for *M. hominis* on DNA extracted from specimens, from broth culture these primers provided for quality amplification products (Fig. 3.4). Clearly defined fragments were obtained with nested Takara-PCR for the detection of *M. hominis*, *M. fermentans* and *Ureaplasma* spp. (Figs. 3.8 and 3.9).



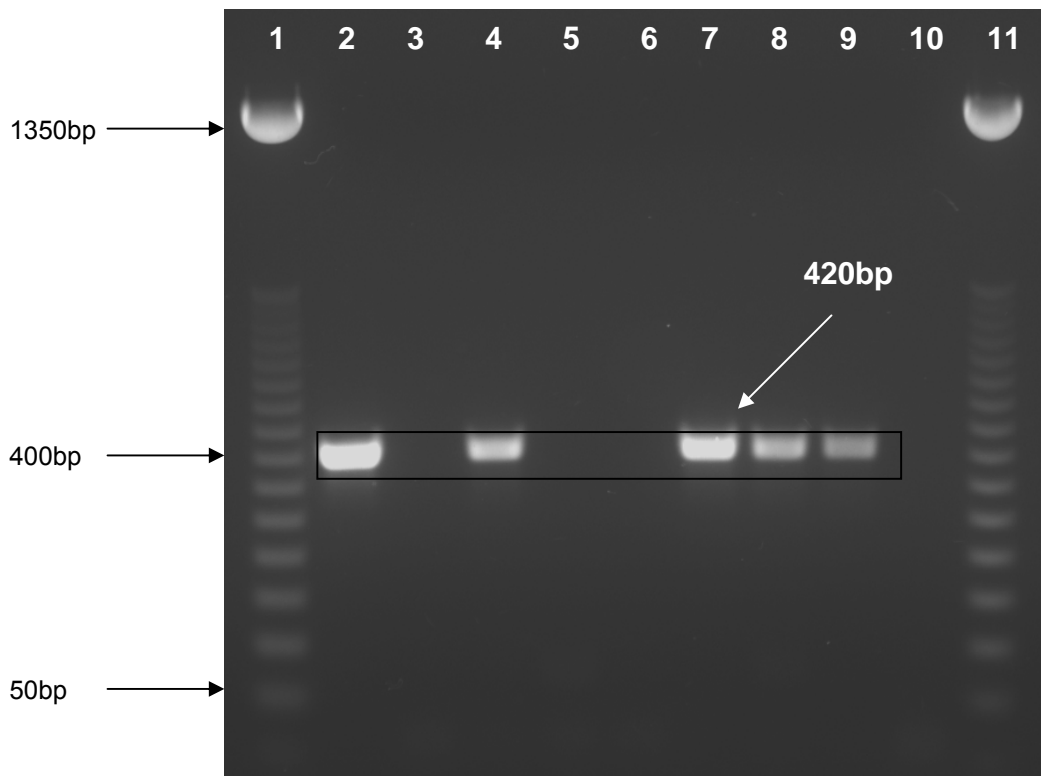
**Figure 3.4:** (A): *M. hominis* detection employing HOM<sup>+</sup> and UNI<sup>-</sup> primers.  
Lane 1: Promega 100 bp DNA ladder, Lane 2: *M. hominis* (ATCC 14027).  
(B): *C. trachomatis* detection employing T1 and T2 primers.  
Lane 1: Promega 100 bp DNA ladder, Lane 2: *C. trachomatis* (ATCC VR-887).



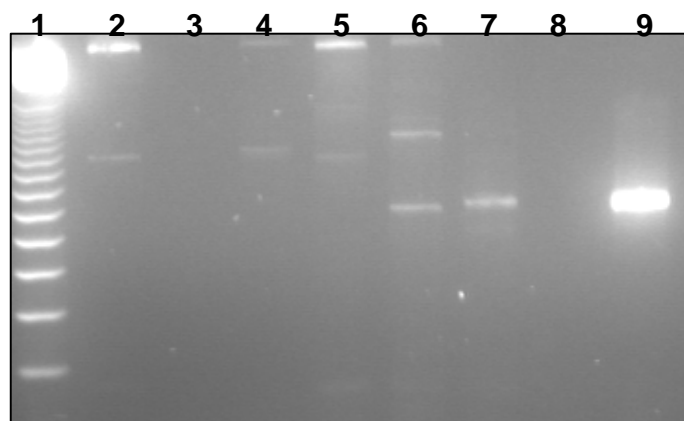
**Figure 3.5:** A: Detection of *M. penetrans* employing a control culture. Lanes: (1) Promega 100bp DNA ladder; Lanes: (2) *M. penetrans* ATCC control.  
 B: Detection of *M. genitalium* employing a control culture. Lanes: (1) Promega 100bp DNA ladder; (2) *M. genitalium* ATCC control.



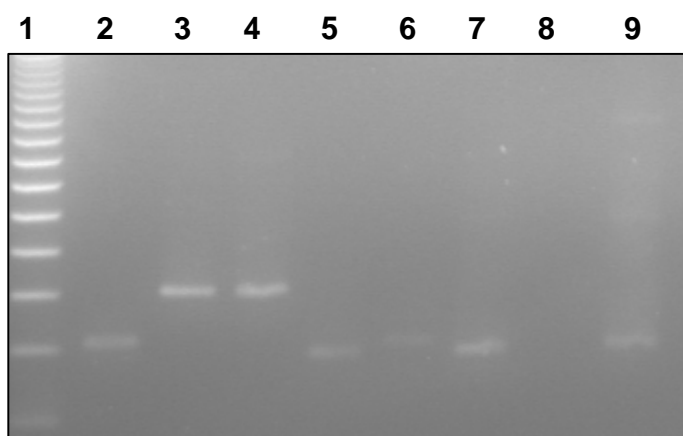
**Figure 3.6:** Representative gel depicting *U. parvum* PCR products using primers UPA and UPS. Lanes 1 and 11: Promega 100 bp DNA ladder; Lane 2: Positive *U. parvum* ATCC 27815 control; Lane 3: Negative control; Lanes 4 – 10: Endocervical samples.



**Figure 3.7:** Representative gel depicting *U. urealyticum* PCR products using primers UUA and UUS. Lanes 1 and 11: Molecular weight markers (Promega 50 bp DNA ladder); Lane 2: *U. urealyticum* ATCC 27618 positive control; Lanes 3 and 10: Negative controls; Lanes 4 – 9: Endocervical samples.



**Figure 3.8:** Representative gel depicting first reaction products employing Takara-PCR. Lanes: (1) Promega 50 bp DNA ladder; (2) *Ureaplasma* spp. (482 bp); (4) *M. fermentans* (491 bp); (5) *Ureaplasma* spp. (482 bp); (6, 7) *M. hominis*; (8) negative control; (9) positive *M. hominis* (370 bp) control.

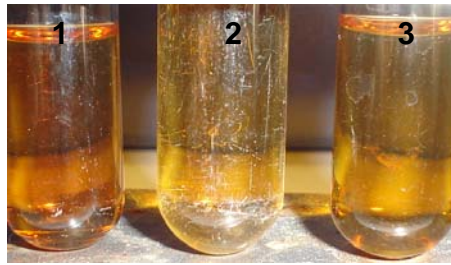


**Figure 3.9:** Representative gel depicting second reaction products employing Takara-PCR. Lanes: (1) Promega 50 bp molecular weight marker; (2) *Ureaplasma* spp. (154 bp); (3, 4) *M. fermentans* (195 bp); (5) *M. hominis* (148 bp); (6) *Ureaplasma* spp.; (7) *M. hominis* (148 bp); (8) negative control; (9) positive *M. hominis* control.

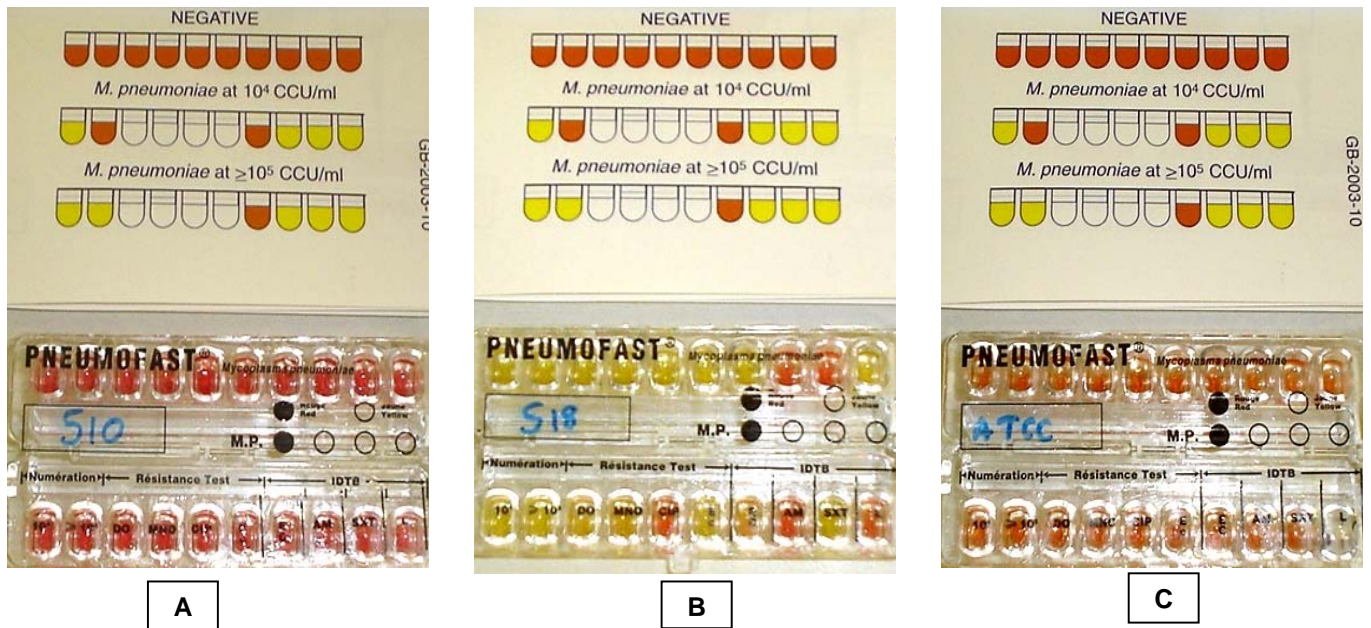
### 3.2.3 PCR Detection: *M. pneumoniae*

Growth of *M. pneumoniae* was not evident from any of the clinical respiratory specimens either on Modified New York City (MNYC) agar plates or by the Pneumofast kit. *M. pneumoniae* ATCC® 29343™ showed growth in #988 ATCC broth with a characteristic colour change from red-brown to yellow after 21 days of incubation (Fig. 3.10).

The Pneumofast kit was also tested using the control *M. pneumoniae* ATCC® 29343™ but after 30 days of incubation a broth colour change to from red to orange and not yellow was recorded, which according to kit criteria is an inconclusive result (Fig. 3.11).



**Figure 3.10:** Characteristic ATCC #988 broth colour change from red-brown to yellow due to growth of *M. pneumoniae* ATCC 29343. Tube 1: Negative control; Tube 2: *M. pneumoniae* ATCC 29343 (1:7 dilution); Tube 3: *M. pneumoniae* ATCC 29343 (1: 12.5 Dilution).

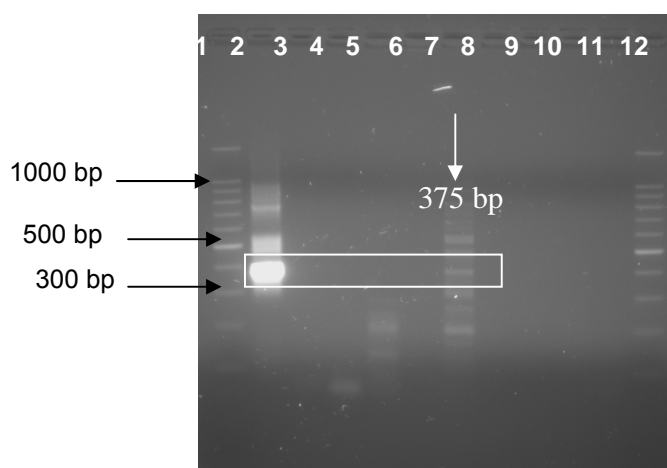


**Figure 3.11:** Detection of *M. pneumoniae* using the Pneumofast kit. (A) Negative (B) False positive and (C) *M. pneumoniae* ATCC 29343 Pneumofast Tray after 30 days.

Employing both P1 adhesin and 16S rRNA PCR primers, *M. pneumoniae* was detected in one sputum sample (Chapter 5). However, this sample could not be

sequenced as there was insufficient DNA and all attempts to repeated PCR and re-extraction failed. In addition, the patient's details were not available at the hospital so the patient could not be traced either for clinical follow-up or for a further specimen to be taken.

Sequence confirmation was performed on the *M. pneumoniae* ATCC 29343 control culture to confirm a P1 adhesin gene PCR product under primers and conditions employed (Fig. 3.12). Sequence alignment and comparison with the Genbank referenced P1 adhesin gene (GenBank accession no: M18639) confirmed the PCR fragment of *M. pneumoniae* ATCC 29343 (Fig.3.13) (Su *et al.*, 1987).



**Figure 3.12:** Detection of *M. pneumoniae* employing P1 adhesin gene primers. Lanes 1 and 12: Promega 100 bp DNA ladder; Lanes 3 and 11: Negative controls; Lanes 4 – 10: Sputa samples (1-7); Lane 2: Positive *M. pneumoniae* ATCC 29343 control.

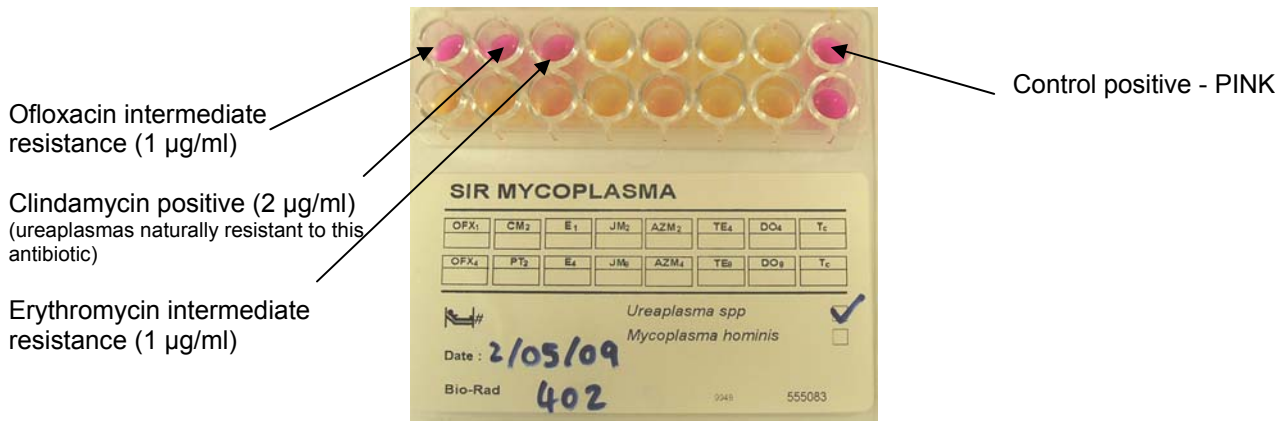
	1	10	20	30	40
<i>M. pneumoniae</i>	GGACACCAAAGTCAAGGCTTTAAAAATAGAGGTGAAAAAG				
Su et al., (1987)	998	1,007	1,017	1,027	1,037
<i>M. pneumoniae</i>	AAATCGTCTCGGGAGGACAATGGTCCACTGCAGTTAGAAA				
Su et al., (1987)	1,047	1,057	1,067	1,077	
<i>M. pneumoniae</i>	AAAATGATCTCGCCAACGCTCCCATTAAGCGGAGCGAGGA				
Su et al., (1987)	1,087	1,097	1,107	1,117	
<i>M. pneumoniae</i>	GTGGGGTCAGTCCCTCCAAACTCAAGGAGGACGATTTTGCC				
Su et al., (1987)	1,127	1,137	1,147	1,157	
<i>M. pneumoniae</i>	ACCGTCCTTTCAGTTCGGGATCAGGCGGCAACTCCAATC				
Su et al., (1987)	1,167	1,177	1,187	1,197	
<i>M. pneumoniae</i>	CCGGTCCCCCACCCTGAAGGCCG				
Su et al., (1987)	1,207	1,223			

**Figure 3.13:** DNA sequence of a 226 bp amplified DNA portion of the P1 adhesin gene from *M. pneumoniae* ATCC 29343 compared to GenBank accession no: M18639 (Su *et al.*, 1987).

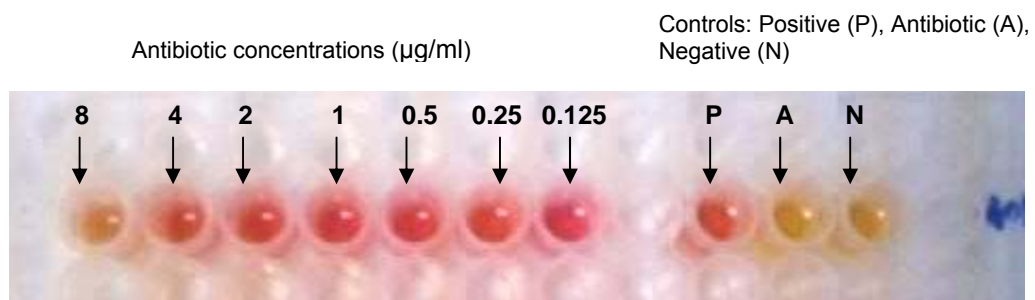
### 3.2.4 SIR Mycoplasma kit and MIC determinations

Specimens in which *U. parvum* and/or *U. urealyticum* had been detected by PCR, were cultured in U9 broth and ATCC recommended media and screened for antimicrobial susceptibility using the SIR kit (Fig. 3.14). As the SIR kit is unable to differentiate between *U. urealyticum* and *U. parvum* and tests only two concentrations of an antibiotic, MICs were also determined using the microbroth dilution method (Fig. 3.15). *Ureaplasma* cultures were constantly checked for any signs of contamination, not only by media clarity but also by plating onto chocolate agar plates with incubation at 37°C in a candle extinction jar for 48 hours. No contamination was detected.





**Figure 3.14:** Antimicrobial susceptibility testing of *U. parvum* strain (Up-402) using the SIR kit. Pink wells indicated that growth had occurred in the presence of an antimicrobial agent and results recorded as intermediate resistance/resistant, while yellow wells indicated no growth and susceptibility to an antibiotic. For intermediate resistance, only the well with low concentration of antibiotic turned pink. For resistance, wells containing both high and low concentration of antibiotics turned pink.



**Figure 3.15:** MIC determinations of ureaplasmas using the microbroth dilution method.

### 3.3 DISCUSSION

Mycoplasmas and ureaplasmas are fastidious, difficult to culture as they are very susceptible to adverse environmental conditions including desiccation, osmotic change, toxic metabolites and fluctuations in temperature (Duffy and Waites, 2008). Poor recovery of *Ureaplasma* spp. from clinical samples has been documented (Waites *et al.*, 2001).

For both gynaecological studies endocervical specimens were eluted by a nurse into microbanks that were stored at -80°C within 30 minutes. Freeze thaw procedures were kept to a minimum. A major challenge during the project was obtaining repeat culture and DNA when initial tests showed insufficient PCR product for sequence confirmation. Of six culture positive presumptive *M. hominis* strains obtained from 2005, endocervical specimens, two were susceptible to all antibiotics tested and four strains were resistant to tetracycline and intermediately-resistant to doxycycline. Unfortunately on trying to repeat susceptibility testing and *M. hominis* confirmation no further cultures were obtainable from the specimens.

Ethics, sample quality and storage and patient information were certainly high on the agenda as studies proceeded. The gynaecological study that was conducted in 2003 (prior to PhD investigations being initiated) was formulated to investigate the effect of *Chlamydia* carriage on preterm labour. After analysing mycoplasma and ureaplasma prevalence on these specimens, information on the HIV status of patients was not available. In order to investigate mycoplasma/ureaplasma prevalence in conjunction with HIV, ethics was approved and an independent nurse was contracted to handle all specimens which were taken in a controlled clinic environment to ensure that the quality/standard of specimens and patient's histories were not compromised. Whilst the majority of patients were willing to give consent during 2005, by 2006 there was a change in attitude and it became progressively more difficult to obtain specimens.

In contrast, the quality/standard of specimens and patient's histories in PE Hospitals presented a major challenge to expanding findings of the respiratory study. The neonate endotracheal aspirate specimens obtained from the hospital were of a poor quality (i.e. some were contaminated with blood). Due to very small specimen volumes, samples had to be flushed out of the endotracheal tubes with sterile water which resulted in sample dilution. Due to circumstances at the hospital, it was not possible for specimens to be transferred into transport media prior to collection and delivery to NMMU for analysis, which may have contributed to negative culture results. These circumstances included refusal of nurses to transfer specimens into media before storage and transport to NMMU for analysis. There were insufficient funds to employ a private nurse in Port Elizabeth hospitals for collection of specimens. Staff at the hospital were reluctant to participate in research projects because of their increased workload. In an effort to remedy the situation the nurses had agreed to store the specimen in a freezer and contact the researcher as soon as a specimen was available. The researcher collected the specimen within an hour of being contacted by the hospital.

*M. hominis* was not detected in endotracheal aspirate samples as has been reported in the majority of other studies (Waites *et al.*, 2005). Unfortunately and certainly for the two neonates in which *U. parvum* was detected, all attempts to obtain information from NICU patient files at the Dora Nginza, on co-infections like HIV, TB, treatment or the type of delivery were unsuccessful.

All cultures were constantly checked for contamination and PCR conducted for genus (mycoplasma vs ureaplasma) verification.

The majority of PCR positive endocervical specimens collected in 2006 were positive for growth of *Ureaplasma* spp. using commercially prepared U9 broth. There was no contamination in any microtiter plate wells indicating that U9 broth can be considered a suitable medium for the culture of *Ureaplasma* spp. prior to

antibiotic susceptibility screening tests. The ATCC recommended broths were specific in differentiating between *U. parvum* and *U. urealyticum* while it should be noted that commercial U9 broth does allow growth of both *U. urealyticum* and *U. parvum*.

The MycoDuo kit results seem to be in agreement with Evans *et al.* (2007), whereby the MycoDuo kit showed a higher detection rate (52%) than culture on A7 agar plates (36%). However, the results of this study were contrary to Cheah *et al.* (2005), which showed an overall agreement of 96% between PCR and Mycoplasma Duo Kit for the detection of *Ureaplasma* spp. in endotracheal aspirates from neonates.

The MycoIST2 kit allows for the detection of *Ureaplasma* spp. and *M. hominis* as well as antibiotic susceptibility testing but a high rate of contamination by other microorganisms from the specimens produced inconclusive results. There have been contrasting reports concerning antibiotic resistance profiles when using the MycoIST2 kit (Smayevsky *et al.*, 1995; Kenny and Cartwright, 2001). Four positive cultures that were inhibited by ciprofloxacin 1 µg/ml using the MycoIST2 kit were on MIC testing found to be inhibited by 2 µg/ml ciprofloxacin (Smayevsky *et al.*, 1995). In addition, false positive results have been reported due to the presence of other bacteria (e.g. *Proteus*, *Klebsiella*) that are capable of hydrolysing urea (Biernat-Sudolska *et al.*, 2006).

Granato *et al.* (1983), reported that MNYC medium was better suited for supporting *M. pneumoniae* growth from clinical specimens than the ATCC 988 medium, as it provided for rapid and specific growth. However, in the current investigation neither MNYC/ATCC 988 agar plates supported growth of a *M. pneumoniae* from a respiratory specimen that was isolated in ATCC broth medium. The Pneumofast kit has been specifically designed for the identification of *M. pneumoniae* from clinical respiratory specimens. Madani and Al-Ghamdi (2001), incubated an ATCC control for 30 days after which a positive result was

recorded. However, after 30 days of incubation the *M. pneumoniae* ATCC 29343 control in the present study only exhibited an orange colour which is considered inconclusive. Waris *et al.* (1998), have reported that use of the Pneumofast kit for the detection of *M. pneumoniae* from PCR positive specimens was significantly low, with only 1/20 PCR positive samples being test positive by the Pneumofast kit. Limitations of the Pneumofast method that have been shown to affect results include sample quality and viability levels of *M. pneumoniae* present within the samples.

Antimicrobial susceptibility testing methods of mycoplasmas and ureaplasmas which has been in constant flux was semi-consolidated at the 17<sup>th</sup> IOM Congress and Mycoplasma Techniques Workshop July 2008, however, even after this international interactive meeting some groups are still reluctant to fully define MIC methodologies and media compositions. The SIR kit for antibiotic susceptibility screening was seen to be an efficient method and has been validated by laboratories in Bordeaux, France and used in preliminary antibiotic resistance screening tests (Bebear *et al.*, 2003; Degrange *et al.*, 2008).

The project did encounter problems due to the prolonged time frame (part-time PhD), with some contributing factors par for the course and others unforeseen. The prolonged time frame meant constantly being aware of updates of changing media, methodologies concerning detection especially *Ureaplasma* speciation division which required all genital specimens collected in 2003 to be re-analysed for *U. parvum*; and antibiotic susceptibility determination preferences of different international groups. It must be said that information finally presented in the thesis benefited from repositioning with extensive comparative and confirmatory aspects incorporated.

## CHAPTER FOUR

### PREVALENCE OF GENITAL MYCOPLASMAS, UREAPLASMAS AND *CHLAMYDIA* IN PREGNANCY

#### 4.1 INTRODUCTION

The possible role of genital mycoplasmas (*Mycoplasma hominis*, *Mycoplasma fermentans*, *Mycoplasma penetrans*, *Mycoplasma genitalium*), *Ureaplasma urealyticum*, *Ureaplasma parvum* and *Chlamydia trachomatis* on the outcome of pregnancy is a topic of great interest and debate and has not been satisfactorily resolved. Studies of women from whom ureaplasmas and *M. hominis* were isolated from the endometrium, placenta or midtrimester amniotic fluid have shown a consistent association with premature rupture of membranes and spontaneous abortion, (Grattard *et al.*, 1995; Kafetzis *et al.*, 2004; Nguyen *et al.*, 2004; Perni *et al.*, 2004; Witt *et al.*, 2005). Other reports have found associations between *U. urealyticum* in amniotic fluid (Yoon *et al.*, 1998; Gerber *et al.*, 2003), cervix (Mitsunari *et al.*, 2005) and vaginal colonisation with bacterial vaginosis (Vogel *et al.*, 2006) to contribute to adverse outcomes on pregnancy. In addition, vaginal colonisation with the newly classified *U. parvum* has been seen to be associated with late abortion or early preterm birth (Kataoka *et al.*, 2006). Although *M. penetrans*, *M. fermentans* and *M. genitalium* have received less attention a) *M. penetrans* has been isolated from the urogenital tracts of individuals with HIV infection (Hussain *et al.*, 1999), b) *M. fermentans* has been reported to be an opportunist in HIV infection, (Ainsworth *et al.*, 2001; Waites and Talkington, 2005) and c) *M. genitalium* has been associated with pelvic inflammatory disease (Taylor-Robinson, 2002; Haggerty *et al.*, 2006), and acute endometritis in women in Kenya (Cohen *et al.*, 2002), but was not seen to affect pregnancy outcomes in women of Guinea-Bissau (Labbe *et al.*, 2002). However, as to whether *M. penetrans*, *M. fermentans* and *M. genitalium* present significant risk factors in pregnancy outcome remains unclear (Taylor-Robinson, 2007).

Spontaneous preterm labour is a major cause of neonatal mortality with a prevalence of 20.3% in the Tygerberg area, accounting for 40% of neonatal deaths (Pattinson and van Zyl, 1991; Odendaal *et al.*, 2002). In a previous study at the Tygerberg Hospital, an association between preterm birth and *M. hominis* and *C. trachomatis* infection was found (Odendaal *et al.*, 2002). Furthermore, a subsequent study reported an association between *C. trachomatis*, lower body mass index (BMI) and younger maternal age with preterm delivery (Odendaal *et al.*, 2006).

A study was designed (including patients enrolled at two time periods, refer to section 2.1) to extend findings on *C. trachomatis* pertaining to preterm delivery by Odendaal *et al.* (2006), by including mycoplasma and ureaplasma prevalence data and to ascertain whether a positive HIV status lends to increased colonisation rates.

## 4.2 RESULTS

### 4.2.1 Mycoplasmas, *Ureaplasma* spp. and *C. trachomatis* according to women's age groups

In 2003, 45% of women sequentially screened were aged 14–20 years, however, in 2005 only 6% were in this age group (Table 4.1). Conversely in 2003, 13% of women were older than 30 years yet in 2005, 36% were in this age group. The number of specimens in which no detection was recorded increased from 16.5% in 2003 to 48% in 2005. Colonisation was seen to be highest in the 14–20 year group from 2003. When compared with the 14-25 age group prevalence of *Ureaplasma* spp. tended to be higher in women  $\geq 26$  years while *C. trachomatis* and *M. hominis* tended to be lower in women  $\geq 26$  years. In women aged  $\geq 21$  years, although co-colonisation remained at 13% there was a shift from co-colonisation with *M. hominis* and *Ureaplasma* spp. in 2003 to other dual/triple combinations in 2005.

**Table 4.1:** Prevalence of mycoplasmas, *Ureaplasma spp.* and *C. trachomatis* according to women's age groups during 2003 and 2005.

Micro-organism detected	Age groups (Years)								Totals			
	14 - 20		21 - 25		26 - 30		>30		(No.)		(%)	
	2003	2005	2003	2005	2003	2005	2003	2005	2003	2005	2003	2005
<i>M. hominis</i>	43	3	28	19	13	13	12	16	96	51	48	23
<i>M. fermentans</i>	1				1				2		1	
<i>Ureaplasma spp.</i>	13		6	2	8	6	4	5	31	13	15.5	6
<i>C. trachomatis</i>	2	3	2	3		10	1	5	5	21	3	10
<i>M. hominis</i> + <i>Ureaplasma spp.</i>	4	1	2	1	6	1	2	5	14	8	7	4
<i>M. hominis</i> + <i>C. trachomatis</i>	9		2	5		4	1	3	12	12	6	5.5
<i>M. fermentans</i> + <i>C. trachomatis</i>	1								1		0.5	
<i>Ureaplasma spp.</i> + <i>C. trachomatis</i>	3			2		1			3	3	1.5	1.5
<i>M. hominis</i> + <i>Ureaplasma spp.</i> + <i>C. trachomatis</i>	1		1	2		0		3	2	5	1	2
No detection	12	6	7	26	8	32	6	42	33	106	16.5	48
Totals	89	13	48	60	36	67	26	79	199	219		



#### 4.2.3 *Mycoplasma* spp. and *Ureaplasma* spp. colonisation and preterm deliveries (2003 study)

There were no significant differences in the presence of *M. hominis* (55% and 63%;  $p = 0.22720$ ), *U. urealyticum* (25% and 25%;  $p=0.70340$ ) and *U. parvum* (50% and 36.9%;  $p= 0.43157$ ) in women that delivered preterm and full term (Table 4.2).

**Table 4.2:** Comparisons of *Mycoplasma* and *Ureaplasma* colonisation with preterm and full term deliveries (2003 study).

Micro-organism detected	Preterm (20 women)		Full term (179 women)	
	No.	%	No.	%
<i>M. hominis</i>	11	55	113	63
<i>M. fermentans</i>	1	5	2	1
<i>U. urealyticum</i>	5	25	45	25
<i>U. parvum</i>	10	50	66	36.9

#### 4.2.4 *M. hominis*, *Ureaplasma* spp. and *C. trachomatis* colonisation and HIV status (2005 study)

Comparisons of colonisation with HIV status are shown in Table 4.3. The 20 samples in the HIV positive group were also screened for the presence of *U. parvum* and only 3 specimens were positive (15%). *M. penetrans* and *M. genitalium* were not detected in the preterm or the HIV positive groups.

**Table 4.3:** Comparisons of *M. hominis*, *Ureaplasma* spp. and *C. trachomatis* colonisation with HIV status (2005 study).

Micro-organism detected	HIV positive (20 women)		HIV negative (199 women)	
	No.	%	No.	%
<i>M. hominis</i>	5	25	71	36
<i>Ureaplasma</i> spp.	2	10	27	14
<i>C. trachomatis</i>	4	20	37	19

### 4.3 DISCUSSION

The number of women in the sexually transmitted infection (STI) high risk group in South Africa 14-20 years age group (Rours *et al.*, 2006a and 2006b), in 2003, was 89 whereas in 2005, only 13 women were in this age group. The 2003 study focussed on low adverse pregnancy outcome risk women, attending an antenatal clinic located near the Tygerberg hospital, while the 2005 study was conducted on women (high risk) referred to the obstetric unit at the Tygerberg hospital. In 2005, in order to capture prevalence in conjunction with HIV status and to conform to high ethical standards, specimens from women attending for gynaecological examination at Tygerberg hospital were studied. In addition, in 2003, there was controversy of HIV testing/consent issues whereas by 2005, community initiatives by activist groups and government had increased awareness to the consequences of sexual behaviour, promoted condom usage and HIV testing, condemned rape and legalised abortion.

It is acknowledged that there were limitations within the study design. The study is mainly laboratory based due to practicalities of carrying out a PhD study part-time, the candidate residing in Port Elizabeth. There is a lack of enriching clinical aspects as no detailed patient epidemiological, clinical history and clinical examination details were available. Despite the lack of opportunity to collect the clinical information, or data on the number of sexual partners, use of condoms, other STIs etc., number of cases missed during the collection periods or how representative the sample group is; this study was an 'initial step' in exploring the prevalence of *Mycoplasmas* and *Ureaplasmas* in pregnant women in SA. Other South African researchers have investigated *M. genitalium* (Black *et al.*, 2008; Lewis *et al.*, 2008) and *M. hominis* (Ballard *et al.*, 2002; Odendaal *et al.*, 2002) but using different categories of sample groups and methods compared to those used in this study.

Colonisation with *M. hominis*, *Ureaplasma* spp. and *C. trachomatis* appeared to be age related. Similar findings have been documented in South Africa (Rours *et al.*, 2006) and other parts of the world (Baczynska *et al.*, 2008) where chlamydial infection was the most prevalent STI in women less than 20 years. Syndromic management protocols for vaginal discharge in South Africa do consider *C.*

*trachomatis* hence a reduction of chlamydial carriage in older women would be expected in that they would have more readily (reduced stigma attachment/previous pregnancy) attended clinics. From 2003 to 2005, there was a general decline across all age groups in the prevalence of single colonisation with *M. hominis* or *Ureaplasma* spp. It is unclear with prevalence data from only two time frames and in the absence of antibiotic resistance development investigations, as to why there was a lower prevalence of *M. hominis* yet higher trend for *Ureaplasma* spp. in women  $\geq 26$  years of age and a shift in co-colonisation types.

The study did not find an association between *M. hominis* and *Ureaplasma* spp. with preterm labour. This is contrary to the findings of other research groups (Grattard *et al.*, 1995; Cultrera *et al.*, 1998; Donders *et al.*, 2000; Perni *et al.*, 2004; Edwards *et al.*, 2006; Kataoka *et al.*, 2006). Grattard *et al.*, (1995) screened 208 women (cervico-vaginal swabs) and their neonates (aspirates) and reported (using MycoFast All-In test kit) *M. hominis* in 11% of women and 1% of neonates respectively while *U. urealyticum* was detected in 47.6% women and 19.2% neonates respectively. *U. urealyticum* detection by PCR was suggested to be associated with HIV infection in 12 out of 84 patients (Cultrera *et al.*, 1998). In 228 women (28 preterm births; 21 early pregnancy losses), 14 weeks gestation, *M. hominis* and *U. urealyticum* remained associated with an increased risk of miscarriage (Donders *et al.*, 2000). The detection of *U. urealyticum* or *M. hominis* in midtrimester amniotic fluids by PCR was shown to be a potential risk factor for subsequent preterm premature rupture of membranes as all women with premature rupture of membranes tested positive for either *U. urealyticum* or *M. hominis* (Perni *et al.*, 2004).

Conflicting findings have been reported as to whether *U. urealyticum* (Kim *et al.*, 2003) or *U. parvum* (Kataoka *et al.*, 2006) be considered the most important pathogen. *U. parvum* was more frequently isolated from amniotic fluid of preterm gestations than *U. urealyticum* in a sample population of 77 women (gestational age < 37 weeks) (Kim *et al.*, 2003). In a prospective cohort study of 877 women, vaginal colonisation with *U. parvum* by PCR-based methods was associated with late abortion or early preterm birth (Kataoka *et al.*, 2006). Studies that were limited to sampling the lower genital tract of women have yielded inconclusive results, mainly

because not all women who are colonised in the lower tract will develop infection in the upper tract (Waites *et al.*, 2005).

In the present study, there was no significant association with *U. parvum* and preterm delivery which is in agreement with other reports (Aaltone *et al.*, 2002; Matovina *et al.*, 2004; Gonzalez Bosquet *et al.*, 2006). However, failure to establish a statistically significant relationship may have been due to the small sample size of the preterm group (Govender *et al.*, 2009). Kacerovsky *et al.*, (2009) found that the prevalence of cervical colonisation by *U. urealyticum* was 68% (152/225) and *M. hominis* was 28% (63/225) in patients with preterm premature rupture of membranes. In the control group *U. urealyticum* was found in 17% (38/225) and *M. hominis* in 15% (35/225) pregnant women.

*M. genitalium* and *M. penetrans* were not detected, which is in agreement with others findings on *M. genitalium* in preterm labour (Lu *et al.*, 2001; Casin *et al.*, 2002; Labbe *et al.*, 2002). Comparisons of colonisation with HIV status also showed no association ( $p=0.36063$ ) between *M. hominis*, *Ureaplasma* spp.; *C. trachomatis* and HIV infection (Govender *et al.*, 2009), contradictory to a study by Cordova *et al.* (2000) that found *M. penetrans* was seen to occur more frequently in HIV infected individuals. Recent reports have associated *M. genitalium* from endocervical swabs and HIV infection/sereopositivity and vaginal discharge syndrome (Napierala and Weiss, 2009; Venter *et al.*, 2009).

## CHAPTER FIVE

### PREVALENCE OF MYCOPLASMAS AND UREAPLASMAS IN ADULTS AND NEONATES PRESENTING WITH PNEUMONIA AT HOSPITALS IN THE PORT ELIZABETH REGION

#### 5.1 INTRODUCTION

*Mycoplasma pneumoniae* is a common cause of acute upper and lower respiratory tract infections particularly in children, young adults and the elderly; and community-acquired pneumonia which often results in hospitalisation due to the severity of the illness (Madani and Al-Ghamdi, 2001; Waites and Talkington, 2004). *Pneumocystis jiroveci* an opportunistic fungus, is the causative agent of *Pneumocystis* pneumonia (PcP) in humans, and is an important cause of morbidity and mortality among immunocompromised persons, AIDS related or patients receiving prolonged corticosteroid or intensive immunosuppressive therapies (Pinlaor *et al.* 2004, Arcenas *et al.* 2006, Kong *et al.* 2007 and Van Oosterhout *et al.* 2007).

*M. hominis*, *U. urealyticum* and *U. parvum* infections of the newborn have been associated with pneumonia, bacteraemia, chronic lung disease (CLD) and central nervous system (CNS) invasion (Waites *et al.*, 2005; Schelonka and Waites, 2007). *Chlamydia trachomatis* (serotypes D – K) have been implicated in neonatal disease, including conjunctivitis and pneumonia of the newborn (Darville, 2005; Rours *et al.*, 2006b).

As delayed treatment of pneumonia increases the risk of morbidity/mortality, rapid and accurate identification is crucial to the establishment of informed prognosis and facilitation of early appropriate treatment (Khanna *et al.*, 2005).

By 2006, provincial hospitals in Port Elizabeth had noted that the incidence of HIV in patients presenting with pneumonia had increased, yet there was no regional information on the extent of concomitant *M. pneumoniae* (as mentioned in section

1.1.4 of Chapter one) or *P. jiroveci*. The respiratory component of the study was conducted in an effort to determine prevalence of *M. pneumoniae* in Port Elizabeth hospitals and at the request of doctors at the hospitals who had observed an increase in pneumocystis pneumonia cases; simultaneous *P. jiroveci* detection was also performed and hence included in the thesis. PCR assays have been found to be very effective in detecting and improving diagnosis of *P. jiroveci*, (Durand-Joly *et al.* 2005; Gupta *et al.* 2007) and *M. pneumoniae*, (Abele-Horn *et al.* 1998; Daxboeck *et al.* 2003) and were employed to investigate the prevalence of *P. jiroveci* and *M. pneumoniae* in patients admitted with pneumonia to two hospitals in Port Elizabeth.

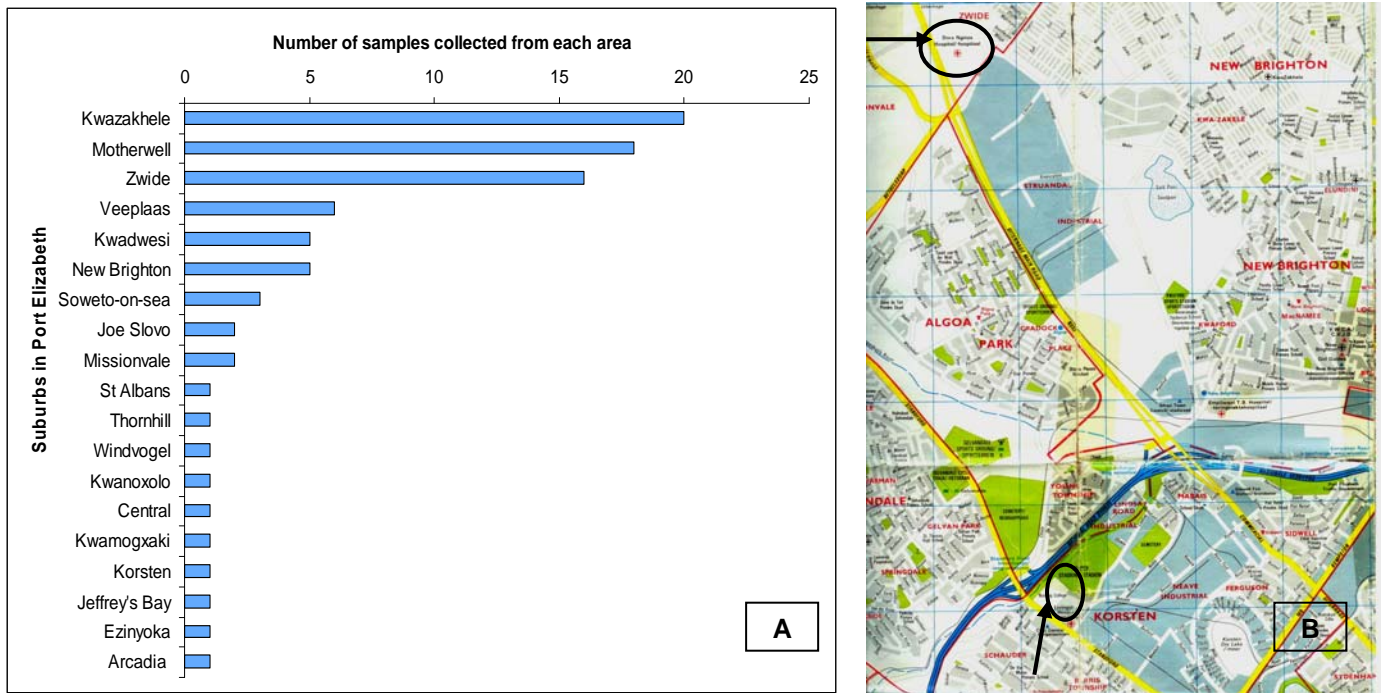
By 2007, an increase in mortality rate in neonates presenting with pneumonia at the Dora Nginza hospital Neonatal Intensive Care Unit (NICU) in Port Elizabeth raised treatment management concerns. Investigations were therefore requested and conducted on the prevalence of atypical pneumonia pathogens such as *U. urealyticum*, *U. parvum* and *M. hominis* in neonates.

## 5.2 RESULTS

### 5.2.1 Demographics of patient population

#### 5.2.1.1 *Pneumonia in adults and detection of M. pneumoniae and P. jiroveci*

The 102 community-acquired pneumonia patients, came from 19 suburbs surrounding the Dora Nginza and Livingstone Hospitals [Fig. 5.9 (A) and (B)]. A substantial number of samples were obtained between March, May and August (2006-2008), but there was a decrease in collection during June and July (2006-2008). The national nurses strike held in 2007, resulted in no samples being collected for both March and April. Patients were admitted and treated with cefuroxime if there were no contraindications or alternatively penicillin/gentamycin/erythromycin.



**Figure 5.1:** Locations from which samples were obtained. (A) 19 suburbs within Port Elizabeth and (B) Hospitals in relation to these suburbs.

Of the 102 patients for whom gender information was available, 85 were female, while 17 were males. The majority of pneumonia cases were seen in the 21-40 years age groups, comprising of 73 patients, with *P. jiroveci* detected in 40 patients. The high prevalence of *P. jiroveci* in women (46/85) was consistent with their high risk profile. Overall, *P. jiroveci* was detected in 54/102 patients; 41 patients were HIV-positive, 3 were HIV-negative and the HIV status of 10 patients was unknown. *M. tuberculosis* and *P. jiroveci* were present together in 12 HIV-positive patients and 1 HIV-negative patient (Table 5.1). *P. jiroveci* was found to be the sole causative agent in 25/71 HIV-positive patients, while *M. tuberculosis* was found alone in only 7 HIV-positive patients. Although closely linked with HIV infection, *P. jiroveci* was not necessarily co-associated with *M. tuberculosis*. Patient history and case information were provided by the hospitals and many patients with *P. jiroveci* pneumonia were found to have other infections. Some of the common co-infections (where patient history was available) included: PcP, TB and HIV (10.78%); PcP and HIV (24.51%) with 20.59% of HIV-positive samples being PcP negative. Both of the young females (<15 years) were HIV positive and one had associated *P. jiroveci* colonisation. *M. pneumoniae* was detected in one patient by 16SrRNA and P1 adhesin gene PCR but

unfortunately HIV status was not known. There was no information on how many patients were receiving Bactrim prophylaxis (which was recommended for all HIV infected patients, along with vitamins, during the time of the study).

**Table 5.1:** Patient history and the presence or absence of *P. jiroveci* (PcP) from sputa samples.

	Number of patients (%)
	Total (n) = 102 <sup>a</sup>
PcP positive and HIV positive	25 (24.51)
PcP positive, HIV positive and TB positive	11 (10.78)
PcP negative, HIV positive and TB positive	7 (6.86)
PcP negative and HIV positive	21 (20.59)
PcP positive and HIV negative	2 (1.96) <sup>b</sup>
PcP positive, HIV negative and TB positive	1 (0.98) <sup>b</sup>
PcP positive and TB positive, HIV status unknown	1 (0.98)
PcP positive, HIV positive and previous TB	1 (0.98)
PcP positive, HIV positive and diabetes	1 (0.98)
PcP positive, HIV positive and epilepsy	1 (0.98)
PcP positive, HIV positive and asthma	1 (0.98)
PcP positive, HIV positive, <i>Klebsiella pneumoniae</i> and pleural effusion	1 (0.98)
PcP negative, HIV positive and pleural effusion	1 (0.98)
PcP negative, HIV positive and anemia/thrombosis	1 (0.98)
PcP positive, HIV status and co-infection history unknown	11 (10.78)
PcP negative, HIV status and co-infection history unknown	16 (15.69)

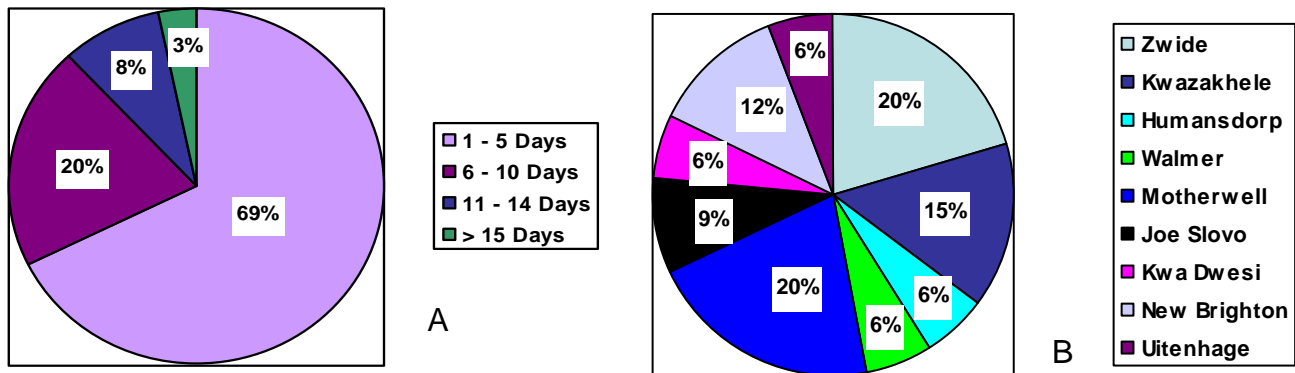
a: HIV positive (n) = 71, HIV negative (n) = 3, HIV status unknown = 28

b: Not tested for HIV RNA by PCR



### 5.2.1.2 Neonatal pneumonia and detection of *M. hominis*, *C. trachomatis* and *Ureaplasma spp.*

Sixty-nine endotracheal aspirate samples from neonates were obtained from the Neonatal Intensive Care Unit (NICU) at Dora Nginza Hospital. Ages ranged from 1-19 days with the majority of neonates in the study being in the 1-5 days old group [Fig. 5.10 (A)] from nine suburbs surrounding Dora Nginza hospital [Fig. 5.10 (B)].



**Figure 5.2:** (A) Age groups of neonates presenting with pneumonia.  
(B) Suburbs in Port Elizabeth from which samples were obtained.

*M. hominis* and *C. trachomatis* were not detected in any of the neonate endotracheal aspirate specimens. Two specimens were positive for *U. parvum* using the UPA and UPS primers. These results were verified with repetition of PCR tests and products were sequence confirmed. Attempts to culture the two positive *U. parvum* neonate specimens on U9 broth and conventional broth (ATCC #1331 and #2616) were unsuccessful. The two endotracheal aspirate samples that were positive for *U. parvum* were negative for *U. urealyticum*. Mothers of the two neonates resided in the Uitenhage and Humansdorp suburbs which were regionally about 150 km apart.

### 5.2.2 Detection of *M. pneumoniae*

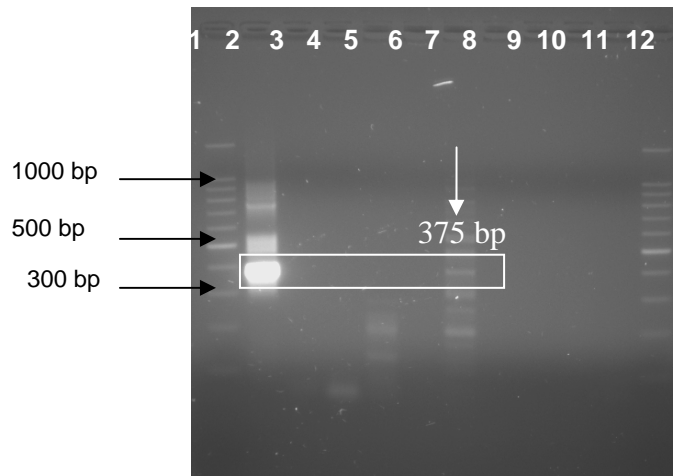
*M. pneumoniae* detection (refer to sections 2.7.2.2 and 2.8) was based on PCR using P1 adhesin and 16S rRNA primers. PCR products in the range of 375 bp, with the P1 adhesin gene, and 277 bp using 16S rRNA primers, for *M. pneumoniae* ATCC 29343 are shown in Figure 5.3. *M. pneumoniae* was detected in only one

sample by both 16S rRNA (results not shown) and P1 adhesin PCR (Fig. 5.3), however culture attempts failed (refer to section 3.2.6). The concentration of *M. pneumoniae* DNA from the clinical sample was very low and repeated DNA extraction and PCR using both P1 adhesin and 16S rRNA primers failed to provide a product that could be forwarded for DNA sequence confirmation. Unfortunately a second specimen could not be obtained, while HIV status and other important clinical data were also not available for this particular patient.

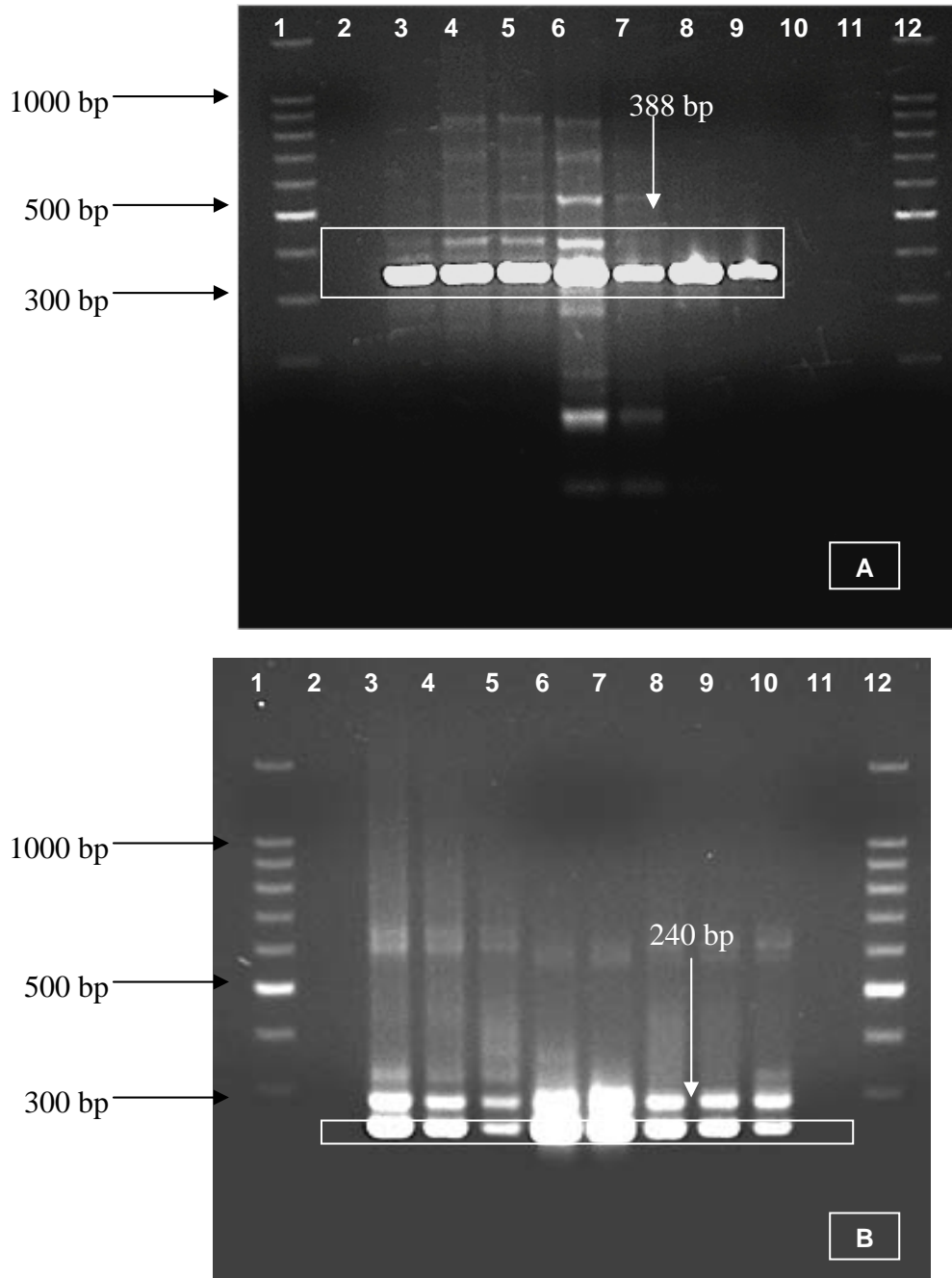
### 5.2.3 Detection of *P. jiroveci* and DHPS/DHFR gene analysis

For most sputa specimens (refer to section 2.2) using mtLSUrRNA primers a distinct 388 bp DNA fragment, was obtained from the first round of PCR amplification [Fig. 5.4(A)] while a 240 bp fragment was produced in the second round of PCR [Fig. 5.4 (B)].

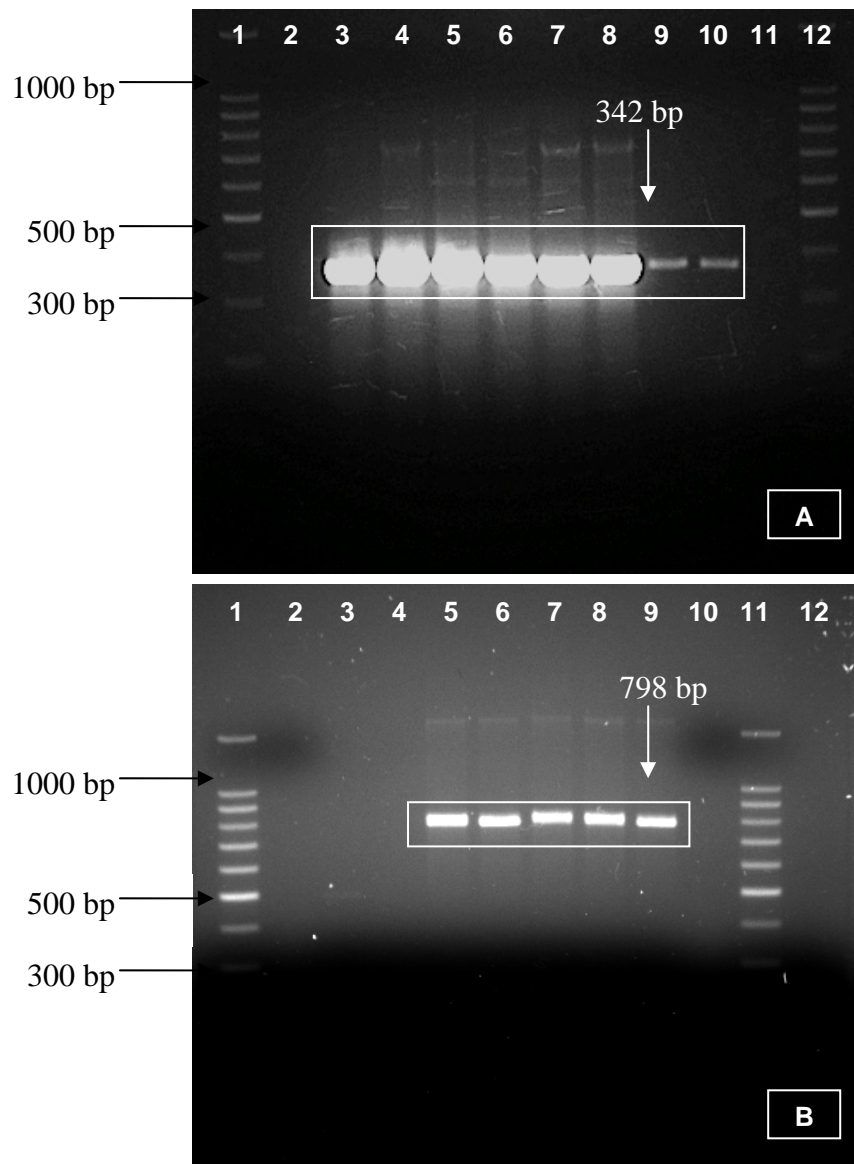
DHPS nested PCR generated a 342 bp fragment [Fig. 5.5 (A)], while PCR amplification using DHFR-specific primers, yielded a fragment of 798 bp [Fig. 5.5 (B)]. DHFR gene PCR did not produce bands for all samples even though they were positive by DHPS or mtSURRNA as has been reported previously by Robberts *et al.*, (2007).



**Figure 5.3:** Detection of *M. pneumoniae* employing P1 adhesin gene primers. Amplified fragments of 375 bp. Lanes 1 and 12: Molecular weight markers [100 bp DNA ladder (Promega)]; Lanes 3 and 11: Negative controls; Lanes 4 – 10: Sputa samples; Lane 2: Positive *M. pneumoniae* ATCC 29343 control.



**Figure 5.4:** Detection of *P. jirovecii* employing nested mtLSUrRNA PCR. (A) First round mtLSUrRNA PCR showing amplified fragments of 388 bp. Lanes 1 and 12: Molecular weight markers [100 bp DNA ladder (Promega)]; Lanes 2, 10 and 11: Negative controls; Lanes 3 – 8: Sputa samples (1-6); Lane 9: Positive control (lung biopsy – PcP positive) and (B) Second round mtLSUrRNA PCR showing amplified fragments of 240 bp. Lanes 1 and 12: Molecular weight markers [100 bp DNA ladder (Promega)]; Lanes 2 and 11: Negative controls; Lanes 3 – 9: Sputa samples (1-7); Lane 10: Positive control (*P. jirovecii* detected from a lung biopsy).



**Figure 5.5:** *P. jiroveci* DHPS and DHFR gene amplification.

(A) Amplified fragments of 342 bp. Lanes 1 and 12: Molecular weight markers [100 bp DNA ladder (Promega)]; Lanes 2 and 11: Negative controls; Lanes 3 – 9: Sputa samples (1-7); Lane 10: Positive control (lung biopsy - 1 in 2 dilution). (B) Amplified fragments of 798 bp. Lanes 1 and 11: Molecular weight markers [100 bp DNA ladder (Promega)]; Lanes 2 and 10: Negative controls; Lanes 3 – 8: Sputa samples (1-6); Lane 9: Positive control (*P. jiroveci* detected from a lung biopsy).

*P. jiroveci* sequencing results on mtLSUrRNA from specimens were compared with the wild type sequence of a 295 bp portion (GenBank accession no: M58605) of the mtLSUrRNA gene from *P. jiroveci* (Sinclair *et al.*, 1991) [Fig. 5.6]. The 20 amplification products from the DHPS nested PCR method were sequenced, and found to be identical to a known DHPS gene sequence (GenBank accession no: U66279) [Fig. 5.7] (Lane *et al.*, 1997). Alignments of DHFR sequences from 17 PCR products were all identical to a DHFR gene (GenBank accession no: AF090368) [Fig. 5.8] (Ma *et al.*, 1999).

	1	10	20	30
Specimen	A	A	T	T
Sinclair et al., (1991)	A	A	T	T
	56	65	75	85
Specimen	A	A	T	T
Sinclair et al., (1991)	A	A	T	T
	40	50	60	70
Specimen	T	A	G	G
Sinclair et al., (1991)	T	A	G	G
	95	105	115	125
Specimen	A	T	T	T
Sinclair et al., (1991)	A	T	T	T
	80	90	100	110
Specimen	A	T	T	T
Sinclair et al., (1991)	A	T	T	T
	135	145	155	165
Specimen	T	A	A	A
Sinclair et al., (1991)	T	A	A	A
	120	130	140	150
Specimen	T	A	A	A
Sinclair et al., (1991)	T	A	A	A
	175	185	195	205
Specimen	G	T	C	G
Sinclair et al., (1991)	G	T	C	G
	160	170	180	190
Specimen	G	T	C	G
Sinclair et al., (1991)	G	T	C	G
	215	225	235	245
Specimen	C	C	C	A
Sinclair et al., (1991)	C	C	C	A
	200	210	220	228
Specimen	C	C	C	A
Sinclair et al., (1991)	C	C	C	A
	255	265	275	283

**Figure 5.6:** Sequence of the amplified region of mtLSUrRNA from sputum specimens compared to *P. jiroveci* DNA sequence (GenBank accession no: M58605) (Sinclair *et al.*, 1991).

	1	10	20	30	40
Specimen	CTGATTC	TTTTTCGAT	GGGGGTG	TCATTCAT	ATGATTCT
Lane et al., (1997)	CTGATTC	TTTTTCGAT	GGGGGTG	TCATTCAT	ATGATTCT
	50	60	70	80	
Specimen	ATATTAAT	TGGATGT	GGAGAAT	TTTATAAA	TGCAGGGGCGAC
Lane et al., (1997)	ATATTAAT	TGGATGT	GGAGAAT	TTTATAAA	TGCAGGGGCGAC
	90	100	110	120	
Specimen	GATAATT	GATATTGGT	GGGCAGT	CTACACGGC	CTGGTTCCAC
Lane et al., (1997)	GATAATT	GATATTGGT	GGGCAGT	CTACACGGC	CTGGTTCCAC
	130	140	150	160	
Specimen	ATGTTGT	TTCTATAG	AGGAAGAG	ATTTCTCG	AGTTATTTCCCT
Lane et al., (1997)	ATGTTGT	TTCTATAG	AGGAAGAG	ATTTCTCG	AGTTATTTCCCT
	170	180	190	200	
Specimen	GCTATAAAA	TATCTCTT	AAAAGTAT	ATCCTGAT	ATTTTAGT
Lane et al., (1997)	GCTATAAAA	TATCTCTT	AAAAGTAT	ATCCTGAT	ATTTTAGT
	210	220	230	240	
Specimen	AAGTG	TAGATACT	TTTTCGTT	CTGAGGTT	GCAGAACAAAGCAA
Lane et al., (1997)	AAGTG	TAGATACT	TTTTCGTT	CTGAGGTT	GCAGAACAAAGCAA
	250	260	270	280	
Specimen	TTAAGGCT	TGGTGCT	AGTCTTGTT	AATGATA	TAAGTGGGGGA
Lane et al., (1997)	TTAAGGCT	TGGTGCT	AGTCTTGTT	AATGATA	TAAGTGGGGGA
	290	300	310	320	322
Specimen	AGGTATGAT	CCAAAAAT	GCTTAAT	TGTGGTT	GCCAA
Lane et al., (1997)	AGGTATGAT	CCAAAAAT	GCTTAAT	TGTGGTT	GCCAA

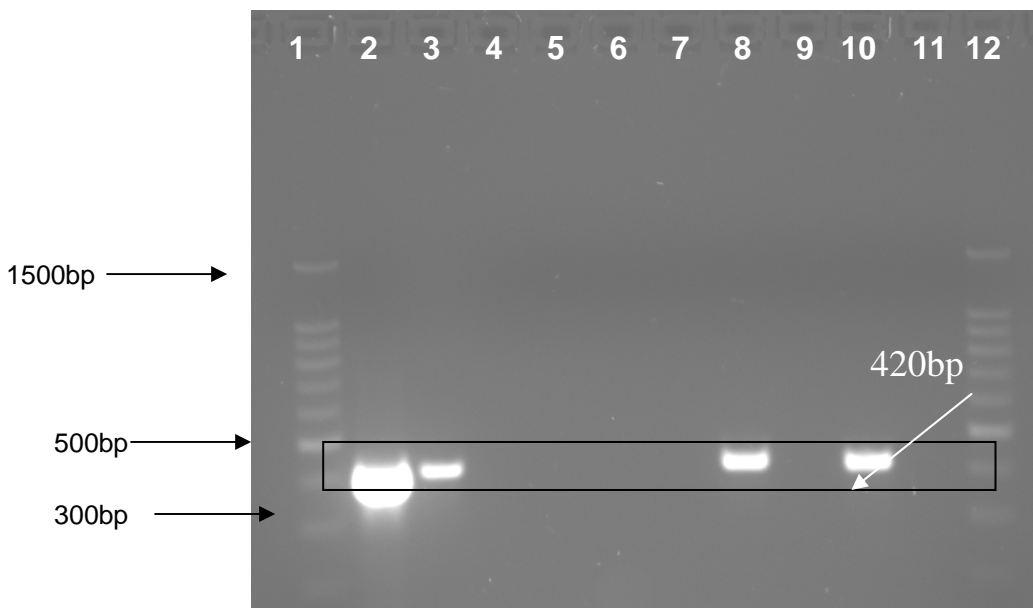
**Figure 5.7:** Sequence of a 322 bp amplified region of the DHPS gene from sputum specimens compared to *P. jiroveci* DNA sequence (GenBank accession no: U66279) (Lane *et al.*, 1997).

	1	10	20	30	40	50	60	70
Specimen	1	10	20	30	40	50	60	70
Ma et al., (1999)	790	799	809	819	829	839	849	859
Specimen	80	90	100	110	120	130	140	
Ma et al., (1999)	80	90	100	110	120	130	140	
Specimen	869	879	889	899	909	919	929	
Ma et al., (1999)	150	160	170	180	190	200	210	
Specimen	150	160	170	180	190	200	210	
Specimen	939	949	959	969	979	989	999	
Ma et al., (1999)	220	230	240	250	260	270	280	
Specimen	220	230	240	250	260	270	280	
Ma et al., (1999)	1,009	1,019	1,029	1,039	1,049	1,059	1,069	
Specimen	290	300	310	320	330	340	350	
Ma et al., (1999)	290	300	310	320	330	340	350	
Specimen	1,079	1,089	1,099	1,109	1,119	1,129	1,139	
Ma et al., (1999)	360	370	380	390	400	410	420	
Specimen	360	370	380	390	400	410	420	
Ma et al., (1999)	1,149	1,159	1,169	1,179	1,189	1,199	1,209	
Specimen	430	440	450	460	470	480	490	
Ma et al., (1999)	430	440	450	460	470	480	490	
Specimen	1,219	1,229	1,239	1,249	1,259	1,269	1,279	
Ma et al., (1999)	500	510	520	530	540	550	560	
Specimen	500	510	520	530	540	550	560	
Ma et al., (1999)	1,289	1,299	1,309	1,319	1,329	1,339	1,349	
Specimen	570	580	590	600	610	620	630	
Ma et al., (1999)	570	580	590	600	610	620	630	
Specimen	1,359	1,369	1,379	1,389	1,399	1,409	1,419	
Ma et al., (1999)	640	650	660	670	680	690	700	
Specimen	640	650	660	670	680	690	700	
Ma et al., (1999)	1,429	1,439	1,449	1,459	1,469	1,479	1,489	
Specimen	710	720	730	740	749			
Ma et al., (1999)	710	720	730	740	749			
Specimen	1,499	1,509	1,519	1,529	1,538			
Ma et al., (1999)								

**Figure 5.8:** Sequence of a 749 bp amplified DNA region of the DHFR gene from sputum specimens compared to *P. jiroveci* (GenBank accession no: AF090368) (Ma et al., 1999).

#### 5.2.4 Detection of *U. parvum* and *U. urealyticum* and sequence confirmation

PCR products (Fig. 5.9) obtained from two endotracheal aspirates (out of a total of 69 specimens) were sequenced (Fig. 5.10) and comparisons made with the 5 696 bp urease gene of *U. urealyticum* and *U. parvum* (GenBank accession number AF085729) (Biernat-Sudolska *et al.* 2006; Kong *et al.* 2000; Kong *et al.*, 1999).



**Figure 5.9:** PCR detection of *U. parvum* using primers UPA and UPS. Neonate specimens: Lanes 1 and 12: Molecular weight marker (Promega 100 bp DNA ladder); Lane 2: Positive *U. parvum* ATCC 2616 control; Lane 3: 1:10 dilution of the positive control; Lanes 4 and 11: Negative controls; Lanes 5 – 7, 9: *U. parvum* negative endotracheal aspirate samples 49 – 51, 53; Lanes 8 and 10: *U. parvum* positive endotracheal aspirate samples 52 and 54.



Kong <i>et al.</i> Specimen 52	GCCGAAATTGTGATGAACGAAGGTAGAGAAGCAAAGTAATCAGCATTAAAA GGTGAAATTGTGATGAATGAAGGTAGAGAGGCAAAGTAATTAGTATTAAAA	612
Kong <i>et al.</i> Specimen 52	ATACTGGTGACCGTCCTATCCAAGTTGGATCACATTTCCACTTATTGAAAC ATACTGGGGACCGTCCTATACAAGTTGGATCACATTTTCACTTGTGTTGAAGT	664
Kong <i>et al.</i> Specimen 52	AAATAGGTCATTAGTATTC TTGATGAAAAAGGAAACGAAGACAAAGAACGT GAATAGTGCATTAGTATTT TTGATGAAAAAGGAAATGAAGATAAAGAACGC	716
Kong <i>et al.</i> Specimen 52	AAAGTTGCTTATGGACGTCGTTTCGATATTCCATCAGGTA CTGCTATTTCGTTT AAAGTTGCTTATGGACGACGTTTCGATATTCCATCAGGTA CTGCTATTTCGTTT	768
Kong <i>et al.</i> Specimen 52	TGAACCAGGAGACAAAAAAGAAGTTTCAGTTATTGATTTAGTCGGAACACGT TGAACCAGGAGATAAAAAAAGAAGTTTCAATTATTGATTTAGCCGGAACACGC	820
Kong <i>et al.</i> Specimen 52	GAAGTTGAGGTGTAAACGGCTTAGTTAACGGAAAACCTTAAAAAATAA GAAGCTTGAGGTGTAAATGGCTTAGTTAATGGAAAACCTTAAAAAATAA	872
-----		
Kong <i>et al.</i> Specimen 54	GCCGAAATTGTGATGAACGAAGGTAGAGAAGCAAAGTAATCAGCATTAAAA GGTGAAATTGTGATGAATGAAGGTAGAGAGGCAAAGTAATTAGTATTAAAA	612
Kong <i>et al.</i> Specimen 54	ATACTGGTGACCGTCCTATCCAAGTTGGATCACATTTCCACTTATTGAAAC ATACTGGGGACCGTCCTATACAAGTTGGATCACATTTTCACTTGTGTTGAAGT	664
Kong <i>et al.</i> Specimen 54	AAATAGTGCATTAGTATTC TTGATGAAAAAGGAAACGAAGACAAAGAACGT GAATAGTGCATTAGTATTT TTGATGAAAAAGGAAATGAAGATAAAGAACGC	716
Kong <i>et al.</i> Specimen 54	AAAGTTGCTTATGGACGTCGTTTCGATATTCCATCAGGTA CTGCTATTTCGTTT AAAGTTGCTTATGGACGACGTTTCGATATTCCATCAGGTA CTGCTATTTCGTTT	768
Kong <i>et al.</i> Specimen 54	TGAACCAGGAGACAAAAAAGAAGTTTCAGTTATTGATTTAGTCGGAACACGT TGAACCAGGAGATAAAAAAAGAAGTTTCAATTATTGATTTAGCCGGAACACGC	820
Kong <i>et al.</i> Specimen 54	GAAGTTTGAGGTGTAAACGGCTTAGTTAACGGAAAACCTTAAAAAATAA GAAGTTTGAGGTGTAAATGGCTTAGTTAATGGAAAACCTTAAAAAATAA	872

**Figure 5.10:** Sequence comparisons of PCR products derived from specimens 52 and 54 with the urease gene (UreA – UreB spacer region; Kong *et al.*, 1999) from *U. parvum*. Shaded areas show point mutations that may indicate different strains of *U. parvum*.

### 5.3 DISCUSSION

The standard diagnostic procedures employed at Port Elizabeth's local hospitals involved determining the HIV and TB status of a patient, using microscopy, chest X-rays and ELISA tests on collected sputum and blood samples. Upon completion of these tests, a second sputum sample may have been requested by doctors if PcP was suspected, based on clinical signs and symptoms, (such as a fever, non-productive cough, shortness of breath), CD<sub>4</sub> count, response to treatment and other data recorded in the patient's file. The clinical detection protocol carried out at NHLS in Port Elizabeth made use of cytochemical staining procedures, for the visualisation of cysts and trophozoites of *P. jiroveci*. All sputa samples submitted to NMMU, Microbiology for molecular detection of *P. jiroveci* were based on patient history and clinical observation of PcP symptoms by the resident physician at the ward.

The patients who participated in the study resided in predominantly poor, rural areas where medical assistance and resources are not widely available. Statistics have shown that the spread of HIV and AIDS related opportunistic infections are favoured due to the community lifestyle and living conditions within these areas, with reports in Port Elizabeth of two to three AIDS related deaths occurring per day ([www.toolkitparticipation.nl/cases/87](http://www.toolkitparticipation.nl/cases/87)). Rama, (2005) showed that New Brighton, Kwazakhele, Motherwell, Zwide, Soweto-on-Sea and Veeplaas accounted for approximately 66% of the HIV-positive population in the Nelson Mandela Metropolitan area, whereas areas such as Bethelsdorp, Kwadwesi and Missionvale housed only 5.38% of the HIV-positive population.

No samples were collected during March and April of 2007, due to serious staff shortages experienced at both provincial hospitals as a result of the national nurse's strike. During this time important patient information was also lost. Unfortunately, these are just some of the continuous challenges that this study and clinical microbiology research faces within the Port Elizabeth region. Seasonal variations in the number of new *P. jiroveci* infections could be as a direct result of an increase during the colder months, or could be as a result of other respiratory tract infections, present in addition to *P. jiroveci* (Varela *et al.*, 2004).

Generalised observations made by hospital personnel concerning the surrounding communities indicated that a) the female population was over-worked and undernourished, and if presenting to hospitals for treatment were in most instances immunosuppressed, with living conditions and lifestyles favouring the spread of opportunistic infections and b) men from the region tended not to access clinic or hospital services (Govender *et al.*, 2008).

Of the 102 patients for whom gender information was available, 85 were female, while 17 were male, whether this is indicative of men in the region just not approaching health care facilities or a lower incidence of pneumonia in males is not clear. The majority of pneumonia cases were seen in the 21-40 year age group, comprising 73 patients, with *P. jiroveci* detected in 40 patients, which is consistent with the demographics of the HIV/AIDS epidemic in South Africa ([www.avert.org/safricastats.htm](http://www.avert.org/safricastats.htm)). The high prevalence of *P. jiroveci* in women (46/85) was reflective of their high risk profile, a young female (12 years) in which *P. jiroveci* was present was HIV positive.

Although closely linked with HIV infection, *P. jiroveci* was not necessarily co-associated with *M. tuberculosis*. Fisk *et al.* (2003), on studying co-infection rates associated with PcP in patients with AIDS in developing countries, found co-infection with *M. tuberculosis* in 13-66% of cases. The finding of HIV positive and *P. jiroveci* negative patients (21%) could suggest a) that in these immunocompromised patients' the immune system had not deteriorated to the extent that placed them at risk for PcP, b) non-exposure or c) prophylactic treatment may have been given. Incomplete patient records/data bases not only compromised interpretation of results but would also hamper community follow-ups and management of family situations.

Treatment protocols for diabetes, epilepsy, asthma and meningitis all rely on the use of extreme drugs and therapies that regularly lead to immunosuppression of the host. A compromised host therefore has a higher risk of acquiring a *P. jiroveci* infection (Prescott *et al.*, 2007). In this study the three patients with underlying conditions of asthma, diabetes and epilepsy were in addition to being HIV-positive also colonised with *P. jiroveci*. It should also be noted that information on one patient

showed that in addition to having PcP, the patient was also being treated for a *Klebsiella pneumoniae* infection.

Of the total samples tested for which the HIV status was known (n=74), two were HIV-negative but positive for *P. jiroveci* (there was no information available on other reasons for immunosuppression or underlying conditions). The study conducted by Robberts, (2005) involved the collection of 636 sputa samples from different regions in South Africa (KwaZulu Natal, Western Cape, Eastern Cape, North West province and Mpumalanga). Of the 148 sputa from the Eastern Cape, five *P. jiroveci* positive results were recorded from HIV-negative patients. In other regions there was a significant association between a positive HIV status and PcP. In Sweden, Mikaelsson *et al.*, (2006) also found that the prevalence of PcP in HIV negative patients had increased.

*P. jiroveci* was detected in 54 of 102 (52.9%) sputum samples, using the mtLSUrRNA nested PCR protocol. According to Robberts *et al.*, (2007) the mtLSUrRNA nested PCR protocol, employed against South African isolates yielded high levels of sensitivity (78%) and specificity (100%). The success of this method is thought to be as a result of it targeting a multi-copy gene, which enhances sensitivity and allows the detection of very low numbers of organisms (Morris *et al.*, 2008; Robberts *et al.*, 2007; Sing *et al.*, 2000 and Torres *et al.*, 2000). The early identification of *P. jiroveci* colonisation is important because: (1) undetected *Pneumocystis* colonisation increases the patient's risk of pneumonia; (2) transmission of the infection to other hosts may occur and (3) latent infections could lead to permanent lung damage (Morris *et al.*, 2005). However, PCR reactions cannot differentiate between viable and non-viable cells (de Oliveira *et al.*, 2007).

Although vastly limited for diagnosis, DHPS and DHFR gene analysis is of major importance for monitoring TMP-SMX resistance development. In this study, no resistance associated mutations were detected in the PCR products of 20 DHPS and 17 DHFR genes. Crewe-Brown *et al.* (2004), reported TMP-SMX-resistant South African isolates had begun to increase at a steady rate from 2000 to 2004. A high prevalence of DHPS mutations were found in the Gauteng Province (38%), while Robberts *et al.*, (2007) and Zar *et al.*, (2004) both showed lower prevalence rates in

DHPS mutations, 1.9% and 13.3% respectively, within the Western Cape. Whilst no resistance mutations to TMP-SMX were demonstrated in the current study, the use of TMP-SMX for PcP prophylaxis has been progressively increasing country wide since 2007. Resistance monitoring should be maintained as PcP prophylaxis to South African patients in the past, has not been well documented (Dini *et al.*, 2006).

*M. pneumoniae* was detected in one specimen. The P1 adhesin primers were found to be more sensitive than the 16S rRNA primers, with the most likely rationale for this occurrence being the high copy number of this gene within the genome of *M. pneumoniae* (Ieven *et al.*, 1996). The prevalence of *M. pneumoniae* within HIV infected adults can range from 1.9 to 30% (Hammerschlag, 2001) although a study by Loens *et al.* (2003) concluded that *M. pneumoniae* did not necessarily appear to be responsible for lower respiratory tract infections in HIV-positive adults and children. It has been suggested that the low prevalence/detection of *M. pneumoniae* may be due to the low concentration of *M. pneumoniae* within samples (Nour *et al.*, 2005).

Highly active antiretroviral therapy is changing the epidemiology of *P. jiroveci* pneumonia in AIDS patients, as globally the incidence of PcP has been seen to be decreasing, and PcP is now mostly prevalent in AIDS patients who are not receiving HAART or those on severe immune suppressive treatments (Bahamondes *et al.* 2006). The recent surge in ARV roll out in South Africa may well similarly reduce the prevalence of *P. jiroveci* in AIDS patients and complex prophylactic management, in that cotrimoxazole could well be administered unnecessarily. It was evident in this study and reported in a community-acquired pneumonia study in Limpopo that without the introduction of rapid microbial diagnostics the mainstay of treatment remains empiric, based on South African guideline approaches (Mpe *et al.*, 2005). Empiric treatment regimens, however, do not promote identification and susceptibility testing of causative organisms and could well perpetuate the prevalence of atypical/less severe pneumonias in the community (Govender *et al.*, 2008). The first line antibiotic administered to patients with pneumonia in the Port Elizabeth hospitals was/is a second generation cephalosporin, cefuroxime. As cefuroxime is not effective against *P. jiroveci* and *M. pneumoniae*; in the absence of a definitive *P. jiroveci* diagnosis, co-trimoxazole prophylaxis was also administered. It is important

that clinicians monitor a patient's progress carefully for cases that are not responding to treatment, e.g. *M. pneumoniae* where a macrolide/ketolide or quinolone should be considered (Govender *et al.*, 2008).

The neonate endotracheal aspirate specimens obtained from the hospital were of a poor quality (i.e. some were contaminated with blood). Due to the very small volume/amount available, the specimens had to be flushed out of the endotracheal tubes with sterile water which caused dilution of the sample material from which DNA was extracted. Due to circumstances at the hospital (as described in section 3.3), it was not possible for specimens to be transferred onto transport media prior to collection and delivery to NMMU for analysis which may have contributed to negative culture results. Unfortunately and certainly for the two neonates in which *U. parvum* was detected, all attempts to obtain information from patient files at the Dora Ngizwa (NICU) on co-infections like HIV, TB, treatment or the type of delivery were unsuccessful. This type of epidemiological study at a provincial hospital in Port Elizabeth was the first of its kind and revealed a total lack of combined approaches within the hospital to address community microbial health issues. It should also be added that staffing conditions at the hospital were quite atrocious; one of the nurses who assisted with the adult study contracted XDR-TB and passed away during the project.

Despite the limitations of hospital records and patient follow-up, new information obtained from this under research region were a) *U. parvum* was demonstrated in two neonates with *M. pneumoniae* detected in one adult patient, and b) *M. pneumoniae* was not seen to be associated with HIV, TB or *P. jiroveci*. In the absence of laboratory diagnoses and other microbial indicators, mycoplasmas and ureaplasmas will remain persistent under the radar and even perpetuate in communities.

## CHAPTER SIX

### ANTIBIOTIC RESISTANCE GENES OF UREAPLASMAS AND INTERGENERIC TRANSFER POTENTIAL

#### 6.1 INTRODUCTION

Antibiotic resistance in addition to ureaplasmas' intrinsic resistance to  $\beta$ -lactams/cephalosporins and clindamycin can occur either by mutation or acquisition of new genes carried by transposons (Bebear and Kempf, 2005). Resistance to macrolides by drug inactivation, active efflux or methylation of the target site have as yet not been reported in ureaplasmas (Pereye *et al.*, 2007a). Mutations in the 23S rRNA or in ribosomal protein L4 and L22 are thought to be associated with macrolide resistance development in *Ureaplasma* spp. (Pereye *et al.*, 2007a). Fluoroquinolone resistance in *Ureaplasma* spp. is associated with mutations in target enzymes DNA gyrase and topoisomerase IV (Bebear *et al.*, 2003). Transposons carrying tetracycline resistance genes (*tetM*) have been recognised in ureaplasmas (Roberts and Kenny, 1986; de Barbeyrac *et al.*, 1996).

As natural exchange of chromosomal DNA between cells of mycoplasmas has only been proposed by Teachman *et al.*, (2002), it is thought that mycoplasmas/ureaplasmas probably do not acquire DNA by conventional transformation processes. The lack of a cell wall in mycoplasmas/ureaplasmas would be expected to facilitate the introduction of exogenous DNA into the cells that may well involve transient fusion of the cell membranes at the zone of contact (Razin *et al.*, 1998). The very small genomes of mycoplasmas/ureaplasmas lack a substantial part of standard recombination mechanisms available in larger bacterial genomes, however, they do contain large numbers of repeat DNA sequences considered vital for adaptation and chronic colonisation of their hosts (Rocha *et al.*, 2005).

Artificial transformation using a polyethylene glycol (PEG) mediated procedure similar to that used to transform Gram-positive bacteria has been used to transform

*M. pulmonis*, *M. mycoides*, *M. capricolum* and *M. gallisepticum*. Electroporation has been used to transform *M. pneumoniae* (Hedreyda *et al.*, 1993), *M. gallisepticum* and *M. mycoides*, but has been ineffective for species such as *M. pulmonis* (Voelker and Dybvig, 1996). Although mycoplasmal phages are known to exist, transduction has not been described. Several mycoplasmas have been shown to acquire the conjugative transposon *Tn916* by mating with an enterococcal donor, but conjugal transfer of any genetic element including *Tn916* from a mycoplasmal donor has not been described. Circumstantial evidence suggests that horizontal gene exchange has occurred between species of *Mycoplasma* with the *IS1221* insertion sequence having been found in *M. hyorhinae*, *M. hyopneumoniae* and *M. flocculare* (Teachman *et al.*, 2002).

It has been reported that high-level tetracycline resistance in *Neisseria gonorrhoeae* resulted from the acquisition of a streptococcal *tetM* determinant found in streptococci/enterococci, *M. hominis* and *U. urealyticum* (Morse *et al.*, 1986; Roberts and Kenny, 1986; Roberts and Kenny, 1987). It is therefore important to investigate whether *Ureaplasma* spp. can act as tetracycline resistance gene pools with the potential to transfer resistance to each other (as population shifts are evident) and to urogenital pathogens especially *N. gonorrhoeae* which is known to be naturally competent and transformable.

The study was designed to determine ureaplasma antibiotic susceptibility profiles and analyse resistance genes/mutations associated with antibiotic resistance development. The transfer potential of tetracycline resistance genes between ureaplasmas and *N. gonorrhoeae* was investigated.

## **6.2 RESULTS**

### **6.2.1 Antimicrobial susceptibility profiling**

Endocervical specimens (132) were cultured from which growth was attained from 66 samples (35 *U. parvum*, 9 *U. urealyticum*, 22 *U. parvum* + *U. urealyticum*) (Table 6.1). Ureaplasma MIC determinations to ofloxacin, erythromycin, tetracycline, doxycycline, azithromycin and josamycin were performed in accordance with recent



recommendations by the The Mycoplasma Chemotherapy Working Team of the IOM. MIC ranges and breakpoints were based on data compiled from multiple published studies (Waites and Duffy, 2008) as there is no CLSI breakpoint information. Throughout procedures, culture from specimens and MIC determinations, ureaplasma broth cultures were checked for contamination. All broth cultures were clear and no microbial growth was detected on chocolate agar plates incubated in 8-10% CO<sub>2</sub> at 37°C for 48 h. Thirty-seven ureaplasma cultures (56.1%) were fully susceptible to all antibiotics tested. Twenty-one cultures (31.8%) showed intermediate resistance to erythromycin, azithromycin and ofloxacin while seven (10.6%) were resistant to tetracycline, three of which were also resistant to doxycycline (Table 6.2). Two cultures (including a tetracycline-resistant strain), were seen to exhibit azithromycin resistance. The cultures showing reduced antimicrobial susceptibilities were species confirmed by PCR and further investigated for genetic mutations in drug target regions or gene acquisition.

**Table 6.1:** Detection of *Ureaplasma* spp.

	No. of specimens producing ureaplasma cultures			
	<i>U. parvum</i>	<i>U. urealyticum</i>	<i>U. parvum</i> + <i>U. urealyticum</i>	Total (n = 191)*
PCR Positive	62	14	56	132
PCR Negative				59
Culture Positive from PCR positive	35	9	22	66

\*= Total number of specimens analysed

**Table 6.2:** Antibiotic profiles of *Ureaplasma* spp.

Antibiotic profiles	<i>U. parvum</i>	<i>U. urealyticum</i>	<i>U. parvum</i> + <i>U. urealyticum</i>	Total (n = 66)*
<b>Susceptible</b>	16	1	20	<b>37</b>
<b>Intermediate (I)</b>	12	6	3	<b>21</b>
OFX	4	3		7
E			1	1
OFX + E	6	3	2	11
OFX + E + AZM	2			2
<b>Resistant</b>	7	1		<b>8</b>
TET	3			3
TET + DOX	1	1		2
TET + AZM + E (I)	1			1
AZM + E (I)	1			1
DOX + TET + OFX (I)	1			1

\*= Total number of culture positive specimens. OFX: ofloxacin; E: erythromycin; AZM: azithromycin; TET: tetracycline; DOX: doxycycline; (I): intermediate

## 6.2.2 Target Gene Mutations in Antibiotic -Intermediate/ -Resistant Strains

### 6.2.2.1 Quinolone resistance determining region (QRDR): PCR amplification and sequence analysis

The *gyrA*, *gyrB*, *parC* and *parE* genes were amplified and sequenced to determine whether there were any mutations in one ofloxacin-intermediate resistant *U. urealyticum* and six *U. parvum* strains.

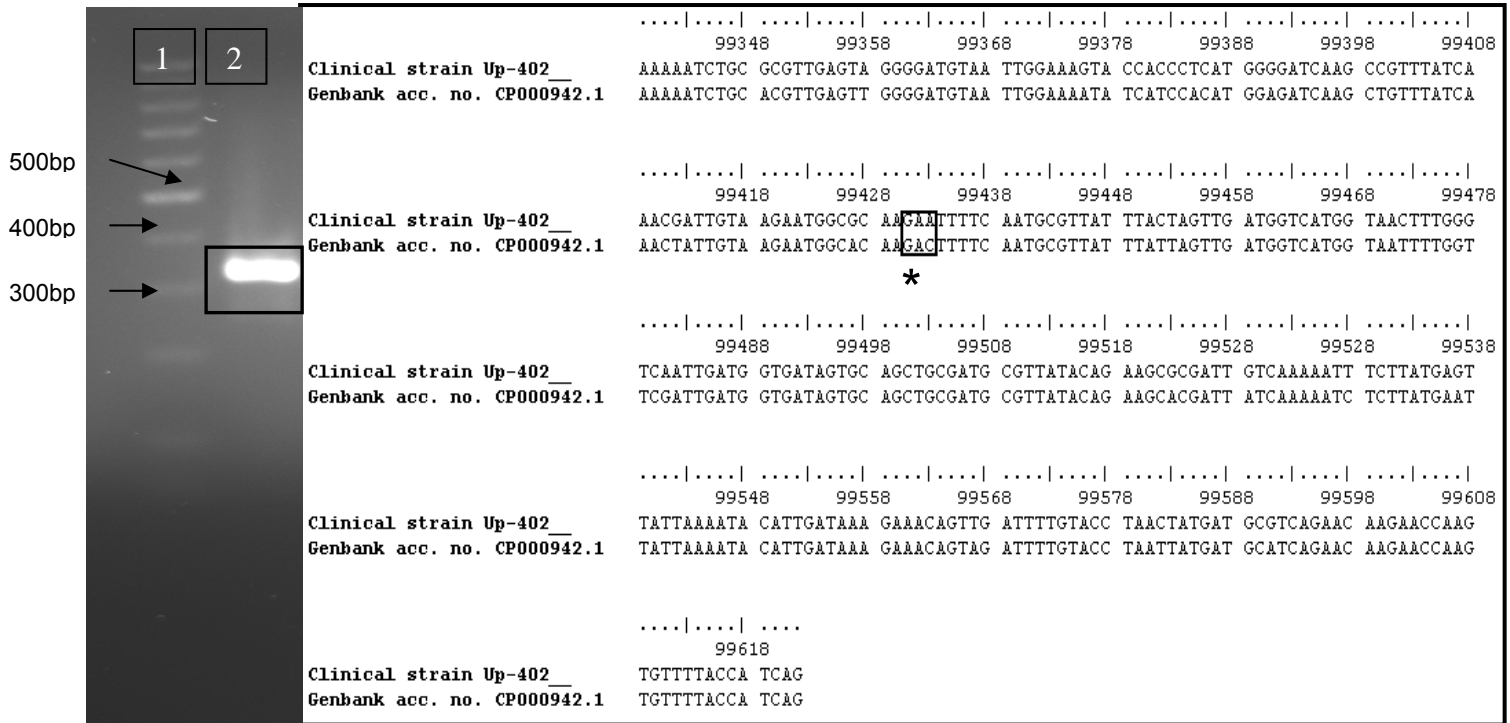
Amplification of the *gyrA* gene produced a 320 bp fragment, while *gyrB*, *parC* and *parE* genes produced 300 bp fragments. Sequence analyses were performed by BLAST software comparisons against the amplified sequence of *U. parvum*; Serovar 3, ATCC 27815 (GenBank accession number CP000942.1). PCR amplification and sequence alignments revealed that there were no mutations in *gyrB* and *parE* genes of strains under investigation, however mutations were observed in *gyrA* and *parC* genes (Figs. 6.1 and 6.2, Table 6.3). A substitution of Ser83Leu in ParC was demonstrated in one intermediately-resistant strain (MIC 4 µg/ml) whilst a triple substitution of Asp112Glu in GyrA along with Ala125Thr and Ala136Thr in ParC was

found in six further intermediately-resistant strains. No background mutations were found in any cultures with MICs 1 µg/ml.

**Table 6.3:** Ofloxacin MICs with *gyrA* and *parC* sequence analysis

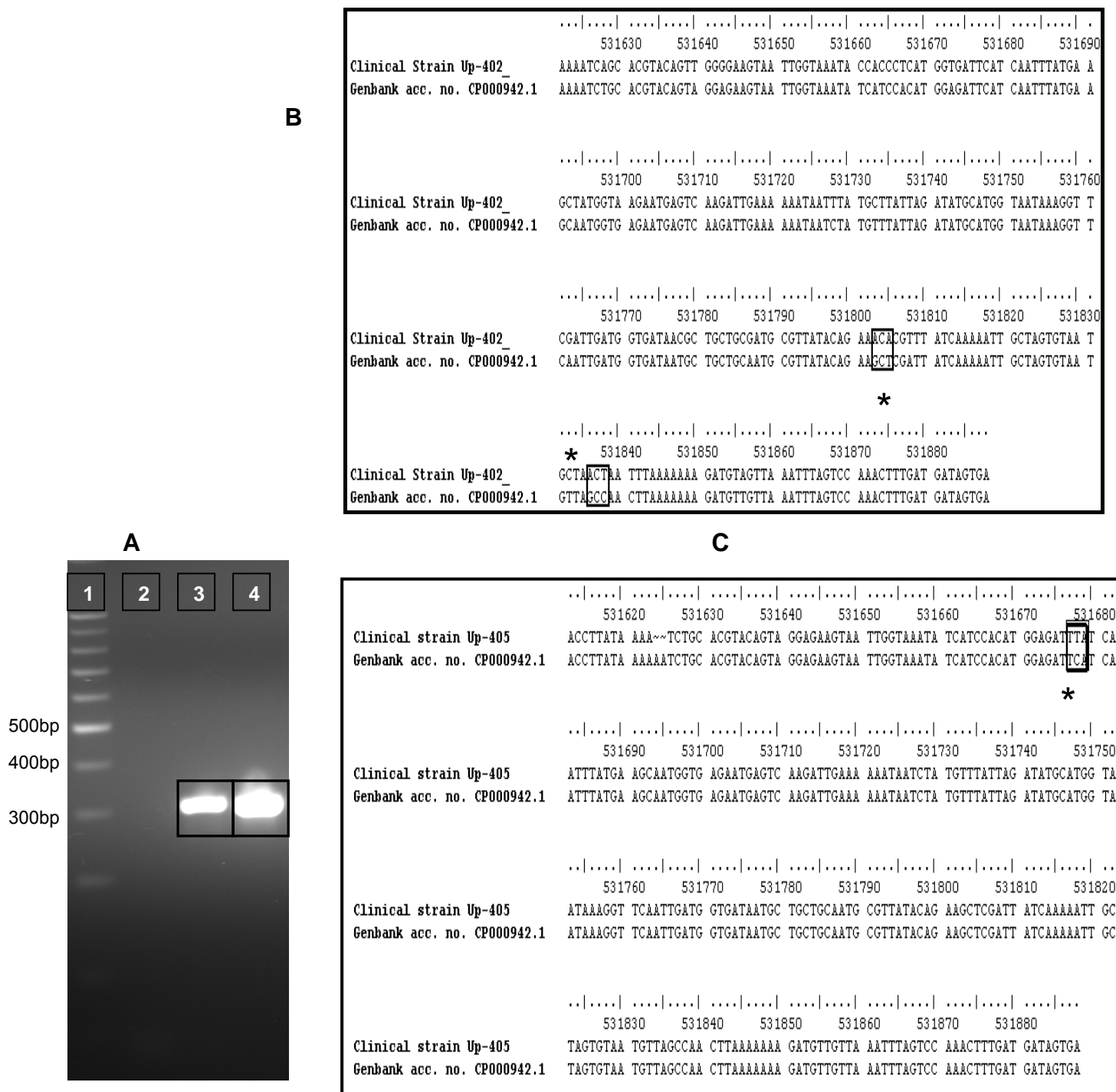
Strain	Amino acid change in QRDR		MIC with ATCC Broth (µg/ml)
	<i>gyrA</i>	<i>parC</i>	
Up-367	Asp112Glu	Ala125Thr Ala136Thr	OFX: 8 (I) <sup>a</sup>
Up-372	Asp112Glu	Ala125Thr Ala136Thr	OFX: 8 (I)
Uu-384	Asp112Glu	Ala125Thr Ala136Thr	OFX: 8 (I)
Up-392	Asp112Glu	Ala125Thr Ala136Thr	OFX: 4 (I)
Up-402	Asp112Glu	Ala125Thr Ala136Thr	OFX: 4 (I)
Up-405	None	Ser83Leu	OFX: 4 (I)
Up-417	Asp112Glu	Ala125Thr Ala136Thr	OFX: 4 (I)

a: MIC ≤ 8 µg/ml (Bebear *et al.*, 2008)



**A** **B**

**Figure 6.1:** (A): PCR amplification the QRDR of the *gyrA* gene. Lane 1: 100 bp DNA ladder, (Promega); Lane 2: *U. parvum* (strain Up-402).  
(B): Sequenced region of *gyrA* gene of strain Up-402 compared to *gyrA* gene sequence that corresponded to bases 99339 – 99622 of the reference strain (*U. parvum*, serovar 3, ATCC 27815). \*Mutation at position 99433 resulted in amino acid alteration at codon 112: Asp to Glu. Positive and negative controls were employed but are not shown.



**Figure 6.2:** (A): PCR amplification of the QRDR of the *parC* gene. Lane 1: 100 bp DNA ladder; Lane 2: negative control; Lane 3: *U. parvum* strain Up-402; Lane 4: *U. parvum* strain Up-405. Positive control employed not shown.

(B): Sequenced region of *parC* gene of *U. parvum* strain Up-402 (corresponding to base position 531622 - 531888) compared to *parC* gene sequence of reference strain (*U. parvum*, serovar 3, ATCC 27815). \*Mutations at base position: 531803, 531805, 531836 and 531838 resulted in amino acid substitutions at codons 125 and 136: Ala to Thr.

(C): Sequenced region of *parC* gene *U. parvum* strain 405 (corresponding to position 531622 -531888) compared to *parC* gene sequence of reference strain *U. parvum*, serovar 3, ATCC 27815.

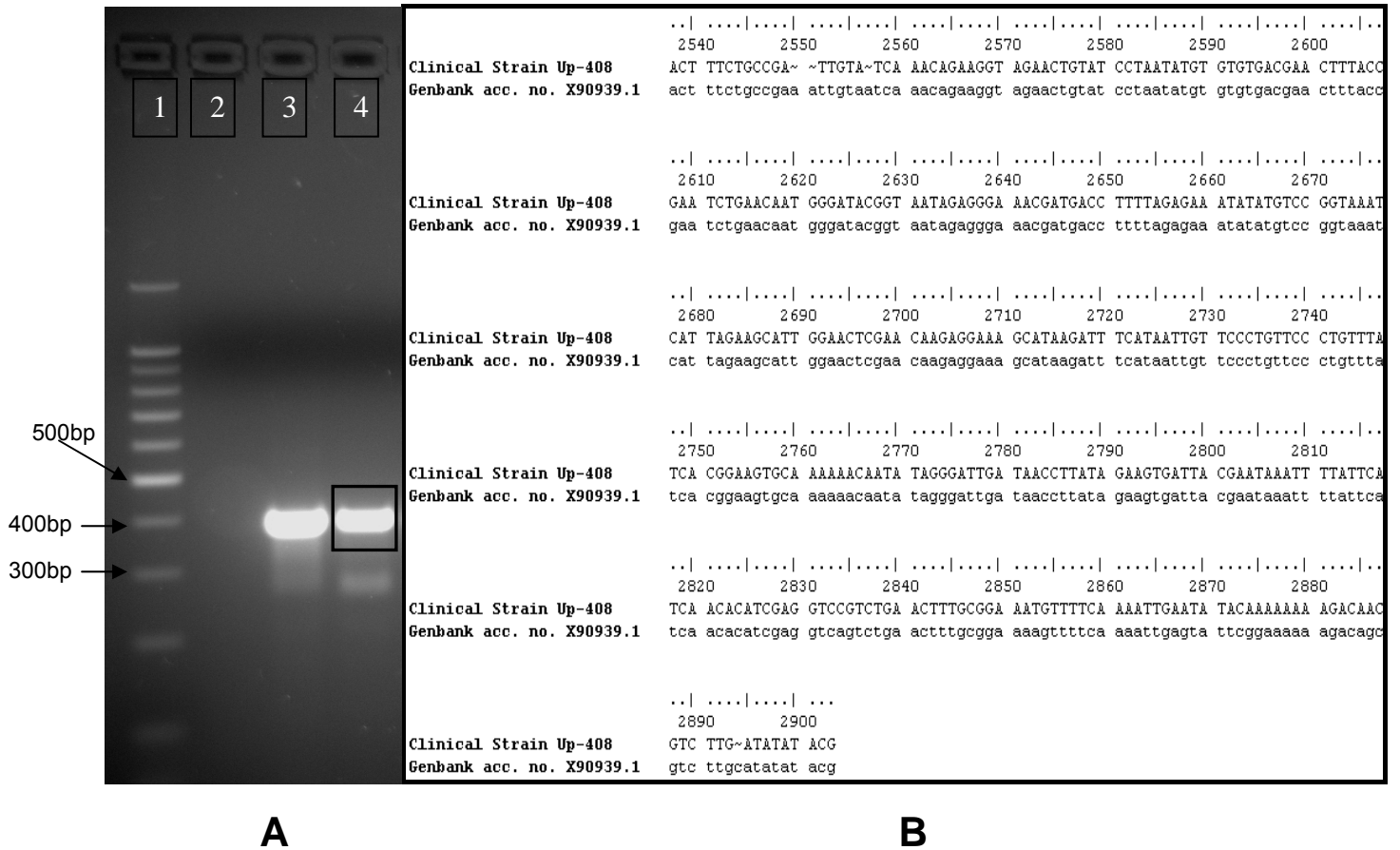
\*Mutation at position 531678 resulted in amino acid alteration at codon 83: Ser to Leu.

### **6.2.2.2 Erythromycin/ azithromycin resistance gene targets: 23S rRNA, L4 and L22 proteins**

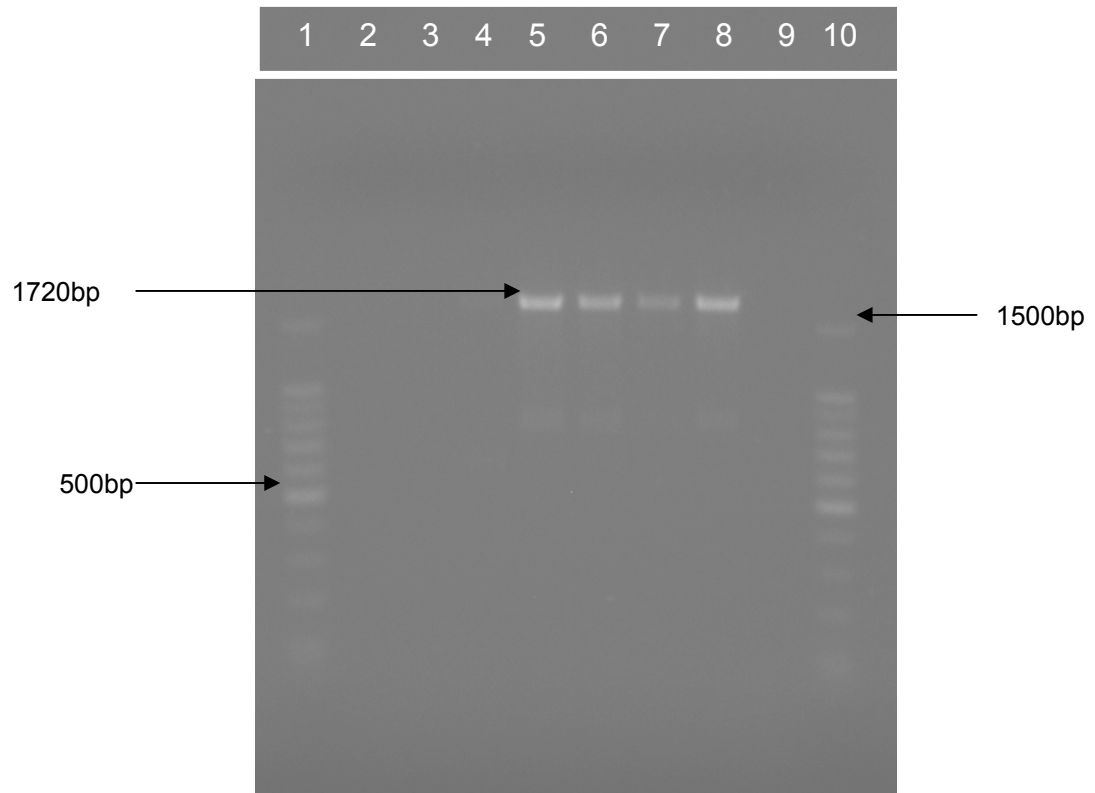
Although no erythromycin-resistant strains were found, 14 strains exhibited intermediate erythromycin resistance ( $\text{MIC} \leq 1 \mu\text{g/ml}$ ) and 2 out of the 14 were also intermediately resistant to azithromycin ( $\text{MIC} \leq 4 \mu\text{g/ml}$ ). One strain was seen to be resistant to azithromycin ( $\text{MIC} \leq 16 \mu\text{g/ml}$ ) and tetracycline with intermediate resistance to erythromycin while another strain was resistant to azithromycin ( $\text{MIC} \leq 16 \mu\text{g/ml}$ ) with intermediate resistance to erythromycin (Table 6.2). On amplification and sequence analyses of the two operons (OP1 and OP2 in domain V) of 23S rRNA, L4 and L22 proteins of these strains, no mutations were detected.

### **6.2.2.3 Tetracycline resistance gene analysis**

Seven ureaplasmas exhibited tetracycline resistance. The presence of *tetM* genes in all resistant strains was then investigated using primers directed at the 397 bp (Fig. 6.3) and 1.7 kb *tetM* region (Fig. 6.4) and *int*-Tn gene (Fig. 6.5). Screening for *tetM* and *int*-Tn gene regions was extended to include all ureaplasmas whether tetracycline-susceptible, -intermediate or -resistant (Table 6.4). All tetracycline resistant strains contained 1.7 kb and 397 bp regions of *tetM* and the *int*-Tn gene. PCR was repeated on all susceptible strains to verify no amplicon contamination had occurred. Eleven susceptible strains whilst possessing *int*-Tn gene lacked a large region of the *tetM* gene. The remaining 48 susceptible strains did not possess the *int*-Tn protein region (integrase) of transposon Tn 1545. No plasmids were detected in the seven tetracycline-resistant strains using the Pure Yield plasmid miniprep kit (section 2.13.1). Of the three tetracycline- + doxycycline- resistant strains, two possessed *tetM* mutations at positions 2750: A to C and 2751: C to T resulting in histidine to proline amino acid substitutions while one tetracycline strain had only the *tetM* mutation at position 2751: C to T (Table 6.5).



**Figure 6.3:** (A): PCR amplification of the *tetM* gene. Lane 1: 100 bp DNA ladder; Lane 2: negative control; Lane 3: Up-408; Lane 4: Up-417.  
 (B): Sequences corresponding to position 2538-2904 (of 397bp band) of the *tetM* gene in strain Up-408 compared to *S. pneumoniae tetM* gene sequence (accession number = X90939.1).  
 A positive control was employed but not shown.



**Figure 6.4:** PCR products of the 1.7 kb *tetM* gene region using primers TetMF and TetMR-2123.

Lanes 1 and 10: Molecular weight markers (Promega 100 bp DNA ladder); Lane 2: Negative control; Lane 3-9: Samples 251, 266, 354, 366, 385, 408, 417

Results of sequence comparisons are discussed in Section 6.2.2.4.

A positive control was employed not shown.





**Table 6.4:** Screening of tetracycline-susceptible, -intermediate (to other antibiotics) and -resistant *U. parvum* and *U. urealyticum* strains for *tetM* (397 bp and 1.7 kb regions) and *int*-Tn region.

Tetracycline	No. of strains with regions associated with tetracycline resistance			
	397 bp	397 bp + 1.7 kb	397 bp + <i>int</i> -Tn gene	397 bp + 1.7 kb + <i>int</i> -Tn gene
Susceptible (n=59)	45	3	11	
Resistant (n=7)				7

**Table 6.5:** Tetracycline and doxycycline MICs and *tetM* sequence analysis.

Strain	MIC broth ( $\mu\text{g/ml}$ )		<i>TetM</i> detected (397 bp + 1.7 kb)	Sequence analysis Base changes: position 2750, 2751
	Tet	Doxy		
Up-251	32	4	Yes	No
Up-266	16	4	Yes	No
Uu-354	16	8	Yes	Yes (2750: A to C) + (2751: C to T) *
Up-366	16	8	Yes	Yes (2751: C to T) (silent)
Up-385	8	4	Yes	No
Up-408	64	4	Yes	No
Up-417	32	8	Yes	Yes (2750: A to C) + (2751:C to T) *

\* Amino acid change = Histidine to proline

#### **6.2.2.4 *Ureaplasma spp. tetM* sequence comparisons with *S. pneumoniae*, *N. gonorrhoeae* and *ureaplasma* GenBanked sequences**

The *tetM* gene sequences were aligned as shown in Table 6.6. Alignments were performed against the *tetM* sequence of *N. gonorrhoeae* strain 6418 (GenBank accession number: L12241) [USA, 1993, plasmid pOZ100] (Gascoyne-Binzi *et al.*, 1993) base numbers corresponding to nucleotides 674 – 2174. Only nucleotide positions that differed are shown across the strains. The genes aligned are: *S. pneumoniae*: X90939: [Italy, 1995, Transposon Tn5251 (Provvedi *et al.*, 1996) nucleotides 2553 – 4040 and *U. urealyticum* SV9-Seattle: U08812 [1988, Transposon Tn916 doxycycline-susceptible (Sanchez-Pescador *et al.*, 1988) nucleotides 2436 – 3877. The leader 600 nucleotides of the *tetM* gene exhibited considerable diversity between strains and the region was schematically represented in Figure 6.6.

#### **6.2.2.5 *Ureaplasma spp. int-Tn* sequence comparisons with a *S. pneumoniae* GenBanked sequence**

Sequences of the seven tetracycline-resistant ureaplasma *int-Tn* genes were aligned with *S. pneumoniae* Tn916 *int-Tn* (X61025) nucleotides 758-1337 (Table 6.7). On examining alignments, based on common nucleotide alterations there were three major *int-Tn* types: Type 1, Deletions were observed yet other than a short region (bases 981-1009) nucleotide alterations to those of *S. pneumoniae* Tn916 *int-Tn* were not evident for strains Up-408, Up-417, Up-366 and Uu-354; Type 2, nucleotide changes throughout (bases 1-1135) common to strains Up-251 and Up-385; Type 3, no initial nucleotide alterations (bases 1-914) then specific alterations for strain Up-266.

**Table 6.6:** *TetM* sequence alignments of the seven tetracycline-resistant *Ureaplasma* spp. strains and GenBank *tetM* genes.

Sequence comparisons were performed against the *tetM* sequence of *N. gonorrhoeae*: L12241 (Gascoyne-Binzi *et al.*, 1993). Only nucleotide positions that differed from *N. gonorrhoeae* L12241 are shown across the strains.

*N. gonorrhoeae* strain 6418: L12241 [USA, 1993, plasmid pOZ100] (Gascoyne-Binzi *et al.*, 1993). Base numbers correspond to nucleotides 674 –2174.

The *tetM* genes aligned are:

Sp= *S. pneumoniae*: X90939: [Italy, 1995, Transposon Tn5251 (Provvedi *et al.*, 1996). Base numbers correspond to nucleotides 2553 – 4044.

Uu-S= *U. urealyticum* SV9: U08812 [Seattle, 1988, Transposon Tn916 doxycycline-susceptible (Sanchez-Pescador *et al.*, 1988). Base numbers correspond to nucleotides 2436 – 3877.

Up-251= *U. parvum* current study: doxycycline-susceptible.

Up-266= *U. parvum* current study: doxycycline-susceptible.

Up-385= *U. parvum* current study: doxycycline-susceptible.

Up-408= *U. parvum* current study: doxycycline-susceptible.

Up-417= *U. parvum* current study: doxycycline-resistant.

Up-366= *U. parvum* current study: doxycycline-resistant.

Uu-354= *U. urealyticum* current study: doxycycline-resistant.

W=nucleotide wobble, strains exhibiting excessive problems were re-sequenced. d=Nucleotide deletion

-= Not determined for strains Up-251 and Up-266 nucleotides 343-378, as sequence interpretation was not possible.





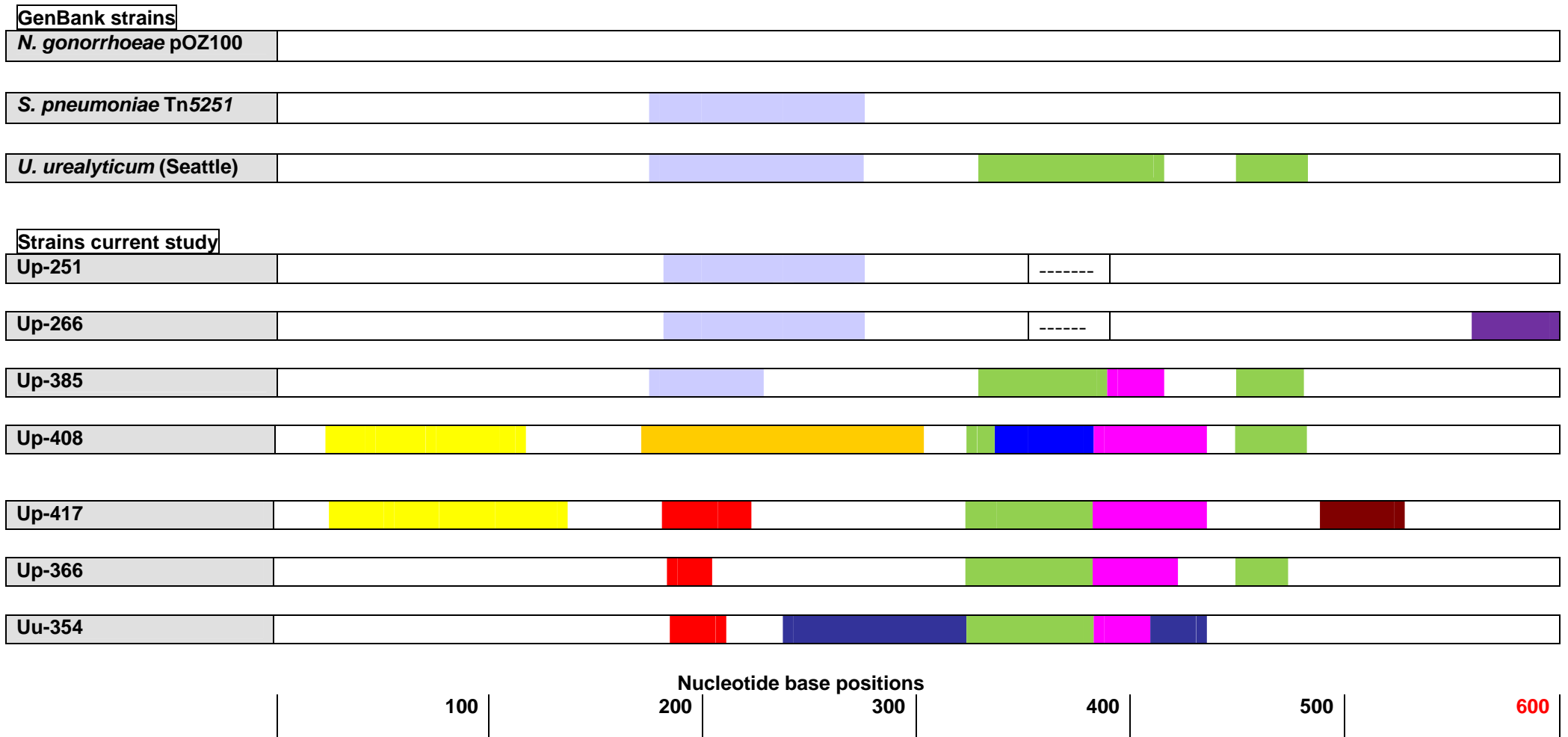






	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	
	3	4	4	4	4	4	5	5	5	6	6	6	6	6	7	7	7	7	8	8	8	8	9	
	7	2	3	4	5	7	1	6	9	0	3	4	7	9	2	3	5	7	0	2	3	4	6	7
Ng	t	a	t	t	g	t	a	a	a	a	t	g	t	t	t	t	a	a	t	t	t	a	a	t
Sp																								
Uu-S																								
Up-251	d				-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	
Up-266											-	-	-	-	-	-	-	-	-	-	-	-	-	
Up-385		w	w			C	d	w	d			d	d		G									
Up-408			w	d			C	d	d		d	d	d							C				
Up-417					A			w			w	w		d	d		w	w	d	G	w	w	w	
Up-366			d	d							-	-	-	-	-		-	-	-					
Uu-354							d		d		d		d	d	d		d		C					

A schematic diagram of *tetM* sequence comparisons (Fig 6.6) was prepared from bases 1-600 with the format: clear sections identical to *N. gonorrhoeae*; blue: *S. pneumoniae*; green: *U. urealyticum* SV9, other colours: are independent to *Ureaplasma* spp strains from current study.



**Figure 6.6:** Schematic representation of aligned *tetM* gene sequences from the different tetracycline-resistant *Ureaplasma* spp strains from current study.





### 6.2.3 Antibiotic Resistance Gene Transfer

#### 6.2.3.1 Screening of *N. gonorrhoeae* cultures for tetracycline resistance prior to transformation experiments

Four *N. gonorrhoeae* strains were screened for tetracycline recipient suitability. Strains were screened employing Etests and included if they exhibited inhibitory concentrations of  $\leq 0.25$   $\mu\text{g/ml}$ .

#### 6.2.3.2 Transformation/culture co-incubation type experiments

The four *N. gonorrhoeae* recipients were broth culture optimised to fulfil transformation criteria for DNA addition/uptake, exponential ( $1 \times 10^7$  cfu/ml) to early stationary phase ( $1 \times 10^8$  cfu/ml). Donor DNA from Up-417 resistant to tetracycline (MIC 32  $\mu\text{g/ml}$ ) and possessing *int-Tn* and *tetM* 1.7 kb regions was added to *N. gonorrhoeae* when cultures had reached approximately  $1 \times 10^7$  cfu/ml. After a two hour uptake period, viability was approximately  $1-2 \times 10^8$  cfu/ml. Fresh media was then added to ensure the broth could sustain growth whilst any DNA that had undergone recombination could be expressed. At the end of this expression period, culture viability was approximately  $1-3 \times 10^8$  cfu/ml. No tetracycline-resistant *N. gonorrhoeae* transformants (frequency  $< 3-9 \times 10^{-7}$ ) were detected after performing Etests.

In order to increase detection frequencies after transfer and expression periods recipient cultures were challenged with suitable concentrations of tetracycline. No tetracycline-resistant *N. gonorrhoeae* transformants were obtained using a co-culture type protocol between *N. gonorrhoeae* recipients and tetracycline-resistant *Ureaplasma* donors (culture and DNA). Incubation of a susceptible *U. parvum* recipient (tetracycline MIC  $\leq 0.25 \mu\text{g/ml}$ ) with DNA from tetracycline-resistant *U. parvum* (3 strains) and *U. urealyticum* (1 strain) produced transformants with MICs ranging between 4-8  $\mu\text{g/ml}$ . PCR analysis on these low-level tetracycline-resistant transformants revealed that the 1.7kb region of the *tetM* gene was not present.

Transformation was also conducted with *E. coli* JM109 competent cells. *E. coli* JM109 commercially available competent cells (Promega) were transformed by donor DNA (pGEM-3Z vector) which served as a positive control and two colonies on LB agar plates with ampicillin were recovered. In contrast no transformants were obtained with donor *tetM* DNA from the *Ureaplasma* spp strains.

### 6.2.3.3 Electroporation

Electroporation (refer to section 2.14.4) using *N. gonorrhoeae* plasmid containing *tetM* and DNA from tetracycline-resistant *U. parvum* and *U. urealyticum* strains into *N. gonorrhoeae* recipients was unsuccessful.

Electroporation was also conducted employing an *E. coli* control strain DH5 $\alpha$  as the recipient. *E. coli* DH5 $\alpha$  bacteria were transformed with control pUC18 plasmid DNA resulting in growth of *E. coli* colonies (n=8) on LB with ampicillin. However, no transformation occurred using the control *E. coli* cells with DNA from *Ureaplasma* strains on transfer to LB agar plates containing tetracycline. No transformants resulted on employing an *E. coli* DH5 $\alpha$ -pUC18 transformant as the recipient when DNA from *Ureaplasma* spp. was added, with selection performed on LB plates containing ampicillin and tetracycline.

## 6.3 DISCUSSION

One *U. urealyticum* and seven *U. parvum* strains exhibited resistance to one or more of the antimicrobial agents tested (ofloxacin, azithromycin, tetracycline and doxycycline) and were seen to have MICs greater than normal ranges previously published by Waites *et al.*, (2005), Bebear *et al.*, (2008) and Bebear and Kempf (2005). All strains were susceptible to josamycin.

Twenty ureaplasmas were found to be intermediately-resistant to ofloxacin, based on MIC ranges for *Ureaplasma* documented by Bebear *et al.*, (2008), of which 13 had MICs  $\leq$  1-2  $\mu$ g/ml; three strains had MICs of 8  $\mu$ g/ml while the other four strains showed MICs of 4  $\mu$ g/ml. No isolates were resistant to erythromycin with only

intermediate resistance (MIC  $\leq 4$   $\mu\text{g/ml}$ ) observed. Two strains (Up-276, Up-266) were resistant to azithromycin (MICs 16  $\mu\text{g/ml}$ ). Three tetracycline-resistant strains (Uu-354; Up-366; Up-417) were concurrently resistant to doxycycline with four tetracycline-resistant strains exhibiting intermediate resistance to doxycycline. Four *U. parvum* strains were resistant/intermediately-resistant to more than one antibiotic, Up-266 azithromycin and tetracycline; Up-367 ofloxacin erythromycin and azithromycin; Up-402 ofloxacin and azithromycin; Up-417 ofloxacin and tetracycline.

Putative molecular mechanisms of antibiotic resistance were investigated based on target genes identified by previous investigators (Bebear *et al.*, 1997; Bebear *et al.*, 2000; Blanchard *et al.*, 1992; de Barbeyrac *et al.*, 1996; Pereyre *et al.*, 2007a; Beeton *et al.*, 2009b). Ofloxacin was used as the representative fluoroquinolone that targets DNA gyrase and topoisomerase IV, mutations in *gyrA*, *parC* and *parE* genes having been shown to be associated with fluoroquinolone resistance (Bebear *et al.*, 1999; 2000; 2003; Bebear and Kempf, 2005; Duffy *et al.*, 2006; Zhang *et al.*, 2002). A substitution of Ser83Leu in ParC was identified in one intermediate-resistant strain Up-405 (MIC=4  $\mu\text{g/ml}$ ) while a triple substitution of Asp112Glu in GyrA along with Ala125Thr and Ala136Thr in ParC all within the QRDR was found in six intermediately-resistant strains (Up-367, Up-372, Up-384; MICs 8  $\mu\text{g/ml}$ . Up-392, Up-402, Up-417; MICs 4  $\mu\text{g/ml}$ ).

In contrast, Beeton *et al.*, (2009a), reported that the triple mutation of Asp112Glu in GyrA along with Ala125Thr and Ala136Thr in ParC was not related to a resistance phenotype, but had resulted from species-specific polymorphism which are found in all *U. parvum* and *U. urealyticum*. However, Ser83Leu is one mutation that remains a candidate for fluoroquinolone resistance development, with further multiple substitutions being required to mediate a resistant phenotype. Investigators found that strains with Ser83Leu substitutions in ParC showed varying degrees of resistance to fluoroquinolones (Bebear *et al.*, 1999; 2000; 2003; Bebear and Kempf, 2005; Duffy *et al.*, 2006; Zhang *et al.*, 2002) while others found that only isolates with the triple and Ser83Leu substitutions exhibited full resistance (Bebear *et al.*, 2003; Zhang *et al.*, 2002). Six strains from the current study had only the triple mutation not related to a resistance phenotype (species-specific polymorphism) while one had the Ser83Leu mutation alone.

Bebear *et al.*, (2003) showed that isolates with ofloxacin MICs 4-8 µg/ml and ciprofloxacin MICs 16-128 µg/ml, contained the triple mutation either with/ without a Ser83Leu substitution. On examining species specific polymorphisms proposed by Beeton *et al.*, (2009a) no unique resistance candidate mutations appear to be evident. The ParC Ser83Leu substitution is homologous to resistance mediating mutations identified in many other fluoroquinolone-resistant bacteria, including *S. aureus* and *S. pneumoniae* (Piddock, 1999).

Fluoroquinolones are known to have preferential sites of action: gyrase for Gram-negative bacteria and topoisomerase IV for Gram-positive bacteria. The proposed evolution of ureaplasmas from Gram-positive progenitors may be responsible for the higher incidence of mutations within topoisomerase IV relative to gyrase. Resistance mechanisms and gene mutations have still to be clarified (Beeton *et al.*, 2009a).

No erythromycin-resistant strains were found and no mutations were detected in OP1 and OP2 in domain V of 23S rRNA, L4, L22 proteins in intermediate-resistant strains or in two strains which exhibited azithromycin resistance. Pereyre *et al.* (2007) have shown that *in vitro*, *U. parvum* mutants derived after passage in increasing concentrations of erythromycin produced mutations in 23SrRNA operons and L4, L22 ribosome associated proteins. Characterisation of *in vitro*-selected mutants of *U. parvum* that were resistant to macrolides revealed no significant difference in MICs, with or without reserpine indicating the absence of a putative efflux mechanism (Pereyre *et al.*, 2007a). The methyltransferase enzyme (Erm family) or enzymes modifying macrolides have not been identified in mycoplasmas/ureaplasmas (Bebear and Kempf, 2005). As no mutations were found, the azithromycin intermediate resistance observed may even be due to media pH change that has been shown to produce false resistance profiles (Waites and Duffy, 2008). Dongya *et al.* (2008), identified point mutations in 23S rRNA operons of *U. urealyticum* isolates that were resistant to macrolides: josamycin, clarithromycin, roxithromycin and azithromycin, unfortunately erythromycin was not tested. Beeton *et al.* (2009b), on sequence analysis of an isolate with an MIC ≥ 64 µg/ml, found no mutations in 23S rRNA operons or L22, only a 6-bp deletion in L4. Jensen *et al.* (2008) detected three different mutations at positions 2058 and 2059 (*E. coli*



numbering) in 23S rRNA domain V of *M. genitalium* that was resistant to azithromycin and erythromycin (MICs >8µg/ml).

Tetracycline resistance in *Mollicutes* is mediated by transferable genetic elements/transposons containing *tetM* genes (Roberts and Kenny, 1986). In the current study, seven strains with MICs 8-64 µg/ml were seen to harbour a 1.7 kb *tetM* gene region and an *int*-Tn gene that encodes integrase and excision functions of transposon Tn1545. Eleven-susceptible strains contained *int*-Tn gene regions but lacked a large portion of the *tetM* gene as only a 397-bp fragment of *tetM* was detected. The remaining 48 strains only contained a 397-bp region of *tetM*. Degrange *et al.* (2008), identified two *tetM* containing *M. hominis* isolates that were tetracycline susceptible, one isolate had a 1260-bp insertion in the leader peptide sequence which prevented successful transcription while no mutation in *tetM* of the other isolate was found. Beeton *et al.* (2009b), also found no explanation for the presence of *tetM* in a susceptible isolate and attributed the finding to possible amplicon contamination.

Three of the seven tetracycline-resistant strains were concurrently resistant to doxycycline. Blanchard *et al.* (1992), have similarly reported that *tetM* in *M. hominis* and *U. urealyticum* conferred resistance to tetracycline but not necessarily to doxycycline; 8/21 tetracycline-resistant strains were resistant to doxycycline, 2 had intermediate resistance to doxycycline and 11 were susceptible. It was proposed by Oggioni *et al.* (1996), that amongst *tetM* genetic mosaics different spectra of activity to different tetracyclines may be present, although further investigations were advocated. Beeton *et al.* (2009b) recently conducted *tetM* sequence comparisons between tetracycline- + doxycycline-susceptible strains with tetracycline-resistant and tetracycline- + doxycycline-resistant strains of ureaplasmas and found no amino acid alterations that could be attributed to either tetracycline or doxycycline resistance. Induction of *tetM* gene transcription by doxycycline has been considered not to be as strong as tetracycline, yet Degrange *et al.* (2008) on investigating *M. hominis* strain passages to increasing sub-inhibitory concentrations of doxycycline observed for one strain that doxycycline enhanced *tetM* transcription. It was therefore suggested that *tetM* gene expression in *M. hominis* is regulated by transcription attenuation that involves activation or inhibition of transcription

termination at a position between the promoter and the *tetM* gene (Degrange *et al.*, 2008). Beeton *et al.* (2009b), just placed a statement that the *tetM* tetracycline-resistant *U. parvum* strain investigated was additionally resistant to doxycycline with no comment on expression.

On comparing *tetM* gene sequences of the seven tetracycline-resistant strains with GenBank sequences of *tetM* genes from *N. gonorrhoeae*, *S. pneumoniae* and *U. urealyticum* SV9-Seattle, the genes of five strains were seen to be highly mosaic. It was not possible to compare sequences with a previously described doxycycline-resistant *Ureaplasma* strain SV9-Vancouver that had been registered in GenBank, as the sequence had been withdrawn without explanation. Sequence analysis was problematic in that although a quality 1.7 kb PCR product was produced, baseline was noisy over some regions and nucleotide wobbles evident particularly in strain Uu-354. Sequencing was conducted twice with 1.7 kb primers, once with 319 bp primers and once with 1.38 kb primers. As the *tetM* gene of some stains was found to contain highly diverse regions primer incompatibility may well have been responsible for sequence inconsistencies. Unfortunately primers of Beeton *et al.* (2009b) that have been shown to produce 901 bp *tetM* fragments failed amplify the region for the seven strains.

On aligning sequences, in the *tetM* leader region, blocks both similar and different to those of the three GenBank strains were evident and unique regions were also noted in three strains from the present study. Three recombination hot spot sites across strains that also corresponded to *S. pneumoniae* and/or *U. urealyticum* SV9-Seattle were seen around bases 172, 300 and 425. A fourth recombination region between bases 376-392 was common only to five study strains (Up-385, Up-408, Up-417, Up-366 and Uu-354). The terminal end whist containing few nucleotide alterations was seen to exhibit multiple deletions.

The three tetracycline- + doxycycline-resistant strains Up-417, Up-366 and Uu-354 exhibited a region between bases 172-244 that was similar to the *N. gonorrhoeae* but with a tight cluster of base differences between 198-214 that were similar to *Enterococcus faecalis* and *Staphylococcus aureus* (Oggioni *et al.*, 1996). Oggioni *et al.* (1996), also recognised that the only significant differences between these *E.*

*faecalis* and *S. aureus* strains were at the ribosomal binding site 115 nucleotides upstream of the structural gene. The doxycycline-resistant *U. parvum* strain of Beeton *et al.* (2009b) was reported to be similar to the *U. urealyticum* SV9-Seattle strain. For reasons unknown, no comparisons or comment was made by Beeton *et al.* (2009b) to mosaic structures of *U. urealyticum* reported by Soroka *et al.* (2002). Soroka *et al.* (2002), on investigating *tetM* determinants in urogenital *U. urealyticum* isolates, described three *tetM* genes that were identical to *tetM* of a GenBanked *Ureaplasma* sequence (U08812) and two that showed new allelic variants. One of these strains had a clipped *U. urealyticum* block which was also evident in the present study.

As no plasmids were demonstrated in the present study or by other investigators, *tetM* genes in the ureaplasmas were considered chromosomally located. Oggioni *et al.* (1996), showed that the *tet* locus and Tn917 exhibited vast differences in sensitivity to restriction endonucleases, Tn917 with few restriction sites whilst on integration, *tetM* like other chromosomally located gene loci could be subjected to recombination events generating mosaic structures. According to Beeton *et al.* (2009b) as mutations/mosaics within *tetM* are similar in doxycycline-susceptible and -resistant strains, expression of *tetM* appears key to doxycycline resistance.

On sequence alignment of *int*-Tn genes from the seven tetracycline-resistant strains with *S. pneumoniae* Tn1545 *int*-Tn, three types of *int*-Tn genes were characterised. It was interesting to note that for the four strains in which *tetM* mosaic structure was the most evident the *int*-Tn genes (Type 1) were the least diverse. As *int*-Tn is a functional gene, integrase with incision and some excision properties, it appears in the three strains where nucleotide alterations had occurred most extensively that *tetM* genetic exchange may have been compromised. Of the four strains possessing a Type 1 *int*-Tn upstream of *tetM*, three were the tetracycline- doxycycline-resistant strains and one a tetracycline-resistant strain.

The mosaic *tetM* genes described both in the current study and that of Soroka *et al.* (2002) and *int*-Tn of the current may well predetermine which upstream transposon promoter/insertion regions would affect survival under selective pressure. In a study investigating doxycycline-resistant populations of streptococci before and after

doxycycline treatment a novel transposon CTn6002 was demonstrated (Warburton *et al.*, 2007). CTn6002 was described as a composite transposon containing Tn916, with a leader region similar to Tn5251, open reading frames similar to lactobacilli, a novel open reading frame, and it was also capable of *in vitro* conjugation. Roberts (2005), commented that there was no clear role for mosaic *tet* genes other than to be a unique feature of a small group of organisms/those from the same environment, but that mosaic genes would certainly influence PCR/probe detection techniques. Whilst Warburton *et al.* (2007), made comment that on performing PCR detection of *tetM* and *int*-Tn genes that thorough characterisation and prevalence determinations be performed for the identification of individual members of Tn916. Mycoplasmas and ureaplasmas are constantly minimising their genomic sizes which would account for lost *tetM* regions. Such essential excision processes could well generate recombination sites at which different *tetM* and *int*-Tn types and leader transposon regions/promoters could integrate when strains are again challenged with tetracycline/ doxycycline.

Although preliminary *in vitro* reciprocal transformation attempts to achieve high-level tetracycline/doxycycline resistance were unsuccessful, ureaplasmas may donate or receive DNA to/from other microorganisms during co-infection/co-colonisation at the sites of infection. Bacterial genomes are prone towards deleting non-essential DNA, and the small, reduced genomes of host-dependent bacteria, attest to the tendency for bacteria to delete the most expendable sequences from their genomes. Hence, bacterial genomes are sampling rather than accumulating sequences and counterbalancing gene acquisition with gene loss (Ochman *et al.*, 2000).

In a clinical setting, cells are under stress due to antimicrobial treatment, and capsules may be damaged/disintegrated and cell lysis stimulated which could facilitate DNA uptake. Other factors regulating gonococcal transformation that are important for efficient DNA uptake are (a) type IV pilus and proteins associated with pilus; pilliated frequencies can exceed 20% whilst in non-pilliated variants frequencies are far reduced,  $1 \times 10^{-7}$  and (b) donor DNA that possesses a ten base sequence (GCCGTCTGAA) called the DNA uptake sequence (DUS) that is frequently present in neisserial chromosomes (Hamilton and Dillard, 2006). It has been shown that *N. gonorrhoeae* strains can lose piliation and high frequency

competence uptake due to multiple non-selective passages *in vitro* (Bogdan *et al.* 2002). Transformation with plasmids can be 1000-fold less efficient than chromosomal DNA although it is not clear as to expected frequencies when donor DNA is a transposon/transposon related gene. Transposon located gene integration may be limited, yet transposons are ideally primed for recombinational success especially concerning integration into a resident transposon and transposon genes are readily incorporated into resident gene cassettes. Filter mating experiments on *S. pneumoniae* with donor DNA from transposons resulted in frequencies of  $8 \times 10^{-8}$  for Tn1545 and  $5 \times 10^{-7}$  for Tn916 (McDougal *et al.*, 1998).

Electroporation using *N. gonorrhoeae* plasmid containing *tetM* and DNA from tetracycline-resistant *U. parvum* and *U. urealyticum* strains into susceptible *N. gonorrhoeae* recipients were unsuccessful. It has been reported that many strains of *Neisseria* are fragile and unable to survive the high voltage discharge generated across their membrane by electroporation (Bogdan *et al.*, 2002). No tetracycline-resistant *N. gonorrhoeae* transformants were obtained using co-culture/DNA protocols between *N. gonorrhoeae* recipients and tetracycline-resistant *Ureaplasma* donors.

One *U. parvum* recipient, on exposure to donor DNA from one tetracycline-resistant *U. urealyticum* and three *U. parvum* strains, produced transformants with low-level tetracycline resistance (MICs 4-8  $\mu\text{g/ml}$ ). PCR analysis performed on these transformants did not detect 1.7 kb *tetM* gene regions. Tetracycline resistance can occur by four different mechanisms: ribosomal protection (RP) mechanism, tetracycline efflux, enzymatic inactivation of tetracycline and modification of the ribosomal target (Kazimierczak *et al.*, 2008). The absence of *tetM* genes in the transformants obtained in this investigation suggests that other genetic element(s) conferred low-levels of tetracycline resistance, such as the expression of a tetracycline efflux mechanism. Non-specific transformation as a result of horizontal gene transfer may occur through a complicated process (Neela *et al.*, 2009), and low transfer frequencies observed with certain bacterial species could be due to the presence of restriction systems that destabilise exogenous DNA (Courvali, 1994).

The *tetM* genes of ureaplasmas are highly diverse, clearly showing that acquisition of different regions has occurred from various sources. In addition three *int*-Tn genes of different Tn916-Tn1545 origins appear evident. These findings may in part have affected genetic transfer studies. *In vivo* exchange is feasible as genital microorganisms possess a variety of transposon located tetracycline resistance genes that include a) ribosomal protection genes: mosaic *tetM* in *N. gonorrhoeae*, mycoplasmas, ureaplasmas, lactobacilli and *Gardnerella vaginalis*; *tetS* in lactobacilli; *tetO* in *N. gonorrhoeae*; *tet36* in lactobacilli and b) efflux gene: *tetK* in lactobacilli (Roberts, 2005; Soroka *et al.*, 2002).

## CHAPTER SEVEN

### CONCLUSIONS

In the medical arena, mycoplasmas and ureaplasmas have been termed “stealth pathogens” as they are difficult to culture and have therefore remained under the radar as they may well have been missed as causes of disease. In the agricultural arena, mycoplasmas have for years been recognised as major pathogens. In addition, recently great strides have been made to detect and track tetracycline resistance in livestock, feed and produce. With new interest from commercial companies and the introduction of PCR techniques into diagnostic laboratories more information on prevalence and antibiotic resistance profiling is becoming available.

During the study period, detection of mycoplasmas and ureaplasmas employing conventional culture was problematic in that quality controlled commercially prepared agar was expensive and not consistently available. Commercial kits for detecting mycoplasmas and ureaplasmas did not perform well when compared to PCR. Due to workloads, the introduction of culture techniques would be challenging for diagnostic laboratories in South Africa and it is recommended that for screening, especially respiratory specimens, that PCR diagnostics be implemented. The Takara kit was excellent but yet again expensive and based classification of ureaplasmas on the previous system whereby both *U. parvum* and *U. urealyticum* species were detected as *U. urealyticum* (biotypes 1 and 2). The primers employed in the study had been extensively used in the research setting and can be recommended. However, it would be important for multiplex-PCR techniques to be assessed against South African isolates before incorporation into diagnostic standard operating procedures.

Without an agar culture format and international recommendations, susceptibility testing of mycoplasmas and ureaplasmas is reliant on selective broth procedures. It should be noted that whilst commercial U9 broth is a suitable medium for culture *Ureaplasma* spp., it does allow the growth of both *U. parvum* and *U. urealyticum* and therefore prior to susceptibility testing, specific ATCC recommended broths should be employed and species determined by PCR. The best performing antibiotic susceptibility screening kit was found to be the SIR kit. PCR for antibiotic resistance gene detection is well developed but for quinolones (mutation detection) sequencing is necessary and whilst the *tetM* primers provided quality amplified fragments sequencing was not ideal. In addition a set of primers that had been shown to amplify a region of *tetM* failed to produce a fragment. As *tetM* was seen to be highly mosaic in strains of the current study, it is recommended that at least two primer pairs are used. Further investigations should be conducted to determine whether mycoplasmas and ureaplasmas possess other tetracycline resistance gene classes that encode ribosome protection proteins, by employing degenerate primers.

The study on endocervical specimens revealed single and co-colonisation with mycoplasmas, ureaplasmas and *C. trachomatis* appeared to be age related. Microbial population shifts were evident but as susceptibility monitoring is not conducted in the general population, consequences as a result of syndromic sexually transmitted disease antibiotic management are not known. There was no association found between *M. hominis* and *Ureaplasma* spp. and preterm labour in the population group studies and also no significant association with *U. parvum*. Failure to establish a statistically significant relationship between *U. parvum* and preterm delivery lends to further investigations as the sample size was too small. Comparisons of colonisation with HIV status in the current study showed no association between *M. hominis*, *Ureaplasma* spp.; *C. trachomatis* and HIV infection. In the South African public sector, diagnostic testing is not performed for *C. trachomatis*, mycoplasmas or ureaplasmas and therefore surveillance on prevalence/co-colonisation and antimicrobial resistance



development of these micro-organisms against changing syndromic management programmes requires further monitoring.

The findings on community-acquired pneumonia in adults and the presence of *U. parvum* DNA in the two cases of neonatal pneumonia in the Port Elizabeth region requires further prevalence and epidemiological based investigations and extension of diagnostic approaches to provide for informed therapeutic decisions. It was evident that robust integrated hospital and pathology laboratory patient data systems suitable for South Africa must be designed and implemented, as efficient health management systems they are vital to ensure that community health issues pertaining to both respiratory illness and sexually transmitted infections can be responsibly addressed.

The overall prevalence of *Mycoplasma* spp. and *Ureaplasma* spp. from endocervical specimens collected during 2005 were 34% and 13.5% respectively. However, the follow on series in 2006 collected for antimicrobial susceptibility testing showed dramatic population shifts in that *Ureaplasma* spp. were found in 69% of specimens and no *Mycoplasma* spp. were detected. This could not have been due to the inherent resistance of ureaplasmas to clindamycin, (as clindamycin is not routinely used at all in South Africa) or to quinolone/erythromycin/azithromycin resistance as high-level resistance to these antibiotics was not demonstrated. The one AZM resistant strain may be considered a false positive as *Ureaplasma* spp. are very sensitive to pH changes in the media. Although high-level quinolone resistance was not detected in ureaplasmas, on analysing intermediate-resistant strains, one strain had the Ser83Leu mutation alone which is related to a low level resistant phenotype.

The findings of *tetM* genes/remnants in all isolates both tetracycline-resistant and –susceptible, was of major interest and have not been described before at this level. Seven strains were resistant to tetracycline with dual doxycycline resistance observed in three strains. To-date, only two mycoplasmas and two

ureaplasmas exhibiting tetracycline and doxycycline resistance have been documented. On sequence alignment, *tetM* genes from seven tetracycline-resistant strains against GenBank sequences of *N. gonorrhoeae*, *Streptococcus pneumoniae* and *U. urealyticum*, leader regions were seen to be highly mosaic in structure. Some of these regions were similar to those of the GenBank strains and others unique to strains in current study. Interestingly, a tight cluster of base differences 198-214, was found only in the three doxycycline-resistant strains and were seen to be similar to *Enterococcus faecalis* and *Staphylococcus aureus*. Four hot spot recombination sites were identified that could certainly have influenced the formation of the mosaic structures, upstream insertion sequences/open reading frames and leader transposon regions that regulate expression. On sequence characterisation of *int*-Tn genes three profile types were evident. The type most resembling that of *S. pneumoniae* appeared linked to the *tetM* genes that exhibited highly mosaic structures.

Further studies are required to a) determine whether positive *Ureaplasma* selective pressure is still present or whether mycoplasmas have re-emerged, even possibly as a consequence of tetracycline/doxycycline gene resistance transfer from ureaplasmas and b) elucidate *tetM* gene expression that results in doxycycline resistance. A revised South African Sexually Transmitted Infection Treatment Guidelines includes doxycycline for treatment of conditions such as urethritis and vaginal discharge syndrome, where ureaplasmas have been implicated (Lewis and Maruma, 2009). It will therefore be of great interest to assess if continued usage of doxycycline impacts on *Ureaplasma* and *Mycoplasma* prevalence and resistance development/acquisition.

The finding of a number of tetracycline-resistant/intermediate-resistant and even susceptible strains harbouring the *int*-Tn gene (Tn916 - Tn1545 family) and *tetM* regions in conjunction with *tetM* diversity, indicates that ureaplasmas are involved in tetracycline and doxycycline resistance interchange. Although high-level tetracycline resistance gene transfer was not seen to occur from

ureaplasmas to *N. gonorrhoeae*, this does not exclude the possibility of *in vivo* transfer from other microorganisms that may originally have acquired resistance genes from ureaplasmas. Further work should be conducted on reciprocal antibiotic resistance gene transfer between urogenital microflora to assess impact of integrated transposons from different origins on antibiotic resistance expression, stability and influence on genomic evolutionary restructuring.

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## LIST OF PUBLICATIONS

### Congress Presentation (Paper)

**Govender, S.** H.J. Odendaal, and L.J. Chalkley. 2007. Prevalence of Mycoplasma and Chlamydia in pregnancy. *Proceedings; International Conference on Chlamydia and Mycoplasma Human Infections*. Ferrara (Italy), April 19<sup>th</sup> – 20<sup>th</sup>.

### Congress Presentations (Poster)

1. **Govender, S.**, S.J. du Plessis, G.S. Ocana and L.J. Chalkley. 2007. Prevalence of *Pneumocystis jiroveci* and *Mycoplasma pneumoniae* in patients presenting with pneumonia at hospitals in Port Elizabeth. FIDSSA II Congress, Cape Town, October 29<sup>th</sup> – 31<sup>st</sup>.
2. **Govender, S.**, M.C. Scheckle and L.J. Chalkley. 2010. Antibiotic susceptibilities and resistance genes of *Ureaplasma* spp. 50<sup>th</sup> Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), Boston, USA, September 12<sup>th</sup> – 15<sup>th</sup>.

### Publications

1. **Govender, S.**, G.B. Theron, H.J. Odendaal, and L.J. Chalkley. 2009. Prevalence of genital mycoplasmas, ureaplasmas and *Chlamydia* in pregnancy. *J Obstet Gynaecol.* **29**:698-701.
2. **Govender, S.**, S.J. du Plessis, G.S. Ocana and L.J. Chalkley. 2008. Prevalence of *Pneumocystis jiroveci* and *Mycoplasma pneumoniae* in patients presenting with pneumonia at hospitals in Port Elizabeth. *South Afr J Epidemiol Infect.* **23**:21-24.